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STUDIES OF PROPOSED STEROL INTERMEDIATES IN
THE BIOSYNTHESIS OF CHOLESTEROL.

RICE UNIVERSITY, PH.D., 1977
RICE UNIVERSITY

STUDIES OF PROPOSED STEROL INTERMEDIATES
IN THE BIOSYNTHESIS OF CHOLESTEROL

by

PETER CHANG

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

THESIS DIRECTOR'S SIGNATURE:

[Signature]

HOUSTON, TEXAS

SEPTEMBER, 1977
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And to Robert A. Welch Foundation for their financial support.
TO MY PARENTS, KATHERINE D. LEE,
AND ALL YE CONNOISSEURS OF
CHOLESTEROL
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I. INTRODUCTION

The enzymic conversion of lanosterol to cholesterol (Figure 1) involves three general processes: reduction of the side chain \( \Delta^2 \) double bond, shift of the nuclear double bond from the \( \Delta^8(9) \)-position in lanosterol to the \( \Delta^5 \)-position in cholesterol, and removal of the three 'extra' methyl groups at positions 4\( \alpha \), 4\( \beta \), and 14\( \alpha \) (C-30, C-31, and C-32 respectively).

While the nature of the first two processes has generally been elucidated, the process of oxidative demethylation of the three 'extra' methyl groups remains unclear. The results of early studies by Olson et al. (1957) suggested that each of the three 'extra' methyl groups of lanosterol is converted to carbon dioxide by aerobic processes that require both reduced and oxidized pyridine nucleotides. Incubation of 'methyl-labeled' lanosterol with a 700 x g supernatant fraction of rat liver homogenate yielded results compatible with the formation of three moles of labeled CO\(_2\) per mole of cholesterol formed from lanosterol. No formaldehyde could be detected. The authors proposed that the removal of these 'extra' methyl groups proceeds via an initial oxygen-dependent hydroxylation followed by dehydrogenations (Olson et al., 1957; Pudles and Bloch, 1960) to yield the corresponding aldehydes and carboxylic acids. However, the results of later studies by Miller and Gaylor (1970a) indicated that oxidations, rather than dehydrogenations, are involved in the removal of the 4\( \alpha \)- and 4\( \beta \)-methyl groups of lanosterol.
Figure 1. The enzymic conversion of lanosterol to cholesterol

Figure 2. Facilitation of decarboxylation of sterols with carboxylic function at C-4 by the presence of a β-keto group at C-3.
The decarboxylation of sterols with carboxylic functions at C-4 would be greatly facilitated by the presence of a β-keto group at C-3 (Figure 2). Lindberg et al. (1963) and Swindell and Gaylor (1967) have provided experimental evidence compatible with an intermediary role for 3-ketosteroids in C-4 demethylation. This concept has been supported by later work of Miller et al. (1967) and Swindell and Gaylor (1968).

The process of C-4 demethylation has been studied extensively since the last decade. The evidence available so far indicates that the 4α-methyl group is first oxidized by an attack of methyl sterol oxidase to form the carboxylic acid (Figure 3). The oxidation is stereospecific, i.e., the β-methyl group is not attacked by the oxidase (Miller and Gaylor, 1970b; Rahman et al., 1970). Although oxidation is thought to be stepwise, \( \text{RCH}_3 \rightarrow \text{RCH}_2\text{OH} \rightarrow \text{RCHO} \rightarrow \text{RCOOH} \), each of the three steps appears to be catalyzed by the same mixed function oxidase (Miller et al., 1971). Aside from 4α-carboxylic acid sterol (Hornby and Boyd, 1970; Miller and Gaylor, 1970a and 1970b), other intermediates have not been isolated at the various stages of oxidation. This is not only because the enzyme preparation used in these studies apparently catalyzes the overall conversion to carboxylic acid, but also because the rate of oxidation appears to increase with each successive attack (Miller et al., 1971).

The 4α-carboxylic acid is metabolized by a DPN-dependent decarboxylase. The product is a 4α-methyl 3-ketone (Rahimtula and Gaylor, 1972), which is formed by epimerization of the original 4β-methyl group (Rahman et al., 1970): epimerization appears to be a noncatalytic part of decarboxylation. The 4α-methyl group of the resulting
Figure 3. Proposed mechanism for C-4 demethylation
3-ketosteroid is not oxidized by methyl sterol oxidase (Swindell and Gaylor, 1968). The oxidase, however, does attack the 4α-methyl group of the 3β-hydroxy steroid, 4α-methyl-5α-cholest-7-en-3β-ol (I), which is oxidized to carboxylic acid (Miller and Gaylor, 1970a). Thus a TPNH-dependent 3-ketosteroid reductase that converts the 3-ketone to a 3β-alcohol has been sought and found. Oxidation of the remaining monomethyl product then occurs by sequential attack of oxidase, decarboxylase, and reductase as before. It has been suggested that the same enzymes catalyze oxidation of the mono- and dimethyl sterol substrates (Miller and Gaylor, 1970a; Miller et al., 1971; Miller and Gaylor, 1970b).

In the case of the C-14 demethylation, no adjacent ketone function is available for facilitation of decarboxylation. Hydroxylation and successive dehydrogenations or oxidations of the C-14 methyl group would give rise to a β,γ-unsaturated acid. A reaction analogous to the nonenzymatic decarboxylations of β,γ-unsaturated acids, which proceed with shift of the double bond from the β,γ- to the α,β-position (Arnold et al., 1950; Bigley and Thurman, 1967) would yield a sterol containing a Δ^8(14)-double bond (Figure 4). The isolation of cholest-8(14)-en-3β-ol from rat skin (Lee et al., 1969) and the enzymatic conversion of Δ^8(14)-sterols to cholesterol have been reported (Lee and Schroepfer, 1968; Lee et al., 1969; Fried et al., 1968; Dudowitz and Fried, 1969). In addition, aerobic incubation of [3α-^3H]-lanost-7-en-3β,32-diol (II) and [3α-^3H]-lanost-7-en-3β-ol-32-al (III) reportedly resulted in the formation of 4,4-dimethyl-5α-cholest-8(14)-en-3β-ol (IV) and cholesterol (Fried et al., 1968; Dudowitz and Fried, 1969). Unfortunately, the methodology employed to characterize
Figure 4. Decarboxylation of 3,7-unsaturated sterol acyls with carboxylic function at C-14 proceeds with shift of the double bond from the 3,7- to the α,β-position to yield sterols containing a Δ9(14)-double bond.
the Δ⁸(14) sterol was not rigorous enough to establish its identity unambiguously. It is interesting to note that the diol (II) has been reported to be convertible to the aldehyde (III), but not to a carboxylic acid, upon anaerobic incubation of the diol (II) with rat liver microsomes in the presence of DPN (Dudowitz and Fried, 1969). This finding indicates that the mechanism of removal of 14α-methyl group may be different from that proposed for C-4 demethylation.

On the other hand, evidence against the intermediary role of Δ⁸(14)-sterols in C-14 demethylation has been presented (Piecchi et al., 1970; Alexander et al., 1971; Gibbons, 1974; Gibbons and Mitropoulos, 1975). All these studies involved either failure in attempts to trap the Δ⁸(14)-sterol from incubation of labeled precursor or failure in attempts to trap a later intermediate using labeled Δ⁸(14)-sterol as the substrate. However, as pointed out by Schroepfer et al. (1972), even though a successful trap experiment does provide supporting evidence concerning the intermediary role of the trapped compound in the biosynthetic sequence under investigation, the inability to demonstrate an effective trap would justify only the most cautious interpretation in view of several complications of this type of experiments.

More recent work has shown that the removal of the 32-carbon atom in cholesterol biosynthesis occurs at the oxidation state of an aldehyde resulting in the release of formic acid (Alexander et al., 1972). Cholest-7,14-dien-3β-ol, rather than cholest-8(14)-en-3β-ol, was claimed to be the immediate product of C-14 demethylation (Figure 5). The removal of the C-14 methyl group as formic acid has since been confirmed by Trowbridge et al. (1975). These results placed the status of Δ⁸(14)-
Figure 5. Mechanism of C-14 demethylation as proposed by Alexander et al. (1972).
sterols in an even more doubtful position.

To further clarify the role of $\Delta^{8(14)}$-sterols in the biosynthesis of cholesterol, the metabolism of $[3\alpha-^3\text{H}]$-14$\alpha$-hydroxymethyl-5$\alpha$-cholest-7-en-3$\beta$-ol (V) with rat liver microsomes under various conditions was investigated. The metabolic fate of $[3\alpha-^3\text{H}]$-14$\alpha$-hydroxymethyl-5$\alpha$-cholest-7-en-3$\beta$-ol was also studied using another approach. By incubating this substrate with rat liver homogenate preparations in the presence of trans-1,4-bis(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride (AY-9944), an inhibitor of specific enzymic reactions in cholesterol biosynthesis, it was hoped that intermediates of this biosynthetic sequence would accumulate in quantities sufficient enough to permit their identification by classical chemical techniques.
II. MATERIALS AND METHODS

Labeled Materials

\[ \text{[3α-}^{3}\text{H]}-14α-\text{Hydroxymethyl-5α-choleste}-7\text{-en-3β-ol} \]

\[ \text{[3α-}^{3}\text{H]}-14α-\text{Hydroxymethyl-5α-choleste}-7\text{-en-3β-ol} \]

was prepared by chemical synthesis by Dr. Roger Shaw according to the scheme

shown in Figure 6. The specific activity was 0.274 millicuries per milligram.

\[ \text{[32-}^{3}\text{H]}-14α-\text{Hydroxymethyl-5α-choleste}-7\text{-en-3β-ol} \]

\[ \text{[32-}^{3}\text{H]}-14α-\text{Hydroxymethyl-5α-choleste}-7\text{-en-3β-ol} \]

was also prepared by Dr. Shaw according to the same scheme.

\[ {^3}\text{H]-Acetic Anhydride} \]

Tritiated acetic anhydride (supplied in benzene solution) was purchased from New England Nuclear. The specific activity was 0.49 millicuries per milligram.

Melting Points

Melting points were recorded in open capillary tubes, using a Thomas Hoover capillary melting point apparatus. Melting points were uncorrected.

Ultraviolet Spectrometry

Ultraviolet spectra were recorded on a Varian Techtron UV-VIS Spectrometer (Model 635), using matched quartz cells with a one centimeter light path. Spectra were recorded in absolute ethanol.
Figure 6. Synthetic scheme of 14a-hydroxymethyl-5α-cholest-7-en-3β-ol and its labeled compounds at both [3α-3H] and [32-3H]-positions.
Preparation of Steryl Acetate

Synthetic sterols and sterols recovered from incubation media were acetylated by the method of Johnston and Bloch (1957). A mixture of dry pyridine and acetic anhydride (1:1) was added to the sterol sample, and the reaction was allowed to proceed at room temperature for 24 to 28 hours. After this period, water was added and the resulting mixture was extracted four times with petroleum ether. The organic layer was then washed three times with water and dried over anhydrous sodium sulfate.

Purification of Cholesterol Through Its Dibromide

Radioactive cholesterol recovered from the various incubation experiments was purified by the method of Frantz et al. (1959). The radioactive cholesterol sample, together with about 40 mg of unlabeled cholesterol, was dissolved in a known volume of benzene. Appropriate aliquots, in triplicate, were then removed for determination of radioactivity and mass content. The sample was evaporated to dryness and the residue was redissolved in 2 ml of anhydrous ether in a centrifuge tube cooled in an ice-water bath.

To this resulting solution, bromine was added dropwise until a red-brownish color persisted. The solution was kept at 4°C for 2 hours and 1 milliliter of cooled glacial acetic acid was then added with stirring. The precipitated dibromide was collected by centrifugation at 400 x g for 20 minutes and at 4°C. The pellet was washed twice with 2 ml portions of glacial acetic acid and collected by centrifugation.

The resulting dibromide was dissolved in 5 ml of anhydrous ether and transferred to a three-necked round bottom flask equipped with
magnetic stirrer and condenser. With the flask immersed in a bath of ice water, 25 mg of zinc dust was added and the solution was stirred for 15 minutes. Water was then added dropwise to dissolve the zinc bromide, and the ether layer was washed once with 2 ml of 0.7N HCl, twice with 2 ml portions of water, once with 2 ml of 8% NaOH, and with water again until neutral. The ether layer was evaporated to dryness and the residue was recrystallized from methanol. The recrystallized cholesterol was dissolved in a known volume of benzene and appropriate aliquots, in triplicate, were removed for assay of radioactivity and mass. A schematic diagram of this procedure is shown in Figure 7.

**Colorimetric Assay of Sterols and Steryl Acetates**

Sterols and steryl acetates were quantitated by the method of Liebermann-Burchard colorimetric assay. The Liebermann-Burchard reagent used throughout these studies was prepared according to the modified method described by Abell et al. (1952). A mixture of acetic anhydride and sulfuric acid (20:1) was cooled in ice for 9 minutes, followed by addition of 10 parts of glacial acetic acid. The reagent was then allowed to warm to room temperature. An aliquot (1.8 ml) of this reagent was added to dry sterol or steryl acetate samples in 10 x 75 mm colorimetric tubes. Absorption was read at 620 nm on a Coleman Junior Spectrophotometer. Color was allowed to develop for the appropriate length of time, as shown in Table 1.

**Measurement of Radioactivity**

Radioactivity was measured in a Beckman LS-250 liquid scintil-
Labeled sample

Add unlabeled authentic cholesterol and determine specific activity

Add bromine and keep at $4^\circ$ for two hours

Add acetic acid with stirring

Collect precipitated dibromide by centrifugation

Supernatant (discard)

Pellet

Wash 2x with acetic acid and redissolve in ether

Add zinc dust and stir for 15 minutes

Add water to dissolve zinc bromide and extract with ether

Ether layer

Wash 1x with 0.7 N HCl
2x with water
1x with 8% NaOH
2x with water

Recrystallize from methanol

Determine specific activity

Figure 7. Schematic diagram of procedures for purification of cholesterol through its dibromide.
Table 1

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Development Time (Minutes)</th>
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<tr>
<td><strong>Monoenes</strong></td>
<td></td>
</tr>
<tr>
<td>$\Delta^8(14)$</td>
<td>13</td>
</tr>
<tr>
<td>$\Delta^8(9)$</td>
<td>4.5</td>
</tr>
<tr>
<td>$\Delta^7$</td>
<td>1.5</td>
</tr>
<tr>
<td>$\Delta^{14}$</td>
<td>8</td>
</tr>
<tr>
<td>$\Delta^5$</td>
<td>30</td>
</tr>
<tr>
<td><strong>Dienes</strong></td>
<td></td>
</tr>
<tr>
<td>$\Delta^7,^{14}$</td>
<td>1.25</td>
</tr>
<tr>
<td>$\Delta^8,^{14}$</td>
<td>7</td>
</tr>
<tr>
<td>$\Delta^5,^7$</td>
<td>1.5</td>
</tr>
<tr>
<td>$\Delta^5,^{24}$</td>
<td>22</td>
</tr>
</tbody>
</table>

*Development times for free sterols or steryl acetates of the same double bond positions are equivalent.

Scintillation system, using a 2.5 gain setting and plug-in iso-sets. Two types of scintillation counting solution were used. For counting of labeled compounds in dry state or in small volume of organic solvent (usually benzene or hexane), a toluene counting solution containing 0.4% 2,5-diphenyloxazole (PPO from Beckman Instruments, Inc.) and 0.005% 1,4-bis-[{a-(5-phenyloxazoyl)}]-benzene (POPOP from Packard Instrument Company, Inc.) was used. Counting of tritium-labeled compounds was carried out at 50-60% efficiency, while carbon $^{14}$-labeled compounds were counted at 90-95% efficiency. For counting of labeled compounds in small volume of aqueous solution, an ethanol-
toluene scintillation counting solution prepared by dissolving 15 g of PPO in a solution of toluene (2.5 liters) and absolute ethanol (1.25 liters) was used. The tritium-labeled compounds were counted at 25-30% efficiency in this counting solution. A constant volume (10 ml per vial) of scintillation counting solution was used throughout these studies.

**Thin-Layer Chromatography**

Silica Gel G thin-layer plates (250 μm thickness, without fluorescent indicator) and Silica Gel GF thin-layer plates (250 μm thickness, with fluorescent indicator) were used as purchased from Analtech, Inc. (Newark, Delaware). For the separation of free sterols (mono-hydroxy and other more polar sterols) or 3-ketosterols, mixtures of chloroform and acetone (95:5, v/v) or ethyl acetate and benzene (5:2, v/v) were used as the solvent system. For the separation of acetates and diacetates, solvent system of either chloroform or hexane-ether (9:1, v/v) was used.

Preparative Silica Gel G thin-layer plates of 500 μm thickness were sometimes used in quantitative isolation of a desired product. Silica Gel G (Type 60, for TLC acc. to STAHL, EM Laboratories Inc., Elmsford, New York) was shaken thoroughly with two volumes of water, and thin-layer plates were prepared by the method of Kammereck et al. (1967). The plates were allowed to dry in air for about an hour and then in oven (100-120°C) for another hour.

Spots were visualized by spraying with a 5% molybdic acid solution (prepared by mixing 20 g of ammonium molybdate with 25 ml of concentrated sulfuric acid, warmed to dissolve, then cooled and diluted to 400 ml with water), followed by heating for 5-10 minutes
in a drying oven maintained at 100-120°.

**Thin-Layer Radiochromatography**

Labeled samples were spotted on Silica Gel G thin-layer plates along with suitable unlabeled standards, and were developed in the appropriate solvent system.

Assay of radioactivity was accomplished by scraping off the adsorbent (in 1 cm increments) of the thin-layer plates directly into counting vials. Measurement of radioactivity was made as described previously.

**Gas-Liquid Chromatography**

A Hewlett-Packard 402 High Efficiency Gas Chromatograph unit equipped with a flame ionization detector was used in all gas-liquid chromatographic analyses.

For the separation of some of the steryl acetates encountered in these studies, a 6 ft by 4 mm (internal diameter) silanized, U-shaped glass column of 3% OV-17 on Gas-Chrom Q (100-120 mesh Gas-Chrom Q coated with 3% phenyl-substituted methyl silicone rubber OV-17) was used. Column operating conditions were as follows: injector temperature, 310°; column temperature, 270°; flame detector temperature, 350°; gas (helium) flow rate, 66 ml per minute.

**Gas-Liquid Radiochromatographic Analysis**

When determination of the radioactive purity or identity of a labeled sample was required, the distribution of its radioactivity throughout the gas-liquid chromatogram was measured by collecting 1 minute fractions of the effluent gas in 30 x 3 mm (internal diameter) glass tubes. Recovery of radioactivity varied from 40-80%.
The collected material was rinsed several times with the toluene counting solution into counting vials and the radioactivity was measured as described previously. A delay of approximately 1.5 minute was observed between the mass curve reading and the radioactivity detected at the outlet.

**Column Chromatography**

**Silicic Acid-Super Cel Column Chromatography**

Silicic acid-Super Cel columns for separation of monohydroxy and more polar sterols were prepared according to Clayton et al. (1963). Silicic acid (100 mesh analytical reagent, Mallinckrodt Chemical Works) and Hyflo Super-Cel (Johns-Manville Corporation) were mixed in a ratio of 2:1 (w/w) and slurried in the appropriate solvent (usually benzene). The slurries were poured into columns (1 x 100 cm) and packed under nitrogen pressure (5 psi). Sterol samples were applied in small volume (2-3 ml) of the eluting solvent (usually 10% ether in benzene). The eluates were collected in test tubes using an automatic fraction collector (LKB Radi-Rac, type 3403B or ISCO Model 273 Fraction Collector). The contents of each fraction were dried under nitrogen using an analytical evaporator (N-EVAP of Organomation Associates Inc.), and the residues were redissolved in a constant volume (usually 2 ml) of benzene. Aliquots were then removed for determination of radioactivity and/or mass content.

**Silica Gel G-Super Cel-Silver Nitrate Column Chromatography**

Silica Gel G-Super Cel-silver nitrate columns were used for the separation of monoenes from dienes and of various dienes from each other, in the form of their acetate derivatives.

A modification of the method of Galli and Paoletti (1966) was
used. Silica Gel G (20 g; Type 60 for TLC acc. to STAHL, Cat. 7731, EM Laboratories, Inc., Elmsford, New York) was thoroughly mixed with Hyflo Super-Cel (20 g) in a one liter lyophilization flask. A solution of silver nitrate (8 g) in distilled water (150 ml) was added and the resulting slurry shaken vigorously. The mixture was frozen in a dry ice-acetone bath and lyophilized for 45-50 hours. The resultant white powder was used immediately or was stored in a vacuum dessicator over Drierite in the dark until used. The prepared column material was slurried in hexane-benzene (7:3, v/v), and columns (1 x 50 cm) were packed under nitrogen pressure as described above. Steryl acetates were applied in small volumes (2 ml) of the eluting solvent; elutates were collected and analyzed as described previously. Typical separations of the steryl acetates on this column are shown in Figure 8. The amount of column material made as above was enough for two columns (1 x 50 cm).

Alumina-Super Cel-Silver Nitrate Column Chromatography

Alumina-Super Cel-silver nitrate columns were used in the separation of various sterol monoene s from each other, in the form of their acetate derivatives.

The method described by Paliokas et al. (1968) was modified in the following manner. Neutral alumina AG-7 without binder (35 g; aluminium oxide for TLC, 2-44 micron particle size, Bio-Rad Laboratories, Richmond, California) was thoroughly mixed with Hyflo Super-Cel (17.5 g) in a one liter lyophilization flask. Silver nitrate (10.5 g) dissolved in deionized water (87.5 ml) was added, and the resulting slurry was shaken vigorously to insure thorough mixing. The mixture was frozen in an acetone-dry ice bath and lyophilized for 24-28 hours. The resulting white powder was slurried in hexane-benzene (9:1, v/v), and
Figure 8. Chromatographic separation of the acetates of cholesterol, cholesta\textsubscript{7,14-dien-3β-ol}, cholesta\textsubscript{8,14-dien-3β-ol}, and cholesta\textsubscript{5,7-dien-3β-ol} on a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, eluted with a mixture of hexane and benzene (7:3, v/v). \textDelta-\textDelta, Steryl acetates measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to cholesta\textsubscript{7,14-dien-3β-ol}, the third is due to cholesta\textsubscript{8,14-dien-3β-ol}, and the fourth is due to cholesta\textsubscript{5,7-dien-3β-ol}. 
columns (1 x 100 cm) were packed under nitrogen pressure. Steryl acetate samples were applied to the column in small volumes (2 ml) of the eluting solvent, and the eluates were collected and analyzed as described previously. Typical separations of monounsaturated steryl acetates are shown in Figure 9.

**Incubation Studies**

**Preparation and Incubation of Cell-Free Rat Liver Homogenate**

Female or male rats (Sprague-Dawley strain; 150-250 g each) were maintained on Purina Laboratory Chow. After decapitation with a miniature guillotine, the bodies were drained of excess blood and the livers were quickly removed. The livers were minced into small pieces with a pair of scissors and then homogenized in ice-cold potassium phosphate buffer (2.5 ml/g of liver; 0.1 M, pH 7.4) using a loose-fitting teflon-on-glass Dounce homogenizer connected to a Talboys Instrument Corporation Motor, Model 101. The homogenate was centrifuged at 500 x g for 15 minutes (4°) to remove large debris, liver chunks, nuclei, and whole cells. The resulting supernatant was centrifuged for 30 minutes at 10,000 x g and 4° to remove mitochondria. This 10,000 x g supernatant fraction was used as the enzyme source for all subsequent incubation studies that did not involve the determination of cofactor requirements (Bucher, 1956; Tchen and Bloch, 1957).

To each Erlenmeyer flask (125 ml) containing 14 ml of the 10,000 x g supernatant fraction of the rat liver homogenate, either a solution of potassium phosphate buffer (15 ml; 0.1 M, pH 7.4) or a solution of inhibitor AY-9944 (15 ml to give a final concentration of 10⁻⁴ M; Ayerst Laboratories Inc., New York, New York) was added. This was followed by the addition of appropriate cofactors in 1 ml of potassium
Figure 9. Chromatographic separation of the acetates of cholesterol, cholest-8(14)-en-3β-ol, cholest-7-en-3β-ol, cholest-8(9)-en-3β-ol, and cholestanol on a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, eluted with hexane-benzene (9:1, v/v). α-α, 14C radioactivity due to [1-14C]-cholesteryl acetate. △-△, Steryl acetate measured colorimetrically. The first peak is due to cholest-8(14)-en-3β-ol, the second is due to cholest-8(9)-en-3β-ol, the third is due to cholest-7-en-3β-ol, and the fourth is due to cholesteryl acetate.
phosphate buffer (0.1 M, pH 7.4) to give final concentrations of 1 mM of DPN (Sigma Chemical Company, Lot 27B-7521-1), 1 mM of TPN (Calbiochem, San Diego, California, Lot 200476), and 3 mM of glucose-6-phosphate (Sigma Chemical Company). The labeled substrate in propylene glycol (0.05-0.1 ml) was added last.

Aerobic incubations were carried out for 3 hours in an air atmosphere at 37°, in a Precision Scientific constant temperature water-bath shaker. At the end of the incubation period, a volume of 15% ethanolic KOH equal to that of the incubation mixture was added and the mixture was heated under reflux for 3 hours. The sterols were extracted with petroleum ether and diethyl ether. The petroleum ether and the diethyl ether extracts were pooled separately, each of them washed three times with water, and dried over anhydrous sodium sulfate. Appropriate aliquots were then removed from both aqueous and organic phases for assay of radioactivity.

**Preparation and Incubation of Rat Liver Microsomes**

Female or male rats (Sprague-Dawley strain; 150-250 g each) were maintained on Purina Laboratory Chow. After decapitation with a miniature guillotine, the bodies were drained of excess blood and the livers were quickly removed. The livers were homogenized in ice-cold potassium phosphate buffer (10 ml/g of liver; 0.1 M, pH 7.4) and the 10,000 x g supernatant fraction was isolated by differential centrifugation as described previously. The 10,000 x g supernatant fraction was then centrifuged at 105,000 x g for 60 minutes in a Beckman L2-65B Ultracentrifuge in polycarbonate screw-capped tubes. The pellets were removed and resuspended in fresh buffer (half of the volume of buffer used in homogenization), using a teflon-on-glass homogenizer operated
manually. The suspension was recentrifuged at 105,000 x g for 60 minutes. The washed microsomal pellets were again removed and resuspended in ice-cold potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride, in a volume that would give a final protein concentration of 15-20 mg/ml as determined by the method of Lowry et al. (1951).

To each test tube containing 2 ml (30–40 mg of protein) of this microsomal preparation, appropriate cofactors (DPN, TPN, or TPNH generating system) and/or AY-9944 inhibitor solution were added to give final concentrations of 1.5 mM of DPN, 1.5 mM of TPN, 1.2 mM of TPNH, and $10^{-4}$ M of AY-9944, respectively. The labeled substrate in propylene glycol (0.05–0.1 ml) was added last.

The TPNH generating system was prepared as follows: TPN (24.37 mg) and DL-isocitric acid (84.39 mg; Sigma Chemical Company, Lot 20C-2710) were dissolved in 6 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride. The pH was adjusted to 7.4 with a few drops of dilute KOH. An aliquot (0.75 ml) of this mixture was added to each incubation sample, followed by isocitrate dehydrogenase (0.15 ml, 10 mg/ml; Sigma Chemical Company, Lot 20C-8090) to give a total volume of 0.9 ml/incubation sample. The final concentration of each component of the TPNH generating system in the incubation mixture (3 ml) was 1.2 mM of TPN, 11.6 mM of isocitric acid, and 0.5 mg of isocitrate dehydrogenase per ml of incubation mixture.

The control for these incubations was prepared by boiling the microsomal enzyme suspension at 100°C for 20 minutes, to which were then added the appropriate cofactors and the labeled substrate.
Aerobic incubations were carried out for 30-120 minutes at 37°C in a Precision Scientific constant temperature water-bath shaker. At the end of the incubation period, acetone (40 ml) was added and the mixture was homogenized using a loose-fitting teflon-on-glass Dounce homogenizer connected to a Talboys Instrument Corporation Motor, Model 101. The resulting coagulum was removed by filtration; the filter paper and the coagulum were washed several times with fresh acetone (30 ml/wash). The combined filtrates (~300 ml) were then evaporated to dryness under reduced pressure, and the residue was subjected to chromatographic analysis.

A schematic sketch of procedures for the incubation of substrate and the subsequent analysis of incubation products is shown in Figure 10.
Rat livers

Homogenize and centrifuge at 500 x g for 15 minutes

Supernatant → Centrifuge at 10,000 x g for 30 minutes

Pellet (discard) → Supernatant (10,000 x g supernatant fraction of rat liver homogenate)

Centrifuge at 105,000 x g for 60 minutes

Pellet (discard) → Supernatant (Washed rat liver microsomes)

Resuspend in fresh buffer and centrifuge at 105,000 x g for 60 minutes

Supernatant (discard) ← Labeled substrate +/- cofactors +/- inhibitors

Incubate at 37° for 3 hours

Saponify with 15% ethanolic KOH for 3 hours

Extract labeled sterols with ether and petroleum ether

Chromatographic analysis

- continued on next page -
Figure 10. Schematic diagram of procedures for the preparation of rat liver homogenate and washed rat liver microsomes, the incubation of labeled substrate, and the subsequent analyses of labeled incubation products.
III. AEROBIC INCUBATION OF [3α-3H]-14α-
HYDROXYMETHYL-5α-CHEST-7-EN-3β-OL
WITH RAT LIVER HOMOGENATE PREPARA-
TIONS IN THE PRESENCE OF AY-9944

PART I. INCUBATION STUDIES

INTRODUCTION AND DISCUSSION

[3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol has previously been demonstrated to be convertible to cholesterol upon incubation, under aerobic conditions, with the 10,000 x g supernatant fraction of rat liver homogenate preparations (Trowbridge et al., 1975). However, intermediates involved in this conversion were either not detected (under the conditions employed) or were present in such minute quantities that further isolation and characterization would be very difficult. One approach to induce the accumulation of intermediates in metabolic studies is to use inhibitors of specific enzymic reactions. It must be recognized, however, that employment of these inhibitors does not permit a distinction between the importance of multiple potential pathways. And conversely, intermediates may not accumulate if an equilibrium exists between the intermediate and its earlier precursor.

The specific inhibitor used in present studies is trans-1,4-bis-
(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride (AY-9944). The
inhibition of the reduction of the Δ⁷-double bond of Δ⁵,⁷ sterols by AY-9944 is well documented (Dvornik et al., 1963; Kraml et al., 1964; Dvornik et al., 1964; Chappel et al., 1964; Niemiro et al., 1965; Dvornik et al., 1966; Scallen et al., 1969). Dvornik et al. (1963) first found that AY-9944 at a concentration of 10⁻⁶ M, prevented the incorporation of [2-¹⁴C]-mevalonate into cholesterol by liver homogenates of rats, monkeys, and dogs. The following year, in vivo incubation of [4-¹⁴C]-cholesta-5,7-dien-3β-ol in the presence of AY-9944 (10⁻⁶ M) resulted in an 80% inhibition of conversion of this sterol to cholesterol (Kraml et al., 1964). Later experiments carried out by Dvornik et al. (1966) demonstrated that incubation of [4-¹⁴C]-cholesta-7-en-3β-ol with rat liver preparations, in the presence of AY-9944 (10⁻⁵ M), resulted in the accumulation of labeled cholesta-5,7-dien-3β-ol. Essentially no radioactivity was associated with cholesterol. More recent work has shown that AY-9944, at a concentration of 10⁻⁴ M, resulted in a 99 and 98% inhibition of the reduction of the Δ¹⁴-double bond of cholesta-8,14-dien-3β-ol and cholesta-7,14-dien-3β-ol, respectively (Lutsky et al., 1975). In this respect, the effect of AY-9944 on sterol biosynthesis in rat liver is similar to that in at least some species of plants. It has been shown that Δ⁸,¹⁴ sterols accumulated during Δ⁵ and Δ⁷ sterol biosynthesis in vivo when the green algae Chlorella ellipsoides and Chlorella emersonii were grown in the presence of AY-9944 (Dickson et al., 1972; Dickson and Patterson, 1972; Dickson and Patterson, 1973). Dickson and Patterson (1973) also reported the accumulation of several 14α-methyl sterols when the alga Chlorella emersonii was cultured in the presence of AY-9944 (20 ppm). They interpreted this accumulation of 14α-methyl sterols in treated cultures
to mean that AY-9944 is an effective inhibitor of C-14 demethylation in *Chlorella emersonii*.

Aerobic incubations of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with rat liver homogenate preparations in the presence of AY-9944 (10^-4 M) were carried out under two different conditions: with added cofactors (DPN, TPN, and glucose-6-phosphate) and without added cofactors. The nomenclature 'without added cofactors' may be a little misleading. It does not mean that the incubation mixture contains no cofactors (rat liver homogenate itself contains some cofactors to start with), but rather that no additional cofactors are added to the incubation mixture. These experiments yielded a total of four major unknown compounds, one of them (Unknown C) occurring exclusively in incubations with no added cofactors. These unknowns will be examined in more detail in Part II of this chapter. It is sufficient to say here that the use of AY-9944 does cause the accumulation of certain unknown compounds, although it is too early to say whether or not these unknown compounds are true intermediates in the overall conversion to cholesterol.

**EXPERIMENTAL**

Thin-layer radiochromatographic analyses of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol on plates of Silica Gel G, with solvent systems of chloroform-acetone (95:5, v/v; Figure 11) and ethyl acetate-benzene (5:2, v/v; Figure 12), showed a single labeled component corresponding in mobility to that of authentic 14α-hydroxymethyl-5α-cholest-7-en-3β-ol. The radiopurity of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol was judged to be in excess of 98% on the basis of above chromatographic analyses.
Figure 11. Thin-layer radiochromatogram of \([3\alpha-^3\text{H}]-14\alpha\)-hydroxymethyl-5α-cholestan-7-en-3β-ol on a plate of Silica Gel G, developed twice in chloroform-acetone (95:5, v/v). Authentic 14α-hydroxymethyl-5α-cholestan-7-en-3β-ol and cholesterol are shown at 3.5 cm and 7.5 cm, respectively.
Figure 12. Thin-layer radiochromatogram of $[3\alpha^3H]-14\alpha$-hydroxymethyl-5α-cholesta-7-en-3β-ol on a plate of Silica Gel G, developed twice in ethyl acetate–benzene (5:2, v/v). Authentic 14α-hydroxymethyl-5α-cholesta-7-en-3β-ol and cholesterol are shown at 9.5 cm and 12.5 cm, respectively.
A. IN THE PRESENCE OF ADDED COFACTORS

Experiment 1

Four female rats (Sprague-Dawley strain; 150-200 g each) were killed by decapitation with a miniature guillotine and the livers (32 g) were removed and homogenized in ice-cold potassium phosphate buffer (80 ml; 0.1 M, pH 7.4) as described previously. The 10,000 x g supernatant fraction of the rat liver homogenate was isolated by centrifugation at 4°C for 30 minutes.

A solution of AY-9944 (2.0 x 10^{-4} M) was prepared by dissolving 9.3 mg of the inhibitor in 100 ml of potassium phosphate buffer (0.1 M, pH 7.4).

To an Erlenmeyer flask (125 ml) containing 14 ml of the supernatant prepared as described above, a solution of AY-9944 (15 ml; to give a final concentration of 10^{-4} M) was added (Incubation II). To another Erlenmeyer flask (125 ml) containing 14 ml of the same rat liver homogenate preparations, a solution of potassium phosphate buffer (15 ml; 0.1 M, pH 7.4) was added (Incubation I). Cofactors in 1 ml of potassium phosphate buffer (0.1 M, pH 7.4) were then added to each of the two incubation samples, giving final concentrations of 1 mM of DPN, 1 mM of TPN, and 3 mM of glucose-6-phosphate. [3α-3H]-14α-Hydroxy-methyl-5α-cholest-7-en-3β-ol (3.43 x 10^6 cpm, 11 μg) in propylene glycol (0.1 ml) was added last. Aerobic incubation was carried out for 3 hours at 37°C with constant shaking.

At the end of the incubation period, both samples were saponified for 3 hours, using 30 ml of freshly-prepared 15% ethanolic KOH per sample. After cooling to room temperature, the solutions were each extracted with three 50 ml portions of petroleum ether and two 40 ml portions of diethyl ether. The petroleum ether and the diethyl ether
extracts were pooled separately, each washed with three 150 ml portions of water, and dried over anhydrous sodium sulfate. Appropriate aliquots were then removed from each solutions for determination of radioactivity. Percent recovery of radioactivity of the various solutions is given in Table 2.

Table 2

Percent Recovery of Various Solutions from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol with Rat Liver Homogenate in the Presence of Cofactors

<table>
<thead>
<tr>
<th>Incubation Sample</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation I - Control</td>
<td></td>
</tr>
<tr>
<td>Aqueous phase</td>
<td>9.8%</td>
</tr>
<tr>
<td>Petroleum ether extract</td>
<td>90.8%</td>
</tr>
<tr>
<td>Diethyl ether extract</td>
<td>0.8%</td>
</tr>
<tr>
<td>Total recovery</td>
<td>101.4%</td>
</tr>
<tr>
<td>Incubation II - 10^{-4} M AY-9944</td>
<td></td>
</tr>
<tr>
<td>Aqueous phase</td>
<td>35.1%</td>
</tr>
<tr>
<td>Petroleum ether extract</td>
<td>58.6%</td>
</tr>
<tr>
<td>Diethyl ether extract</td>
<td>2.7%</td>
</tr>
<tr>
<td>Total recovery</td>
<td>96.4%</td>
</tr>
</tbody>
</table>

Analysis of Incubation I - Control

The recovered nonsaponifiable radioactive material (1.70 x 10^6 cpm) from petroleum ether extract of Incubation I was chromatographed as free sterols on a column (1 x 100 cm) of silicic acid-Super Cel, along with 7 mg of unlabeled cholesterol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 90), fractions 3.2 ml in volume (30 minutes per fraction; 0.107 ml/min) were collected. The contents of each tube were evaporated to dryness under nitrogen and the resulting residues were redissolved in 2 ml of benzene. Aliquots were taken for radioactivity
determination (0.05 ml) and for colorimetric assay (0.2 ml). The results of this column chromatography, shown in Figure 13, indicated that most (94%) of the recovered radioactivity corresponded chromatographically to the mobility of cholesterol (fractions 25-35).

The contents of fractions 25-35 from previous column were pooled and acetylated with dry pyridine-acetic anhydride (2 ml; 1:1, v/v) as described in Methods. The resulting labeled steryl acetates (1.15 x 10^6 cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8^14^-cholestadiene (4 mg), 3β-acetoxy-Δ^5^7^-cholestadiene (5 mg), and cholesteryl acetate (7 mg). Using a mixture of hexane and benzene (7:3, v/v) as the eluting solvent, fractions 4.2 ml in volume (20 minutes per fraction; 0.21 ml/min) were collected. The resulting chromatogram is shown in Figure 14. Essentially all (98%) of the eluted radioactivity corresponded chromatographically to the mobility of authentic cholesteryl acetate (fractions 18-30).

Fractions 18-30 were pooled and applied (9.30 x 10^5 cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8(14)^-cholestene (4 mg), 3β-acetoxy-Δ^7^-cholestene (5 mg), and cholesteryl acetate (5 mg). Using hexane-benzene (9:1, v/v) as the eluting solvent, fractions 3.2 ml in volume (20 minutes per fraction; 0.16 ml/min) were collected. The results, shown in Figure 15, indicated that most (94%) of the recovered radioactivity corresponded in chromatographic mobility to that of cholesteryl acetate (peak centered at fraction 105). A small portion (6%) of the eluted radioactivity showed the chromatographic mobility of 3β-acetoxy-Δ^7^-cholestene (peak at fraction 63).
Figure 13. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with rat liver homogenate preparations in the presence of DPN, TPN, and glucose-6-phosphate. o-o, Tritium radioactivity. Δ-Δ, Cholesterol measured colorimetrically.
Figure 14. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with rat liver homogenate preparations in the presence of DPN, TPN, and glucose-6-phosphate. o-o, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ8,14-cholestadiene, and the third is due to 3β-acetoxy-Δ5,7-cholestadiene.
Figure 15. Alumina-Super Cel-silver nitrate column chromatogram of the acetate derivatives of monounsaturated sterols recovered from aerobic incubation of \([3\alpha^3H]-14\alpha\text{-hydroxymethyl}-5\alpha\text{-cholest-7-en-3\beta\text{-ol}}\) with rat liver homogenate preparations in the presence of DPN, TPN, and glucose-6-phosphate. ☐-☐, Tritium radioactivity. ▲-▲, Steryl acetate measured colorimetrically. The first peak is due to \(3\beta\text{-acetoxy-\(\Delta^6\)(14)\text{-cholestene}}\), the second is due to \(3\beta\text{-acetoxy-\(\Delta^7\)-cholestene}\), and the third is due to cholesteryl acetate.
The contents of fractions 100-140 were pooled, hydrolyzed to free sterol by refluxing in 10 ml of 15% ethanolic KOH, and further characterized via the dibromide purification procedure as described in Methods. The results, given in Table 3, showed that the specific activities of the cholesterol before and after dibromide purification were essentially the same.

Table 3

Dibromide Purification Results of [3α-3H]-Cholesterol Recovered from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol with Rat Liver Homogenate in the Presence of Cofactors

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before dibromide purification</td>
<td>4,840</td>
</tr>
<tr>
<td>After dibromide purification</td>
<td>4,800</td>
</tr>
</tbody>
</table>

Analysis of Incubation II – 10⁻⁴ M AY-9944

The recovered nonsaponifiable radioactive material (2.01 x 10⁶ cpm) from both the petroleum ether and diethyl ether extracts of Incubation II was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 9 mg of unlabeled cholesterol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent, fractions 2.9 ml in volume (20 minutes per fraction; 0.145 ml/min) were collected. The resulting chromatogram is shown in Figure 16. None of the recovered radioactivity corresponded unambiguously in chromatographic mobility to that of cholesterol. A major portion (43%) of the applied radioactivity was eluted right before cholesterol (fractions 19-28). Another 32% of the recovered radioactivity resided in fractions 165-210
Figure 16. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with rat liver homogenate preparations in the presence of DPN, TPN, glucose-6-phosphate, and AY-9944. o-o, Tritium radioactivity. Δ-Δ, Cholesterol measured colorimetrically.
and was shown by thin-layer radiochromatographic analysis to be a compound more polar than the labeled substrate (Figure 17).

The contents of fractions 19-28 were pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (5.32 x 10^5 cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ7,14-cholestadiene (4 mg), 3β-acetoxy-Δ8,14-cholestadiene (5 mg), 3β-acetoxy-Δ5,7-cholestadiene (6 mg), and cholesteryl acetate (8 mg). The column was eluted with hexane-benzene (7:3, v/v) and fractions 5.6 ml in volume (20 minutes per fraction; 0.28 ml/min) were collected. The results (Figure 18) showed that most (95%) of the radioactivity was eluted after cholesteryl acetate but prior to 3β-acetoxy-Δ7,14-cholestadiene (fractions 38-55).

Fractions 38-55 were pooled and applied (2.40 x 10^5 cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ8(14)-cholestene (4 mg), 3β-acetoxy-Δ7-cholestene (6 mg), and cholesteryl acetate (9 mg). Using a mixture of hexane and benzene (9:1, v/v) as the eluting solvent, fractions 3.6 ml in volume (20 minutes per fraction; 0.18 ml/min) were collected. The results of this column chromatography are shown in Figure 19. None of the applied radioactivity was recovered, despite increasing the polarity of the eluting solvent to hexane-benzene (5:5, v/v).

Experiment 2

The livers (36.8 g) of five female rats (Sprague-Dawley strain; 150-200 g each) were homogenized in potassium phosphate buffer (92 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction of the rat liver
Figure 17. Thin-layer radiochromatographic analysis of the contents of fractions 165–210 from the silicic acid-Super Cel column shown in Figure 16. The Silica Gel G thin-layer plate was developed thrice in chloroform-acetone (95:5, v/v). Authentic 14α-hydroxymethyl-5α-cholest-7-en-3β-ol and cholesterol are shown at 6.5 cm and 11.5 cm, respectively.
Figure 18. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with rat liver homogenate preparations in the presence of DPN, TPN, glucose-6-phosphate, and AY-9944. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ7,14-cholestadiene, the third is due to 3β-acetoxy-Δ5,14-cholestadiene, and the fourth is due to 3β-acetoxy-Δ5,14-cholestadiene.
Figure 19. Alumina-Super Cel-silver nitrate column chromatogram of the acetate derivatives of monounsaturated sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with rat liver homogenate preparations in the presence of DPN, TPN, glucose-6-phosphate, and AY-9944. O-O, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to 3β-acetoxy-Δ5(14)-cholestenone, the second is due to 3β-acetoxy-Δ4-cholestenone, and the third is due to cholesteryl acetate.
homogenate was isolated by differential centrifugation.

A solution of AY-9944 (2.0 x 10^{-4} M) was prepared by dissolving 9.3 mg of the inhibitor in 100 ml of potassium phosphate buffer (0.1 M, pH 7.4).

To one Erlenmeyer flask (125 ml) containing 14 ml of the supernatant prepared as described above, a solution of AY-9944 (15 ml; to give a final concentration of 10^{-4} M) was added (Incubation I). To another Erlenmeyer flask (125 ml) containing 14 ml of the same rat liver homogenate preparations, a solution of potassium phosphate buffer (15 ml; 0.1 M, pH 7.4) was added (Incubation II). Cofactors in 1 ml of potassium phosphate buffer (0.1 M, pH 7.4) were then added to each of the two incubation samples, giving final concentrations of 1 mM of DPN, 1 mM of TPN, and 3 mM of glucose-6-phosphate. [3α-^3H]-14α-Hydroxy-methyl-5α-cholest-7-en-3β-ol (3.43 x 10^6 cpm, 11 μg) in propylene glycol (0.1 ml) was added last. Aerobic incubation was carried out for 3 hours at 37°C with constant shaking.

At the end of the incubation period, both samples were saponified for 3 hours, using 30 ml of freshly-prepared 15% ethanolic KOH per sample. After cooling to room temperature, the solutions were each extracted with three 50 ml portions of petroleum ether, the combined extracts were washed with three 150 ml portions of water, and dried over anhydrous sodium sulfate. Appropriate aliquots were then removed from each phases for determination of radioactivity. Percent recovery of radioactivity of the various phases is given in Table 4.

**Analysis of Incubation II - Control**

The recovered nonsaponifiable radioactive material (8.15 x 10^5 cpm) from a portion of the petroleum ether extract of Incubation II
Table 4

Percent Recovery of Various Solutions from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholestan-7-en-3β-ol with Rat Liver Homogenate in the Presence of Cofactors

<table>
<thead>
<tr>
<th>Incubation Sample</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation I - 10⁻⁴ M AY-9944</td>
<td></td>
</tr>
<tr>
<td>Aqueous phase</td>
<td>34.4%</td>
</tr>
<tr>
<td>Petroleum ether extract</td>
<td>65.7%</td>
</tr>
<tr>
<td>Total recovery</td>
<td>100.1%</td>
</tr>
<tr>
<td>Incubation II - Control</td>
<td></td>
</tr>
<tr>
<td>Aqueous phase</td>
<td>11.6%</td>
</tr>
<tr>
<td>Petroleum ether extract</td>
<td>88.3%</td>
</tr>
<tr>
<td>Total recovery</td>
<td>99.9%</td>
</tr>
</tbody>
</table>

was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 7 mg of unlabeled cholesterol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent, fractions 1.4 ml in volume (20 minutes per fraction; 0.07 ml/min) were collected. The resulting chromatogram is shown in Figure 20. Essentially all (98%) of the recovered radioactivity corresponded chromatographically to the mobility of cholesterol (fractions 60-82).

Fractions 60-82 from previous column were pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (5.03 x 10⁵ cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled 3β-acetoxyl-Δ⁷,Δ¹⁴-cholestadiene (4 mg), 3β-acetoxyl-Δ⁵,Δ⁷-cholestadiene (6 mg), and cholesteryl acetate (5 mg). Using hexane-benzene (7:3, v/v) as the eluting solvent, fractions 4.8 ml in volume (20 minutes per fraction; 0.24 ml/min) were collected. The results, shown in Figure 21, indicated that all (100%) of the eluted radioac-
Figure 20. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from second aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with rat liver homogenate preparations in the presence of DPN, TPN, and glucose-6-phosphate. o-o, Tritium radioactivity. Δ-Δ, Cholesterol measured colorimetrically.
Figure 21. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from the second aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesterol-7-en-3β-ol with rat liver homogenate preparations in the presence of DPN, TPN, and glucose-6-phosphate. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ5,14-cholestanol, and the third is due to 3β-acetoxy-Δ5,7-cholestanol.
tivity was associated chromatographically with authentic cholesteryl acetate (fractions 21-40).

The contents of fractions 21-40 were pooled and applied (4.10 x 10^5 cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8(14)-cholestene (4 mg), 3β-acetoxy-Δ^7-cholestene (5 mg), and cholesteryl acetate (5 mg). Using hexane-benzene (9:1, v/v) as the eluting solvent, fractions 3.6 ml in volume (30 minutes per fraction; 0.12 ml/min) were collected. The resulting chromatogram (Figure 22) showed that approximately 93% of the recovered radioactivity corresponded chromatographically to the mobility of cholesteryl acetate (peak at fraction 75). Another 7% cochromatographed with authentic 3β-acetoxy-Δ^7-cholestene (peak at fraction 45).

The contents of fractions 66-99, corresponding to cholesteryl acetate in the previous column, were pooled and hydrolyzed to free sterol by refluxing in 10 ml of 15% ethanolic KOH for 3 hours. The resulting sterol was then purified via formation of the dibromide and subsequent regeneration of cholesterol. The results, shown in Table 5, showed the specific activities of cholesterol before and after dibromide treatment remained essentially unchanged.

**Analysis of Incubation I - 10^{-4} M AY-9944**

The recovered nonsaponifiable radioactive material (1.71 x 10^6 cpm) was chromatographed as free sterols on a column (1 x 100 cm) of silicic acid-Super Cel, along with 10 mg of unlabeled cholesterol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent, fractions 2.2 ml in volume (20 minutes per fraction; 0.11 ml/min) were collected. The results of this column chromatography are given
Figure 22. Alumina-Super Cel-silver nitrate column chromatogram of the acetate derivatives of monounsaturated sterols recovered from the second aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesta-7-en-3β-ol with rat liver homogenate preparations in the presence of DPN, TPN, and glucose-6-phosphate. ○○○, Tritium radioactivity. △△△, Steryl acetate measured colorimetrically. The first peak is due to 3β-acetoxy-Δ5(14)-cholestene, the second is due to 3β-acetoxy-Δ7-cholestene, and the third is due to cholesteryl acetate.
Table 5

Dibromide Purification Results of [3α-3H]-Cholesterol Recovered from Second Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol with Rat Liver Homogenate in the Presence of Cofactors

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before dibromide purification</td>
<td>2,440</td>
</tr>
<tr>
<td>After dibromide purification</td>
<td>2,380</td>
</tr>
</tbody>
</table>

in Figure 23. None of the recovered radioactivity corresponded in chromatographic mobility to that of cholesterol. A significant portion (28%) of the applied radioactivity was eluted prior to cholesterol (fractions 26-35).

The contents of fractions 26-35 were pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (1.70 x 10^5 cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8,14'-cholestadiene (5 mg), 3β-acetoxy-Δ^5,7'-cholestadiene (6 mg), and cholesteryl acetate (8 mg). The steryl acetates were eluted with hexane-benzene (7:3, v/v) and fractions 4.6 ml in volume (20 minutes per fraction; 0.23 ml/min) were collected. The resulting chromatogram is shown in Figure 24. The major peak (78%) was eluted between cholesteryl acetate and 3β-acetoxy-Δ^8,14'-cholestadiene (fractions 34-47). A significant amount (11%) of the recovered radioactivity corresponded chromatographically to the mobility of 3β-acetoxy-Δ^5,7'-cholestadiene (peak centered at fraction 192).

Fractions 34-47 were pooled and stored in freezer for further
Figure 23. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from the second aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with rat liver homogenate preparations in the presence of DPN, TPN, glucose-6-phosphate, and AY-9944. o-o, Tritium radioactivity. Δ-Δ, Cholesterol measured colorimetrically.
Figure 24. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from the second aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesterol-7-en-3β-ol with rat liver homogenate preparations in the presence of DPN, TPN, glucose-6-phosphate, and AY-9944. o-o, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ5,7-cholestadiene, and the third is due to 3β-acetoxy-Δ5,7-cholestadiene.
study. This material was labeled as Unknown A.

**Experiment 3**

The livers (38 g) of five female rats (Sprague-Dawley strain; 150–200 g each) were homogenized in ice-cold potassium phosphate buffer (95 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction of the rat liver homogenate was isolated by differential centrifugation.

A solution of AY-9944 (2.0 x 10^{-4} M) was prepared by dissolving 9.3 mg of the inhibitor in potassium phosphate buffer (100 ml; 0.1 M, pH 7.4).

To two Erlenmeyer flasks (125 ml) each containing 14 ml of the homogenate prepared as described above, a solution of AY-9944 (15 ml; to give final concentration of 10^{-4} M) was added to each of the two flasks. This was followed by the addition of cofactors dissolved in potassium phosphate buffer (1 ml; 0.1 M, pH 7.4) to give final concentrations of 1 mM of DPN, 1 mM of TPN, and 3 mM of glucose-6-phosphate. [3α-^3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol (3.43 x 10^6 cpm, 11 μg) in propylene glycol (0.1 ml) was added last. Aerobic incubation was carried out for 3 hours at 37°C with constant shaking.

At the end of the incubation period, both samples were saponified for 3 hours, using 30 ml of 15% ethanolic KOH per sample. After cooling to room temperature, the solutions were each extracted with three 100 ml portions of petroleum ether and two 75 ml portions of diethyl ether. The petroleum ether and the diethyl ether extracts were pooled separately, washed three times with 300 ml (for petroleum ether extract) or 150 ml (for diethyl ether extract) portions of water, and dried over anhydrous sodium sulfate. Appropriate aliquots were then removed from each solutions for assay of radioactivity. Percent re-
covery of radioactivity of the various solutions is given in Table 6.

Table 6

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous phase</td>
<td>29.7%</td>
</tr>
<tr>
<td>Petroleum ether extract</td>
<td>63.8%</td>
</tr>
<tr>
<td>Diethyl ether extract</td>
<td>6.6%</td>
</tr>
<tr>
<td>Total recovery</td>
<td>100.1%</td>
</tr>
<tr>
<td>Aqueous Phase</td>
<td>39.9%</td>
</tr>
<tr>
<td>Petroleum ether extract</td>
<td>52.8%</td>
</tr>
<tr>
<td>Diethyl ether extract</td>
<td>4.3%</td>
</tr>
<tr>
<td>Total recovery</td>
<td>97.0%</td>
</tr>
</tbody>
</table>

Analysis of Incubation I

The recovered non-saponifiable radioactive material (1.94 x 10^6 cpm) from petroleum ether extract of Incubation I was chromatographed as free sterols on a column (1 x 100 cm) of silicic acid–Super Cel, along with 9 mg of unlabeled cholesterol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent (changed to benzene–ether (8:2, v/v) at fraction 336), fractions 1.4 ml in volume (20 minutes per fraction; 0.07 ml/min) were collected. Two major radioactive peaks were observed in the resulting column chromatogram (Figure 25). Neither of these corresponded chromatographically to the mobility of cholesterol. Approximately 30% of the radioactivity was eluted before cholesterol (peak at fraction 50), and another 21% of the radioactivity was eluted right after cholesterol (fractions 58-74).

The contents of fractions 48-55 were pooled and acetylated with
Figure 25. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from the third aerobic incubation of \([3\alpha^3\text{H}]-14\alpha\text{-hydroxymethyl}-5\alpha\text{-cholesten-7-en-3\beta-ol}\) with rat liver homogenate preparations in the presence of DPN, TPN, glucose-6-phosphate, and AY-9944. ○○, Tritium radioactivity. △△△, Cholesterol measured colorimetrically.
pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. Very little of the acetylation product cochromatographed with authentic cholesteryl acetate upon thin-layer radiochromatographic analysis (Figure 26; solvent system, chloroform). A significant portion of the radioactivity was still associated with cholesterol standard. The reaction was judged to be incomplete and the acetylation was repeated under the same conditions described previously. The result of this second acetylation is shown in Figure 27. The amount of radioactivity which cochromatographed with cholesteryl acetate was greater after reacetylation. However, the major regions of radioactivity still did not correspond to either cholesterol or cholesteryl acetate.

**Analysis of Incubation II**

The recovered nonsaponifiable radioactive material (2.02 x 10⁶ cpm) from petroleum ether extract of Incubation II was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel. No unlabeled samples were added so as to obtain the unknown compounds free from any mass contaminants. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 260), fractions 2.5 ml in volume (30 minutes per fraction; 0.083 ml/min) were collected. The elution profile is shown in Figure 28.

The contents of fractions 21-31 from previous column were pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. Thin-layer radiochromatographic analysis of the acetylation product (solvent system: chloroform), shown in Figure 29, indicated several regions of radioactivity. One possible explanation for the bizarre results obtained in this and previous experiment is the instability of Unknown A, leading to facile decomposition or rearrangement under the conditions employed. To test this hypothesis,
Figure 26. Thin-layer radiochromatographic analysis of the first acetylation results. The plate was developed in chloroform. Authentic cholesterol and cholesteryl acetate are shown at 4.5 cm and 12 cm, respectively.
Figure 27. Thin-layer radiochromatographic analysis of the second acetylation products on a plate of Silica Gel G, developed in chloroform. Authentic cholesterol and cholesteryl acetate are shown at 4.5 cm and 12 cm, respectively.
Figure 28. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from the third aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesten-7-en-3β-ol with rat liver homogenate preparations in the presence of DPN, TPN, glucose-6-phosphate, and AY-9944. o-o, Tritium radioactivity. No unlabeled samples were applied to this column.
Figure 29. Thin-layer radiochromatographic analysis of the acetylation products of fractions 21-31 from silicic acid-Super Cel column shown in Figure 28. The Silica Gel G plate was developed in chloroform. Authentic cholesterol and cholesteryl acetate are shown at 4.5 cm and 12 cm, respectively.
a portion of the contents of fractions 21-31 (before acetylation) was applied to a Silica Gel G thin-layer plate and the plate was developed in ethyl acetate-benzene (5:2, v/v). The resulting chromatogram (Figure 30) showed more than one compound, thus supporting the proposed hypothesis. Subsequent studies indicated that the multiple compounds resulted from autoxidation (exposing it to air) and/or heat (a temperature of over 30°C).

Experiment 4

The livers (35 g) of five female rats (Sprague-Dawley strain; 150-200 g each) were homogenized in potassium phosphate buffer (88 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction of the rat liver homogenate was isolated by differential centrifugation.

A solution of AY-9944 (2.0 x 10^{-4} M) was prepared by dissolving 9.3 mg of the inhibitor in 100 ml of potassium phosphate buffer (0.1 M, pH 7.4).

To an Erlenmeyer flask (125 ml) containing 7 ml of the 10,000 x g supernatant fraction of the rat liver homogenate, a solution of AY-9944 (7.5 ml; to give final concentration of 10^{-4} M) was added. This was followed by the addition of cofactors in potassium phosphate buffer (0.5 ml; 0.1 M, pH 7.4) to give final concentrations of 1 mM of DPN, 1 mM of TPN, and 3 mM of glucose-6-phosphate. [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol (1.72 x 10^6 cpm; 5.5 μg) in propylene glycol (0.05 ml) was added last. Aerobic incubation was carried out for 3 hours at 37°C with constant shaking.

At the end of the incubation period, the sample was saponified for 3 hours, using an equal volume of 15% ethanolic KOH (15 ml). After cooling to room temperature, the solution was extracted with
Figure 30. Thin-layer radiochromatographic analysis of Unknown A recovered from fractions 21-31 of the silicic acid-Super Cel column shown in Figure 28. The Silica Gel G plate was developed in ethyl acetate-benzene (5:2, v/v). Authentic 14α-hydroxymethyl-5α-cholesterol-7-ene-3β-ol and cholesterol are shown at 6.5 cm and 12 cm, respectively.
three 90 ml portions of petroleum ether and two 70 ml portions of diethyl ether. The petroleum ether and the diethyl ether extracts were pooled separately, washed three times with 300 ml (for petroleum ether extract) or 150 ml (for diethyl ether extract) portions of water, and dried over anhydrous sodium sulfate. Appropriate aliquots were then removed from each solutions for assay of radioactivity. Percent recovery of radioactivity of the various solutions is given in Table 7.

Table 7

<table>
<thead>
<tr>
<th>Incubation Sample</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous phase</td>
<td>26.4%</td>
</tr>
<tr>
<td>Petroleum ether extract</td>
<td>68.4%</td>
</tr>
<tr>
<td>Diethyl ether extract</td>
<td>5.9%</td>
</tr>
<tr>
<td>Total recovery</td>
<td>100.7%</td>
</tr>
</tbody>
</table>

The recovered nonsaponifiable radioactive material (5.42 x 10^5 cpm) from petroleum ether extract was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 7 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 184), fractions 1.3 ml in volume (16 minutes per fractions, changed to 32 minutes per fraction at fraction 132; 0.08 ml/min) were collected. The resulting chromatogram is shown in Figure 31. None of the three major labeled components recovered corresponded chromatographically to the mobility of cholesterol. Approximately 35% of the radioactivity was
Figure 31. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from the fourth aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholestan-7-en-3β-ol with rat liver homogenate preparations in the presence of DPN, TPN, glucose-6-phosphate, and AY-9944. o-o, Tritium radioactivity. △-△, Cholesterol measured colorimetrically.
eluted prior to cholesterol (fractions 50-55) and another 22% was eluted right after cholesterol (fractions 60-75).

Experiment 5

The livers (54.7 g) of seven female rats (Sprague-Dawley strain; 150-200 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (136.6 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction of the rat liver homogenate was isolated by differential centrifugation.

A solution of AY-9944 (2.0 x 10^{-4} M) was prepared by dissolving 9.3 mg of the inhibitor in 100 ml of potassium phosphate buffer (0.1 M, pH 7.4).

To two Erlenmeyer flasks (125 ml) each containing 14 ml of the 10,000 x g supernatant fraction of the rat liver homogenate, a solution of AY-9944 (15 ml; giving final concentration of 10^{-4} M) was added to each of the two flasks. To one flask (Incubation I), cofactors in potassium phosphate buffer (1 ml; 0.1 M, pH 7.4) were added to give final concentrations of 1 mM of DPN, 1 mM of TPN, and 3 mM of glucose-6-phosphate. To the other flask (Incubation II), cofactors in potassium phosphate buffer (1 ml; 0.1 M, pH 7.4) were added to give final concentrations of 2 mM of DPN, 2 mM of TPN, and 6 mM of glucose-6-phosphate. [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol (3.43 x 10^6 cpm, 11 μg) in propylene glycol (0.1 ml) was added last. Aerobic incubation was carried out for 3 hours at 37°C with constant shaking.

At the end of the incubation period, both samples were saponified for 3 hours, using 30 ml of 15% ethanolic KOH per sample. After cooling to room temperature, the solutions were each extracted with four 80 ml portions of petroleum ether, the combined extracts were washed
with three 300 ml portions of water, and dried over anhydrous sodium sulfate. Appropriate aliquots were then removed from the various phases for determination of radioactivity. Percent recovery of radioactivity of the various solutions is given in Table 8.

Table 8

<table>
<thead>
<tr>
<th>Incubation Sample</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Incubation I</strong></td>
<td></td>
</tr>
<tr>
<td>Aqueous phase</td>
<td>30.8%</td>
</tr>
<tr>
<td>Petroleum ether extract</td>
<td>65.8%</td>
</tr>
<tr>
<td>Total recovery</td>
<td>96.6%</td>
</tr>
<tr>
<td><strong>Incubation II</strong></td>
<td></td>
</tr>
<tr>
<td>Aqueous phase</td>
<td>30.4%</td>
</tr>
<tr>
<td>Petroleum ether extract</td>
<td>67.8%</td>
</tr>
<tr>
<td>Total recovery</td>
<td>98.2%</td>
</tr>
</tbody>
</table>

Analysis of Incubation I

The recovered nonsaponifiable radioactive material \((1.60 \times 10^6\) cpm) was chromatographed as free sterols on a column (1 x 100 cm) of silicic acid-Super Cel, along with 7 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 115), fractions 1.8 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 115; 0.09 ml/min) were collected. The results are shown in Figure 32. None of the five labeled peaks recovered corresponded in chromatographic mobility to that of cholesterol. Approximately 24% of the radioactivity was eluted prior to cholesterol (peak at fraction
Figure 32. Silicic acid–Super Cel column chromatographic analysis of sterols recovered from the fifth aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with rat liver homogenate preparations in the presence of DPN, TPN, glucose-6-phosphate, and AY-9944. o-o, Tritium radioactivity. Δ-Δ, Cholesterol measured colorimetrically.
40), and another 16% was eluted right after cholesterol (peak at fraction 52). A major portion (45%) of the recovered radioactivity was associated with an unknown polar compound (peak at fraction 188).

**Analysis of Incubation II**

The recovered nonsaponifiable radioactive material (1.70 x 10^6 cpm) from the petroleum ether extract of Incubation II was chromatographed on a column (1 x 100 cm) of silicic acid–Super Cel, along with 8 mg of unlabeled cholesterol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent (changed to benzene–ether (8:2, v/v) at fraction 119), fractions 1.3 ml in volume (16 minutes per fraction, changed to 32 minutes per fraction at fraction 97; 0.08 ml/min) were collected. The resulting chromatogram (Figure 33) showed five radioactive peaks, none of which corresponded chromatographically to the mobility of cholesterol. Approximately 36% of the radioactivity was eluted prior to cholesterol (fractions 52-57), and another 11% was eluted immediately after cholesterol (fractions 60-80). A significant portion (28%) of the recovered radioactivity resided in a highly polar region (fractions 180-210).

**B. IN THE ABSENCE OF ADDED COFACTORS**

**Experiment 1**

The livers (35 g) of five female rats (Sprague-Dawley strain; 150-200 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (88 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction of the rat liver homogenate was isolated by differential centrifugation.

A solution of AY-9944 (2.0 x 10^{-4} M) was prepared by dissolving
Figure 33. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from the fifth aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-choleste-7-en-3β-ol with rat liver homogenate preparations in the presence of DPN, TPN, glucose-6-phosphate, and AY-9944. o-o, Tritium radioactivity. Δ-Δ, Cholesterol measured colorimetrically.
9.3 mg of the inhibitor in 100 ml of potassium phosphate buffer (0.1 M, pH 7.4).

To one Erlemeyer flask (125 ml; Incubation I) containing 14 ml of the rat liver homogenate preparations prepared as described above, a solution of potassium phosphate buffer (15 ml; 0.1 M, pH 7.4) was added. This was followed by the addition of cofactors in potassium phosphate buffer (1 ml; 0.1 M, pH 7.4) to give final concentrations of 1 mM of DPN, 1 mM of TPN, and 3 mM of glucose-6-phosphate. To two other flasks (125 ml; Incubations II and III) each containing 15 ml of the 10,000 x g supernatant fraction of the rat liver homogenate, a solution of AY-9944 (15 ml; giving final concentration of 10\(^{-4}\) M) was added. No cofactors were added to these two flasks. \([3\alpha-^3\text{H}]\)-14\(\alpha\)-Hydroxymethyl-5\(\alpha\)-cholest-7-en-3\(\beta\)-ol (3.43 x 10\(^6\) cpm, 11 \(\mu\)g) in propylene glycol (0.1 ml) was added last to each of the three flasks above. Aerobic incubation was carried out for 3 hours at 37\(^\circ\) with constant shaking.

At the end of the incubation period, all three samples were saponified for 3 hours, using 30 ml of 15% ethanolic KOH per sample. After cooling to room temperature, the solutions were each extracted with three 90 ml portions of petroleum ether and two 70 ml portions of diethyl ether. The petroleum ether and the diethyl ether extracts were pooled separately, washed three times with 300 ml (for petroleum ether extract) or 150 ml (for diethyl ether extract) portions of water, and dried over anhydrous sodium sulfate. Appropriate aliquots were then removed from each solution for assay of radioactivity. Percent recovery of radioactivity of the various solutions is given in Table 9.

**Analysis of Incubation I - Control**

The recovered nonsaponifiable radioactive material (1.42 x 10\(^6\) cpm) was analyzed...
Table 9

Percent Recovery of Various Solutions from Aerobic Incubation of [3α-H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol with Rat Liver Homogenate in the Absence of Cofactors

<table>
<thead>
<tr>
<th>Incubation Sample</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Incubation I - Control</strong></td>
<td></td>
</tr>
<tr>
<td>Aqueous phase</td>
<td>13.1%</td>
</tr>
<tr>
<td>Petroleum ether extract</td>
<td>85.3%</td>
</tr>
<tr>
<td>Diethyl ether extract</td>
<td>1.1%</td>
</tr>
<tr>
<td>Total recovery</td>
<td>99.5%</td>
</tr>
<tr>
<td><strong>Incubation II - 10^{-4} M AY-9944</strong></td>
<td></td>
</tr>
<tr>
<td>Aqueous phase</td>
<td>10.8%</td>
</tr>
<tr>
<td>Petroleum ether extract</td>
<td>79.2%</td>
</tr>
<tr>
<td>Diethyl ether extract</td>
<td>3.1%</td>
</tr>
<tr>
<td>Total recovery</td>
<td>93.1%</td>
</tr>
<tr>
<td><strong>Incubation III - 10^{-4} M AY-9944</strong></td>
<td></td>
</tr>
<tr>
<td>Aqueous phase</td>
<td>9.0%</td>
</tr>
<tr>
<td>Petroleum ether extract</td>
<td>85.0%</td>
</tr>
<tr>
<td>Diethyl ether extract</td>
<td>5.2%</td>
</tr>
<tr>
<td>Total recovery</td>
<td>99.2%</td>
</tr>
</tbody>
</table>

cpm) from the petroleum ether extract of Incubation I was applied to a column (1 x 100 cm) of silicic acid-Super Cel, along with 7 mg of unlabeled cholesterol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 90), fractions 3.2 ml in volume (30 minutes per fraction; 0.107 ml/min) were collected. The resulting chromatogram is shown in Figure 34. Essentially all (99.4%) of the eluted radioactivity corresponded chromatographically to the mobility of cholesterol (fractions 24-32).

The contents of fractions 24-32 from previous column were pooled and acetylated with acetic anhydride-pyridine (2 ml; 1:1, v/v) as described previously. The resulting labeled steryl acetates (1.17 x 10^6 cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-
Figure 34. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with rat liver homogenate preparations in the presence of DPN, TPN, and glucose-6-phosphate. o-o, Tritium radioactivity. △-△, Cholesterol measured colorimetrically.
Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ8,14-cholestadiene (4 mg), 3β-acetoxy-Δ5,7-cholestadiene (5 mg), and cholesteryl acetate (7 mg). The column was eluted with hexane-benzene (7:3, v/v) and fractions 3.8 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 135; 0.19 ml/min) were collected. The results (Figure 35) showed that most (98%) of the recovered radioactivity cochromatographed with authentic cholesteryl acetate (fractions 19-32).

Fractions 19-32, corresponding to cholesteryl acetate in previous column, were pooled and applied (8.85 x 10^5 cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ8(14)-cholestene (4 mg), 3β-acetoxy-Δ7-cholestene (5 mg), and cholesteryl acetate (7 mg). The column was eluted with hexane-benzene (9:1, v/v) and fractions 3.6 ml in volume (30 minutes per fraction; 0.12 ml/min) were collected. The results of this column chromatography are shown in Figure 36. Most (92%) of the recovered radioactivity corresponded chromatographically to the mobility of cholesteryl acetate (peak centered at fraction 72), and the rest (8%) corresponded to the mobility of 3β-acetoxy-Δ7-cholestene (peak at fraction 45).

The contents of fractions 66-100, corresponding to cholesteryl acetate in the alumina column, were pooled and hydrolyzed to free sterol by refluxing in 10 ml of 15% ethanolic KOH for 3 hours. The resulting sterol was then purified via formation of the dibromide and subsequent regeneration of cholesterol. The results, given in Table 10, indicated that the specific activities of cholesterol before and after dibromide treatment remained essentially unchanged.
Figure 35. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesterol-7-en-3β-ol with rat liver homogenate preparations in the presence of DPN, TPN, and glucose-6-phosphate. o--o, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ8,14-cholestadiene, and the third is due to 3β-acetoxy-Δ5,7-cholestadiene.
Figure 36. Alumina-Super Cel-silver nitrate column chromatogram of the acetate derivatives of monounsaturated sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesterol-7-en-3β-ol with rat liver homogenate preparations in the presence of DPN, TPN, and glucose-6-phosphate. ○○○, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to 3β-acetoxy-Δ5(14)-cholestenone; the second is due to 3β-acetoxy-Δ4-cholestenone, and the third is due to cholesteryl acetate.
Table 10

Dibromide Purification Results of \([3\alpha-^3H]-\)Cholesterol Recovered from Aerobic Incubation of \([3\alpha-^3H]-14\alpha\)-Hydroxymethyl-\(5\alpha\)-cholest-7-\(\alpha\)-3\(\beta\)-ol with Rat Liver Homogenate in the Absence of Added Cofactors

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before dibromide purification</td>
<td>6,200</td>
</tr>
<tr>
<td>After dibromide purification</td>
<td>6,260</td>
</tr>
</tbody>
</table>

Analysis of Incubation II - \(10^{-4}\) M AY-9944

The recovered nonsaponifiable radioactive material (2.82 x 10^6 cpm) from the petroleum ether extract of Incubation II was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 7 mg of unlabeled cholesterol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 184), fractions 1.2 ml in volume (16 minutes per fraction, changed to 32 minutes per fraction at fraction 132; 0.075 ml/min) were collected. The resulting chromatogram (Figure 37) showed the recovery of several radioactive peaks. None of these corresponded in chromatographic mobility to that of cholesterol. Percent recovery of radioactivity of the four major peaks is shown in Table 11.

Analysis of Incubation III - \(10^{-4}\) M AY-9944

The recovered nonsaponifiable radioactive material (1.72 x 10^6 cpm) from the petroleum ether extract of Incubation III was applied to a column (1 x 100 cm) of silicic acid-Super Cel, along with 7 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 184), fractions 1.2 ml in volume (16 minutes per fraction, changed to 32 minutes
Figure 37. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholestan-7-en-3ß-ol with rat liver homogenate preparations in the presence of AY-9944 but with no added cofactors. ○-○, Tritium radioactivity. Δ-Δ, Cholesterol measured colorimetrically.
Table 11
Percent Recovery of Radioactivity of Major Labeled Peaks Eluted from Silicic Acid-Super Cel Column of Incubation II

<table>
<thead>
<tr>
<th>Fractions</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>54-59 (Unknown A)</td>
<td>13.5%</td>
</tr>
<tr>
<td>63-74 (Unknown D)</td>
<td>11.1%</td>
</tr>
<tr>
<td>81-105 (Unknown C)</td>
<td>47.2%</td>
</tr>
<tr>
<td>230-270 (Unknown B)</td>
<td>15.6%</td>
</tr>
</tbody>
</table>

per fraction at fraction 132; 0.075 ml/min) were collected. The resulting chromatogram is shown in Figure 38. A total of five different peaks were recovered. None of these corresponded chromatographically to the mobility of cholesterol. Percent recovery of radioactivity of these five peaks is shown in Table 12.

Table 12
Percent Recovery of Radioactivity of Major Labeled Peaks Eluted from Silicic Acid-Super Cel Column of Incubation III

<table>
<thead>
<tr>
<th>Fractions</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>58-64 (Unknown A)</td>
<td>5.4%</td>
</tr>
<tr>
<td>67-82 (Unknown D)</td>
<td>15.4%</td>
</tr>
<tr>
<td>86-105 (Unknown C)</td>
<td>63.1%</td>
</tr>
<tr>
<td>240-265 (Unknown B)</td>
<td>8.4%</td>
</tr>
<tr>
<td>195-220</td>
<td>6.1%</td>
</tr>
</tbody>
</table>

Experiment 2
The livers (54.7 g) of seven female rats (Sprague-Dawley strain;
Figure 38. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of \([3\alpha-\text{H}]-14\alpha\text{-hydroxymethyl-5a-cholester-7-en-3\beta-ol}\) with rat liver homogenate preparations in the presence of AY-9944 but with no added cofactors. ○○, Tritium radioactivity. ΔΔ, Cholesterol measured colorimetrically.
150-200 g each) were homogenized in ice-cold potassium phosphate buffer (136.6 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction of the rat liver homogenate was isolated by differential centrifugation.

A solution of AY-9944 (2.0 x 10^{-4} M) was prepared by dissolving 9.3 mg of the inhibitor in 100 ml of potassium phosphate buffer (0.1 M, pH 7.4).

To two Erlenmeyer flasks (125 ml) each containing 15 ml of the 10,000 x g supernatant fraction of the rat liver homogenate prepared as described above, a solution of AY-9944 (15 ml; to give final concentration of 10^{-4} M) was added. [3α-3H]-14α-Hydroxymethyl-5α-cholesta-7-en-3β-ol (3.43 x 10^6 cpm, 11 μg) in propylene glycol (0.1 ml) was added last. Aerobic incubation was carried out for 3 hours at 37° with constant shaking.

At the end of the incubation period, both samples were saponified for 3 hours, using 30 ml of 15% ethanolic KOH per sample. After cooling to room temperature, the solutions were each extracted with four 80 ml portions of petroleum ether, the combined extracts were washed with 300 ml portions of water, and dried over anhydrous sodium sulfate. Appropriate aliquots were then removed from both the aqueous and the organic solutions for assay of radioactivity. Percent recovery of radioactivity of the various solutions is given in Table 13.

**Analysis of Incubation I**

The recovered nonsaponifiable radioactive material (2.02 x 10^6 cpm) was chromatographed as free sterols on a column (1 x 100 cm) of silicic acid-Super Cel, along with 8 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to
Table 13

Percent Recovery of Various Solutions from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholesterol-7-en-3α-ol with Rat Liver Homogenate in the Absence of Added Cofactors

<table>
<thead>
<tr>
<th>Incubation Sample</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation I</td>
<td></td>
</tr>
<tr>
<td>Aqueous phase</td>
<td>15.6%</td>
</tr>
<tr>
<td>Petroleum ether extract</td>
<td>83.1%</td>
</tr>
<tr>
<td>Total recovery</td>
<td>98.7%</td>
</tr>
<tr>
<td>Incubation II</td>
<td></td>
</tr>
<tr>
<td>Aqueous phase</td>
<td>13.7%</td>
</tr>
<tr>
<td>Petroleum ether extract</td>
<td>86.5%</td>
</tr>
<tr>
<td>Total recovery</td>
<td>100.2%</td>
</tr>
</tbody>
</table>

benzene-ether (8:2, v/v) at fraction 116), fractions 2.1 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 116; 0.105 ml/min) were collected. The resulting chromatogram, shown in Figure 39, indicated a total of seven different compounds. None of these corresponded in chromatographic mobility to that of cholesterol. Percent recovery of radioactivity of the major peaks is given in Table 14.

Table 14

Percent Recovery of Radioactivity of Major Labeled Peaks Eluted from Silicic Acid-Super Cel Column of Incubation I

<table>
<thead>
<tr>
<th>Fractions</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>44-55 (Unknown D)</td>
<td>19.0%</td>
</tr>
<tr>
<td>57-75 (Unknown C)</td>
<td>40.6%</td>
</tr>
<tr>
<td>130-149 (Unknown B)</td>
<td>13.1%</td>
</tr>
</tbody>
</table>
Figure 39. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from the second aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholester-7-en-3β-ol with rat liver homogenate preparations in the presence of AY-9944 but with no added cofactors. o-o, Tritium radioactivity. Δ-Δ, Cholesterol measured colorimetrically.
Analysis of Incubation II

The recovered nonsaponifiable radioactive material (2.44 x 10^6 cpm) from the petroleum ether extract of Incubation II was applied to a column (1 x 100 cm) of silicic acid-Super Cel, along with 8 mg of unlabeled cholesterol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 116), fractions 1.6 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 116; 0.08 ml/min) were collected. The results are shown in Figure 40. A total of six radioactive peaks were observed. None of these corresponded in chromatographic mobility to that of cholesterol. Percent recovery of radioactivity of the major peaks is given in Table 15.

Table 15

Percent Recovery of Radioactivity of Major Labeled Peaks Eluted from Silicic Acid-Super Cel Column of Incubation II

<table>
<thead>
<tr>
<th>Fractions</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>44-55 (Unknown D)</td>
<td>20.7%</td>
</tr>
<tr>
<td>57-68 (Unknown C)</td>
<td>34.1%</td>
</tr>
<tr>
<td>136-154 (Unknown B)</td>
<td>13.4%</td>
</tr>
</tbody>
</table>
Silicic acid-Super Cel column chromatographic analysis of sterols recovered from the second aerobic incubation of [3α-3H]14α-hydroxymethyl-5α-cholestanol with rat liver homogenate preparations in the presence of Al-99m, but with no added cofactors. 3α, Tritium radioactivity, A-A, Cholesterol, measured colorimetrically.

Figure 40.
PART II. ANALYSIS OF UNKNOWNS

INTRODUCTION AND DISCUSSION

Analysis of unknown compounds obtained in previous incubations proved to be quite a formidable task. Due to the extremely small mass of the substrate employed, classical chemical techniques, such as infrared, ultraviolet, nuclear magnetic resonance, and mass spectrometry, could not be applied. The analysis was further complicated by the extreme instability of two of the unknowns, making it difficult, if not impossible, to study the unknowns per se. Nevertheless, the high specific activity of the substrate allowed the use of less direct methods in elucidating the chemical structures of these unknown compounds. But due to reasons cited above, rather than identifying the unknowns directly from their spectral data, a more laborious procedure of eliminating by trial-and-error of each of the several possible candidates had to be employed.

Only the four major unknown compounds (designated as Unknowns A, B, C, and D) isolated from previous incubations were subjected to further analysis. The results of these analyses are summarized below:

1. Unknown A
   a. Unknown A is not any of the following monoene sterols: cholest-8(14)-en-3β-ol, cholest-14-en-3β-ol, cholest-8(9)-en-3β-ol, cholest-7-en-3β-ol, or cholesterol.
   b. Unknown A is not any of the following diene sterols: cholesta-7,14-dien-3β-ol, cholesta-8,14-dien-3β-ol, cholesta-5,7-dien-3β-ol, or cholesta-7,9(11)-dien-3β-ol.
   c. Unknown A is unstable.

2. Unknown B
a. Unknown B is convertible to cholesterol upon aerobic incubation with 10,000 x g supernatant fraction of the rat liver homogenate.

b. Unknown B is unaffected by LiAlH₄ treatment.

c. Unknown B is not any of the following sterols: cholest-7-en-3β,15α-diol, cholest-7-en-3β,15β-diol, cholest-8(14)-en-3β,15α-diol, or cholest-8(14)-en-3β,7,15-triol.

d. Unknown B corresponded chromatographically to the mobility of cholest-8(14)-en-3β,15β-diol in thin-layer radiochromatographic analysis (Silica Gel G plate, developed twice in solvent system of chloroform-acetone (95:5, v/v)).

3. Unknown C

a. Unknown C is extremely unstable.

b. Decomposition products of Unknown C consist primarily of cholesta-7,14-dien-3β-ol and Unknown A.

c. Unknown C is transformed, by LiAlH₄ treatment, to a compound with the chromatographic mobility of authentic 14β-hydroxy-methyl-5α-cholesta-7-en-3β-ol.

4. Unknown D

a. Unknown D is found to contain labeled cholesta-7,14-dien-3β-ol, cholesta-8,14-dien-3β-ol, and trace amounts of cholesta-5,7-dien-3β-ol.

EXPERIMENTAL

A. ANALYSIS OF UNKNOWN A

Chromatographic Behaviour

Typical chromatographic behaviour of Unknown A on columns of silicic
acid-Super Cel (Figure 41), Silica Gel G-Super Cel-silver nitrate (Figure 42), and alumina-Super Cel-silver nitrate (Figure 43) has been reported previously. These chromatographic studies indicate that Unknown A is not cholesterol, cholest-7-en-3β-ol, cholest-8(9)-en-3β-ol, cholest-8(14)-en-3β-ol, cholesta-7,14-dien-3β-ol, cholesta-8,14-dien-3β-ol, or cholesta-5,7-dien-3β-ol. Based on these results, Unknown A is most probably a monohydroxy sterol with either one or two double bonds.

**Comparison with Authentic Cholesta-7,9(11)-dien-3β-ol**

**Rationale.** Cholesta-7,9(11)-dien-3β-ol is known to behave in a similar fashion as Unknown A when applied (as its acetate derivative) to a column of Silica Gel G-Super Cel-silver nitrate (personal communication from Schroepfer, G. J., Jr.). It has also been reported that [3α-3H]-cholesta-7,9(11)-dien-3β-ol is converted in high yield into cholesterol by a 10,000 x g supernatant fraction of the rat liver homogenate (Rahimtula et al., 1969; Akhtar et al., 1972). This compound would therefore serve as a good starting point in determining the identity of Unknown A.

**Preparation of Cholesta-7,9(11)-dien-3β-ol.** Cholesta-7,9(11)-dien-3β-ol was prepared by the method of Heusser et al. (1952), as outlined in Figure 44. Choleste-7-en-3β-y1 acetate (230 mg) in chloroform (3 ml) was added to a solution of mercuric acetate (450 mg) in acetic acid (6 ml) and the mixture was stirred in the dark at room temperature for 5 hours. The precipitated mercurous acetate was removed by filtration; the filtrate was diluted with water and extracted with ether. The ether extract was washed successively with water, saturated sodium bicarbonate solution, saturated sodium chloride solution, and water.
Figure 41. Chromatographic behaviour of Unknown A (fractions 36–42) on a column (1 x 100 cm) of silicic acid-Super Cel. The column was eluted with a mixture of benzene and ether (9:1, v/v). o-o, Tritium radioactivity. Δ-Δ, Cholesterol measured colorimetrically.
Figure 42. Chromatographic behaviour of the acetate derivative of Unknown A (fractions 37-55) on a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate. The column was eluted with a mixture of hexane and benzene (7:3, v/v). A-A, Tritium radioactivity. D-A, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ\(^7\),\(17\)-cholestadiene, the third is due to 3β-acetoxy-Δ\(^8\),\(17\)-cholestadiene, and the fourth is due to 3β-acetoxy-Δ\(^5\),\(17\)-cholestadiene.
Figure 43. Chromatographic behaviour of the acetate derivative of Unknown A on a column (1 x 100 cm) of alumina-Super Cel-silver nitrate. The column was eluted with hexane-benzene (9:1, v/v). No Unknown A could be recovered from this column under the conditions employed. o-o, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to 3\(\beta\)-acetoxy-\(\Delta(14)\)-cholestanone, the second is due to 3\(\beta\)-acetoxy-\(\Delta'\)-cholestanone, and the third is due to cholesteryl acetate.
Figure 44. Synthetic scheme for cholesta-7,9(11)-dien-3β-ol (Heusser et al., 1952)
The extract was dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure. The resulting residue was applied to a column (1 x 22 cm) of aluminum oxide (Activity II/III; for chromatographic adsorption analysis acc. to Brockmann, EM Laboratories Inc., Cat. 1097) for further purification. Using benzene as the eluting solvent, fractions 11.6 ml in volume (32 minutes per fraction; 0.36 ml/min) were collected. Fraction 1 was found to contain the unreacted cholest-7-en-3β-yl acetate. Fractions 2-10 contained the desired product. The contents of fractions 2-10 were pooled, evaporated to dryness, and the residue recrystallized three times from ether-methanol to yield as needles 76 mg of cholesta-7,9(11)-dien-3β-yl acetate, m.p. 114-115°C (lit. 114-115°C, Heusser et al., 1952).

Cholesta-7,9(11)-dien-3β-yl acetate (II, 54 mg) was then refluxed in 20 ml of 15% ethanolic KOH for 1.5 hours. After cooling to room temperature, the solution was extracted with four 50 ml portions of ether, the combined extracts washed with water until neutral, and dried over anhydrous sodium sulfate. The residue was crystallized from methanol to yield 42 mg of cholesta-7,9(11)-dien-3β-ol, m.p. 112-113°C (lit. 107-110°C, Fieser and Henz, 1953).

Determination of Optimum Developing Time of Cholesta-7,9(11)-dien-3β-ol in Liebermann-Burchard Colorimetric Assay. Cholesta-7,9(11)-dien-3β-ol (10 mg) was dissolved in 100 ml of benzene from which 0.05 ml, 0.1 ml, 0.15 ml, 0.2 ml, 0.3 ml, 0.4 ml, 0.6 ml, 0.8 ml, and 1 ml were taken out and transferred to 10 x 75 mm culture tubes. The samples were evaporated to dryness under nitrogen and Liebermann-Burchard reagent (1.8 ml) was added. Absorbance was read at 620 nm every 30 seconds. The results, time vs. absorbance, for 40 μg of the sterol sample are shown in Figure 45. The optimum developing time was deter-
Figure 45. Determination of optimum developing time of cholesta-7,9(11)-dien-3β-ol in Liebermann-Burchard colorimetric assay. Absorption was read at 620 nm.
mined to be 1 minute. A standard curve, mass vs. absorbance, of cholesta-7,9(11)-dien-3β-ol was also obtained as shown in Figure 46.

Silica Gel G-Super Cel-Silver Nitrate Column Chromatography. Cholesta-7,9(11)-dien-3β-yl acetate (2 mg) was applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with samples of 3β-acetoxy-Δ8,14-cholestadiene (5 mg) and cholesteryl acetate (5 mg). Using hexane-benzene (7:3, v/v) as the eluting solvent, fractions 5.6 ml in volume (20 minutes per fraction; 0.28 ml/min) were collected. The resulting chromatogram is shown in Figure 47. Cholesta-7,9(11)-dien-3β-yl acetate was eluted between cholesta-8,14-dien-3β-yl acetate and cholesteryl acetate, as expected.

Silicic Acid-Super Cel Column Chromatography. Cholesta-7,9(11)-dien-3β-ol (4 mg) was applied to a column (1 x 100 cm) of silicic acid-Super Cel, along with 7 mg of cholesterol. The column was eluted with benzene-ether (9:1, v/v) and fractions 1.8 ml in volume (20 minutes per fraction; 0.09 ml/min) were collected. The results of this column chromatography are shown in Figure 48. Cholesta-7,9(11)-dien-3β-ol was eluted right after cholesterol in contrast to the chromatographic behaviour of Unknown A on the same column (Unknown A was eluted prior to cholesterol).

Comparison with Authentic Cholest-14-en-3β-ol

Rationale. The chromatographic behaviour of cholest-14-en-3β-yl acetate on columns of alumina-Super Cel-silver nitrate, as shown by previous studies (Schroepfer et al., unpublished work), is strikingly similar to that of Unknown A on this same column. Both could not be eluted off the column under the conditions employed in these studies.

Preparation of Cholest-14-en-3β-ol. Cholest-14-en-3β-ol was
Figure 46. Standard curve of cholesta-7,9(11)-dien-3β-ol. Liebermann-Burchard colorimetric assay was used and absorption was read at 620 nm.
Figure 47. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of cholesta-7,9(11)-dien-3β-ol, cholesta-8,14-dien-3β-ol, and cholesterol. The column was eluted with hexane-benzene (7:3, v/v) and fractions 5.6 ml in volume were collected. o-o, 3β-Acetoxy-Δ^7,9(11)-cholestadiene measured colorimetrically. △-△, Other steryl acetates measured colorimetrically. The first peak is due to cholesteryl acetate and the second is due to 3β-acetoxy-Δ^8,14-cholestadiene.
Figure 48. Silicic acid-Super Cel column chromatographic analysis of cholesta-7,9(11)-dien-3β-ol and cholesterol. The column was eluted with benzene-ether (9:1, v/v) and fractions 1.8 ml in volume were collected. o-o, Cholesta-7,9(11)-dien-3β-ol measured colorimetrically. △-△, Cholesterol measured colorimetrically.
synthesized chemically according to the scheme outlined in Figure 49. Cholesta-5,7-dien-3β-ol (I, 1.0 g) in 100 ml of ethyl acetate-acetic acid (95:5) was hydrogenated at 3 atmospheres pressure (40 lbs/in²) at room temperature for 22 hours using platinum oxide (100 mg) as catalyst. At the end of the reduction, the catalyst was removed by filtration and the solvent was evaporated under nitrogen yielding a yellowish solid. Recrystallization from methanol yielded 710 mg of cholest-8(14)-en-3β-ol (II), m.p. 117° (literature, 120°, Cornforth et al., 1957; 119°, Schenck et al., 1936; 119°, Windaus et al., 1938; 117°, Wieland and Gornhardt, 1947; 120-121°, Lee and Schroepfer, 1968). A second crop from recrystallization of mother liquor yielded another 207 mg of II, m.p. 116°.

Cholest-8(14)-en-3β-ol (II, 710 mg) was dissolved in pyridine (16 ml) and benzoyl chloride (1 ml) was added with shaking. It was left standing at room temperature for 22 hours, after which the mixture was stirred with water (50 ml) for 1 hour and the solid was collected, washed with water and methanol, dried, and recrystallized from acetone. This procedure gave 414 mg of cholest-8(14)-en-3β-y1 benzoate (III), melting at 109-110° to a cloudy liquid which cleared at 136° (literature, 111-113°, cleared at 138-140°, Cornforth et al., 1957). A second crop after recrystallization of mother liquor yielded another 193 mg of III, m.p. 105-106°, cleared at 138-140°.

Cholest-8(14)-en-3β-y1 benzoate (III, 414 mg) was dissolved in chloroform (2 ml) and a gentle stream of dry HCl gas was passed through the solution for 2 hours at -30°. A slight vacuum was then applied to the reaction flask to removed the excess HCl gas. The solution was poured gently into 10 ml of 5% sodium bicarbonate solution and extract-
Figure 49. Scheme for the synthesis of cholest-14-en-3β-ol and its acetate derivative.
ed with two 25 ml portions of ether. The ether layer was washed with three 30 ml portions of water, dried over anhydrous sodium sulfate, and concentrated to dryness under reduced pressure. The compound recrystallized from acetone yielded 105 mg of cholest-14-en-3β-yl benzoate (IV), m.p. 164-165° (literature, 167°, Cornforth et al., 1957). A second crop after recrystallization of mother liquor yielded another 88 mg of IV, m.p. 161-163°.

Cholest-14-en-3β-yl benzoate (IV, 105 mg) was heated under reflux with a 15% ethanolic KOH solution (20 ml) for 2 hours. After the mixture cooled to room temperature, it was extracted with three 30 ml portions of ether, the combined extracts were washed with water, dried over anhydrous sodium sulfate, and the solvent removed under reduced pressure. Recrystallization from methanol yielded 37 mg of cholest-14-en-3β-ol (V), m.p. 128-129° (literature, 131°, Schenck et al., 1936).

**Silicic Acid-Super Cel Column Chromatography.** Cholest-14-en-3β-ol (5 mg) was applied to a column (1 x 100 cm) of silicic acid-Super Cel, along with 6 mg of unlabeled cholesterol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent, fractions 1.6 ml in volume (20 minutes per fraction; 0.08 ml/min) were collected. The resulting chromatogram is shown in Figure 50. Unknown A could not be cholest-14-en-3β-ol since the latter compound was eluted after cholesterol rather than before.

**B. ANALYSIS OF UNKNOWN B**

**Chromatographic Behaviour**

Typical chromatographic behaviour of Unknown B on a column of silicic acid-Super Cel is shown in Figure 51. Its high polarity reces-
sitated the use of a more polar eluting solvent (benzene-ether (8:2,
Figure 50. Silicic acid-Super Cel column chromatographic analysis of cholest-14-en-3β-ol and cholesterol. The column was eluted with benzene-ether (9:1, v/v) and fractions 1.6 ml in volume were collected. o-o, Cholest-14-en-3β-ol measured colorimetrically. △-△, Cholesterol measured colorimetrically.
Figure 51. Chromatographic behaviour of Unknown B (fractions 178-200) on a column (1 x 100 cm) of silicic acid-Super Cel. The column was eluted with a mixture of benzene and ether (9:1, v/v). o-o, Tritium radioactivity. Δ-Δ, Cholesterol measured colorimetrically.
v/v)) to get it off the column. Unknown B was spotted on Silica Gel G thin-layer plate and was developed twice in a solvent system of chloroform-acetone (95:5, v/v). The resulting thin-layer radiochromatogram (Figure 52) indicated that it does not corresponded chromatographically to the mobility of the incubated substrate, [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol. Unknown B is most probably a dihydroxy or trihydroxy sterol.

Conversion of Unknown B to Cholesterol by Rat Liver Homogenate Preparations Under Aerobic Conditions

Thin-layer radiochromatographic analyses of Unknown B (pooled from several different incubations) on plates of Silica Gel G, with solvent systems of chloroform-methanol (95:5, v/v; Figure 53), chloroform-ethyl acetate (8:2, v/v; Figure 54), ethyl acetate-benzene (5:2, v/v; Figure 55), and chloroform-acetone (95:5, v/v; Figure 56), showed its radiopurity to be in excess of 91%. The solvent was removed under nitrogen and the residue was dissolved in 0.2 ml of propylene glycol. The solution was sonicated for 10 minutes to assure complete dissolution. The specific activity of this sample was 3.02 x 10^5 cpm/μg (9.05 x 10^6 cpm/ml).

The livers (54.7 g) of seven female rats (Sprague-Dawley strain; 150-200 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (136.6 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction of the rat liver homogenate was isolated by differential centrifugation.

To one Erlenmeyer flask (125 ml; Incubation II) containing 14 ml of the rat liver homogenate preparations, cofactors in potassium phosphate buffer (1 ml; 0.1 M, pH 7.4) were added to give final concentrations of 1 mM of DPN, 1 mM of TPN, and 3 mM of glucose-6-phosphate.
Figure 52. Thin-layer radiochromatographic analysis of Unknown B on a plate of Silica Gel G, developed twice in chloroform-acetone (95:5, v/v). Authentic 14α-hydroxymethyl-5α-cholest-7-en-3α-ol and cholesterol are shown at 6.5 cm and 11.5 cm, respectively.
Figure 53. Thin-layer radiochromatographic analysis of Unknown B on a plate of Silica Gel G, developed in chloroform-methanol (95:5, v/v). Authentic 14α-hydroxymethyl-5α-cholest-7-en-3β-ol and cholesterol are shown at 7.5 cm and 10.5 cm, respectively.
Figure 54. Thin-layer radiochromatographic analysis of Unknown B on a plate of Silica Gel G, developed in chloroform-ethyl acetate (8:2, v/v). Authentic 14α-hydroxymethyl-5α-cholest-7-en-3β-ol and cholesterol are shown at 4 cm and 8.5 cm, respectively.
Figure 55. Thin-layer radiochromatographic analysis of Unknown B on a plate of Silica Gel G, developed in ethyl acetate-benzene (5:2, v/v). Authentic 14α-hydroxymethyl-5α-cholest-7-en-3β-ol and cholesterol are shown at 7.5 cm and 10.5 cm, respectively.
Figure 56. Thin-layer radiochromatographic analysis of Unknown B on a plate of Silica Gel G, developed twice in chloroform-acetone (95:5, v/v). Authentic 14α-hydroxymethyl-5α-cholest-7-en-3β-ol and cholesterol are shown at 6.5 cm and 11 cm, respectively.
The other Erlenmeyer flask (125 ml; Incubation I) was first heated at 100° for 30 minutes, followed by the addition of cofactors in potassium phosphate buffer (1 ml; 0.1 M, pH 7.4) to give final concentrations of 1 mM of DPN, 1 mM of TPN, and 3 mM of glucose-6-phosphate. [3α-3H]-Unknown B (9.05 x 10^5 cpm, 3 μg) in propylene glycol (0.1 ml) and a solution of potassium phosphate buffer (15 ml; 0.1 M, pH 7.4) were added last to each of the two flasks above. Aerobic incubation was carried out for 3 hours at 37° with constant shaking.

At the end of the incubation period, both samples were saponified for 3 hours, using 30 ml of freshly-prepared 15% ethanolic KOH per sample. After cooling to room temperature, the solutions were each extracted with four 80 ml portions of petroleum ether, the combined petroleum ether extracts were washed with two 300 ml portions of water, and dried over anhydrous sodium sulfate. Appropriate aliquots were then removed from each phases for assay of radioactivity. Percent recovery of radioactivity of the various solutions is given in Table 16.

**Analysis of Incubation I - Control**

The recovered nonsaponifiable radioactive material (6.58 x 10^5 cpm) from the petroleum ether extract of Incubation I was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 6 mg of unlabeled cholesterol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 110), fractions 1.6 ml in volume (16 minutes per fraction, changed to 32 minutes per fraction at fraction 110; 0.08 ml/min) were collected. The resulting chromatogram is shown in Figure 57. Essentially all (99%) of the recovered radioactivity was
Figure 57. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of $[^3H]$-Unknown B with heat-inactivated rat liver homogenate preparations in the presence of DPN, TPN, and glucose-6-phosphate. ○○○, Tritium radioactivity. Δ-Δ, Cholesterol measured colorimetrically.
Table 16

Percent Recovery of Radioactivity of Various Solutions from Aerobic Incubation of \([3\alpha^-H]\)-Unknown B with Rat Liver Homogenate in the Presence of Added Cofactors

<table>
<thead>
<tr>
<th>Incubation Sample</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation I - Control</td>
<td></td>
</tr>
<tr>
<td>Aqueous phase</td>
<td>15.1%</td>
</tr>
<tr>
<td>Petroleum ether extract</td>
<td>83.5%</td>
</tr>
<tr>
<td>Total recovery</td>
<td>98.6%</td>
</tr>
<tr>
<td>Incubation II</td>
<td></td>
</tr>
<tr>
<td>Aqueous phase</td>
<td>29.5%</td>
</tr>
<tr>
<td>Petroleum ether extract</td>
<td>68.0%</td>
</tr>
<tr>
<td>Total recovery</td>
<td>97.5%</td>
</tr>
</tbody>
</table>

eluted in a single peak (fractions 175-192). Thin-layer radiochromatographic analysis of the contents of this peak and Unknown B on plates of Silica Gel G, shown in Figure 58, indicated that the mobility of this recovered material corresponded chromatographically to that of Unknown B.

Analysis of Incubation II

The recovered nonsaponifiable radioactive material (4.81 x 10^5 cpm) from the petroleum ether extract of Incubation II was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 10 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 115), fractions 1.6 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 115; 0.08 ml/min) were collected. The resulting chromatogram (Figure 59) showed that most (90%) of the recovered radioactivity corresponded chromatographically to the mobility of cholesterol (fractions 55-72).
Figure 58. Thin-layer radiochromatographic analyses of the contents of fractions 175-192 recovered from the silicic acid-Super Cel column shown in Figure 57 (left) and a sample of Unknown B (right) on plates of Silica Gel 0, developed twice in chloroform-acetone (95:5, v/v). Authentic 14α-hydroxymethyl-5α-cholestan-7-en-3β-ol and cholesterol are shown at 6.5 cm and 11.5 cm respectively on both chromatograms.
Figure 59. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of $[^3$H]-Unknown B with rat liver homogenate preparations in the presence of DPN, TPN, and glucose-6-phosphate. ••••, Tritium radioactivity. Δ-Δ, Cholesterol measured colorimetrically.
The contents of fractions 55-72 from previous column were pooled and acetylated with acetic anhydride-pyridine (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (3.30 x 10^5 cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ₈,₁⁴-cholestadiene (4 mg), 3β-acetoxy-Δ⁵,²₇-cholestadiene (6 mg), and cholesterol acetate (7 mg). The column was eluted with hexane-benzene (7:3, v/v) and fractions 4.4 ml in volume (20 minutes per fraction; 0.22 ml/min) were collected. The results are shown in Figure 60. All (100%) of the recovered radioactivity corresponded in chromatographic mobility to that of cholesteryl acetate (fractions 19-39).

Fractions 19-39 were pooled and applied (2.50 x 10^5 cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ₈(¹⁴)-cholestene (5 mg), 3β-acetoxy-Δ⁷-cholestene (6 mg), and cholesteryl acetate (5 mg). Using a mixture of hexane and benzene (9:1, v/v) as the eluting solvent, fractions 3.8 ml in volume (14 minutes per fraction; 0.27 ml/min) were collected. The results are shown in Figure 61. Most (95%) of the recovered radioactivity cochromatographed with authentic cholesteryl acetate (fractions 55-95) and another 5% corresponded chromatographically to the mobility of 3β-acetoxy-Δ⁷-cholestene (fractions 34-47).

The contents of fractions 55-95, corresponding to cholesteryl acetate on the alumina column, were pooled and hydrolyzed to free sterol by refluxing in 10 ml of 15% ethanolic KOH for 3 hours. The resulting sterol was diluted with unlabeled cholesterol (40 mg) and purified by way of the dibromide. The results, given in Table 17, indicated that the specific activities of cholesterol before and after
Figure 60. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from aerobic incubation of $[^3H]$-Unknown B with rat liver homogenate preparations in the presence of DPN, TPN, and glucose-6-phosphate. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to $3\beta$-acetoxy-$\Delta^\alpha,\beta$-cholestadiene, and the third is due to $3\beta$-acetoxy-$\Delta^5,\beta$-cholestadiene.
Figure 61. Alumina-Super Cel-silver nitrate column chromatogram of the acetate derivatives of monounsaturated sterols recovered from aerobic incubation of $[^3$H]-Unknown B with rat liver homogenate preparations in the presence of DPN, TPN, and glucose-6-phosphate. o-o, Tritium radioactivity. $\Delta$$\Delta$, Steryl acetate measured colorimetrically. The first peak is due to 3β-acetoxy-$\Delta^9(14)$-cholestene, the second is due to 3β-acetoxy-$\Delta^7$-cholestene, and the third is due to cholesteryl acetate.
dibromide treatment remained essentially unchanged.

Table 17

Dibromide Purification Results of \([3a-^3H]_2\)Cholesterol Recovered from Aerobic Incubation of \([3a-^3H]\)-Unknown B with Rat Liver Homogenate in the Presence of Cofactors

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before dibromide purification</td>
<td>2,260</td>
</tr>
<tr>
<td>After dibromide purification</td>
<td>2,280</td>
</tr>
</tbody>
</table>

**LiAlH₄ Reduction of Unknown B**

A portion of Unknown B recovered from previous incubations was evaporated to dryness under reduced pressure and stored in dessicator for another 10 hours. The resulting residue was dissolved in 5 ml of anhydrous ether, to which LiAlH₄ (20 mg, 0.527 mmole) was added. After stirring at room temperature for 2 hours, the solution was cooled to 0° and ice was slowly added until the remaining LiAlH₄ had decomposed. Water (50 ml) was then added and the mixture was extracted with three 50 ml portions of ether, the combined extracts were washed with three 150 ml portions of water, and dried over anhydrous sodium sulfate. Thin-layer radiochromatographic analysis of the reduction product, shown in Figure 62, indicated that Unknown B was not reduced under the conditions employed. This finding makes it very unlikely that Unknown B contains any 'reducible' functional groups (-CHO, =CO, etc.).

**Comparison of Unknown B with Available Diol and Triol Standards**

Thin-layer chromatographic analysis of Unknown B with cholest-7-en-3β,15α-diol, cholest-7-en-3β,15β-diol, cholest-8(14)-en-3β,15α-diol,
Figure 62. Thin-layer radiochromatographic analyses of Unknown B before (left) and after (right) LiAlH₄ reduction on plates of Silica Gel G, developed twice in chloroform-acetone (95:5, v/v). Authentic 14α-hydroxymethyl-5α-cholest-7-en-3β-ol and cholesterol are shown at 6.5 cm and 11.5 cm respectively on both chromatograms.
cholesterol-8(14)-en-3β,15β-diol, and cholesterol-8(14)-en-3β,7,15-triol (Figure 63) indicated that only cholesterol-8(14)-en-3β,15β-diol has chromatographic mobility similar to that of Unknown B.

C. ANALYSIS OF UNKNOWN C

Chromatographic Behaviour

Typical chromatographic behaviour of Unknown C on a column of silicic acid-Super Cel is shown in Figure 64. It constitutes the major product in incubations of [3α-3H]-14α-hydroxymethyl-5α-cholesterol-7-en-3β-ol with rat liver homogenate preparations carried out aerobically in the presence of AY-9944 but with no added cofactors. Unknown C is extremely unstable and consequently is a most difficult compound to study. A typical thin-layer radiochromatographic analysis of Unknown C, on a plate of Silica Gel G and developed twice in chloroform-acetone (95:5, v/v), is shown in Figure 65. Despite its eluting as a single component on the silicic acid-Super Cel column, subsequent thin-layer radiochromatographic analyses consistently showed several regions of radioactivity.

Analysis of the Decomposition Products of Unknown C

Since it was difficult to study Unknown C per se due to its extreme instability, attempts were made to examine its decomposition products. Unknown C (6.96 x 10⁵ cpm), recovered from previous incubations, was applied to a column (1 x 100 cm) of silicic acid-Super Cel, along with 7 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 100), fractions 3.0 ml in volume (32 minutes per fraction; 0.09 ml/min) were collected. The resulting chromatogram is shown in Figure 66. Approximately 63% of the recovered radioactivity corresponded chromatographi-
Figure 63. Thin-layer radiochromatographic analysis of Unknown B, along with available diol and triol standards, on a plate of Silica Gel G. The plate was developed thrice in chloroform-acetone (95:5, v/v). Authentic cholest-8(14)-en-3β,7,15-triol, cholest-8(14)-en-3β,15β-diol, cholest-7-en-3β,15β-diol, cholest-7-en-3β,15α-diol, cholest-8(14)-en-3β,15α-diol, 14β-hydroxymethyl-5α-cholest-7-en-3β-ol and cholesterol are shown at 1.5 cm, 4 cm, 4.5 cm, 6 cm, 7 cm, 7.5 cm, and 12.5 cm, respectively.
Figure 64. Chromatographic behaviour of unknown C (fractions 86-105) on a column (1 x 100 cm) of silicic acid-Super Cel. The column was eluted with a mixture of benzene and ether (9:1, v/v). C-2, Tritium radioactivity.
Figure 65. Thin-layer radiochromatographic analysis of Unknown C on a plate of Silica Gel G, developed twice in chloroform-acetone (95:5, v/v). Authentic 14α-hydroxymethyl-5α-cholest-7-en-3β-ol and cholesterol are shown at 5.5 cm and 11.5 cm, respectively.
Figure 66. Silicic acid–Super Cel column chromatographic analysis of the decomposition products of Unknown C. The column was eluted with benzene–ether (9:1, v/v) and fractions 3.0 ml in volume were collected. o–o, Tritium radioactivity. Δ–Δ, Cholesterol measured colorimetrically.
ally to the mobility of cholesterol (fractions 37-48).

Another sample of Unknown C (8.25 x 10^5 cpm) was reapplied to a column (1 x 100 cm) of silicic acid-Super Cel, along with 8 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 115), fractions 1.5 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 115; 0.075 ml/min) were collected. The resulting chromatogram (Figure 67) showed that approximately 30% of the recovered radioactivity corresponded in chromatographic mobility to that of cholesterol (peak centered at fraction 63). A greater amount of polar constituents was recovered in this case, and this was attributed to the effect of autooxidation since this analysis was carried out a month later than the previous one. It is also worthwhile to note that in both cases, approximately 6% of the recovered radioactivity had chromatographic mobility similar to that of Unknown A.

Fractions from two previous columns (fractions 37-48 from the first column and fractions 55-71 from the second column), with chromatographic mobility corresponding to that of cholesterol, were pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (1.75 x 10^5 cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ7,14-cholestadiene (5 mg), 3β-acetoxy-Δ8,14-cholestadiene (5 mg), 3β-acetoxy-Δ5,7-cholestadiene (5 mg), and cholesteryl acetate (6 mg). The column was eluted with hexane-benzene (7:3, v/v) and fractions 6.0 ml in volume (20 minutes per fraction; 0.3 ml/min) were collected.
Figure 67. Silicic acid-Super Cel column chromatographic analysis of the decomposition products of Unknown C. The column was eluted with a mixture of benzene and ether (9:1, v/v) and fractions 1.5 ml in volume were collected. o-o, Tritium radioactivity. Δ-Δ, Cholesterol measured colorimetrically.
The results, shown in Figure 68, indicated that most (80%) of the recovered radioactivity was associated chromatographically with 3α-acetoxy-Δ7,14-cholestadiene (peak at fraction 99). No significant amount of other dienes or monoenes could be detected.

The material corresponding chromatographically to 3α-acetoxy-Δ7,14-cholestadiene on the previous column (fractions 85-110) was combined and subjected to repetitive cocrystallization with 20 mg of authentic unlabeled 3α-acetoxy-Δ7,14-cholestadiene from methanol and from acetone-water. The results, given in Table 18, showed essentially a single component cocrystallizing with the added carrier.

Table 18

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>820</td>
</tr>
<tr>
<td>Once from methanol</td>
<td>680</td>
</tr>
<tr>
<td>Twice from methanol</td>
<td>610</td>
</tr>
<tr>
<td>Once from acetone-water</td>
<td>640</td>
</tr>
<tr>
<td>Twice from acetone-water</td>
<td>660</td>
</tr>
</tbody>
</table>

LiAlH4 Reduction of Unknown C

The livers (42.2 g) of four female rats (Sprague-Dawley strain; 150-200 g each) were homogenized in potassium phosphate buffer (105.5 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction of the rat liver homogenate was isolated by differential centrifugation.

A solution of AY-9944 (2.0 x 10^-4 M) was prepared by dissolving
Figure 68. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from silicic acid-Super Cel columns shown in Figures 66 and 67. The column was eluted with hexane-benzene (7:3, v/v) and fractions 6.0 ml in volume were collected. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ7,14-cholestadiene, the third is due to 3β-acetoxy-Δ8,14-cholestadiene, and the fourth is due to 3β-acetoxy-Δ8,14-cholestadiene.
9.3 mg of the inhibitor in 100 ml of potassium phosphate buffer (0.1 M, pH 7.4).

To two Erlenmeyer flasks (125 ml) each containing 15 ml of the rat liver homogenate preparations, a solution of AY-9944 (15 ml; to give a final concentration of $10^{-4}$ M) was added. $[3\alpha-^3H]-14\alpha$-Hydroxymethyl-5α-cholest-7-en-3β-ol (1.72 x $10^6$ cpm, 5.5 μg) in propylene glycol (0.05 ml) was added last. Aerobic incubation was carried out for 3 hours at 37°C with constant shaking.

At the end of the incubation period, the samples were saponified for 3 hours, using 30 ml of 15% ethanolic KOH per sample. After cooling to room temperature, the solutions were each extracted with three 100 ml portions of petroleum ether and two 100 ml portions of diethyl ether. The petroleum ether and the diethyl ether extracts were combined, washed with three 500 ml portions of water, and dried over anhydrous sodium sulfate. Appropriate aliquots were then removed from each phases for assay of radioactivity. Percent recovery of radioactivity of the various solutions is given in Table 19.

The recovered nonsaponifiable radioactive material (1.26 x $10^6$ cpm) of Incubation I was applied to a column (1 x 100 cm) of silicic acid–Super Cel, along with 7 mg of unlabeled cholesterol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 80), fractions 2.2 ml in volume (30 minutes per fraction; 0.073 ml/min) were collected. The resulting chromatogram is shown in Figure 69. Approximately 24% of the recovered radioactivity corresponded chromatographically to the mobility of cholesterol (peak at fraction 42). Another 48% had the chromatographic mobility of Unknown C (peak at fraction 53), and 9%
Figure 69. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesta-7-en-3β-ol with rat liver homogenate preparations in the presence of AY-9944 but with no added cofactors. □-□, Tritium radioactivity. △-△, Cholesterol measured colorimetrically.
Table 19

Percent Recovery of Various Solutions from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholesterol-7-en-3β-ol with Rat Liver Homogenate in the Absence of Added Cofactors

<table>
<thead>
<tr>
<th>Incubation Sample</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation I</td>
<td></td>
</tr>
<tr>
<td>Aqueous phase</td>
<td>23.5%</td>
</tr>
<tr>
<td>Combined extracts</td>
<td>73.2%</td>
</tr>
<tr>
<td>Total recovery</td>
<td>96.7%</td>
</tr>
<tr>
<td>Incubation II</td>
<td></td>
</tr>
<tr>
<td>Aqueous phase</td>
<td>16.4%</td>
</tr>
<tr>
<td>Combined extracts</td>
<td>74.9%</td>
</tr>
<tr>
<td>Total recovery</td>
<td>91.3%</td>
</tr>
</tbody>
</table>

of the eluted radioactivity cochromatographed with the substrate (fractions 130-150).

The combined extracts from Incubation II were evaporated to dryness under reduced pressure and then in a dessicator for another 8 hours. The resulting residue was dissolved in anhydrous ether (10 ml) and LiAlH₄ (120 mg, 3.15 mmole) was added. After stirring at room temperature for 2 hours, the reaction mixture was cooled to 0°C and ice was slowly added until the remaining LiAlH₄ had decomposed. Water (80 ml) was then added and the mixture was extracted with three 80 ml portions of ether, the ether extracts were combined and washed with three 200 ml portions of water, and dried over anhydrous sodium sulfate.

The labeled reduction products of Incubation II (1.13 x 10⁶ cpm) were applied to a column (1 x 100 cm) of silicic acid-Super Cel, along with 7 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v))
at fraction 80), fractions 2.1 ml in volume (30 minutes per fraction; 0.07 ml/min) were collected. The results (Figure 70) showed that the amount of recovered radioactivity associated with cholesterol (fractions 37-47) stayed approximately the same (26%). However, the amount of eluted radioactivity corresponding to the mobility of Unknown C (fractions 48-80) decreased dramatically from 48% to 23%, and this was compensated by a concomitant increase in the radioactivity associated with the substrate (increased from 9% to 32%; peak at fraction 136). The identity of this latter peak was further established by thin-layer radiochromatographic analysis (Figure 71).

The results of this experiment provide additional support for my speculation that Unknown C is \( [3^{3a}-\text{H}]-14\alpha\text{-formyl-5a-cholest-7-en-38-ol} \), but conclusive establishment of its identity still awaits comparison of Unknown C with chemically-synthesized \( 14\alpha\text{-formyl-5a-cholest-7-en-38-ol} \).

D. ANALYSIS OF UNKNOWN D

Chromatographic Behaviour

Typical chromatographic behaviour of Unknown D on a column of silicic acid-Super Cel is shown in Figure 72. Its chromatographic mobility corresponds very closely to that of cholesterol. It is relatively stable and can be kept in a frozen state for several months without any significant decomposition.

Further Column Chromatographic Analyses

Unknown D, recovered from several previous incubations, was pooled and acetylated with a mixture of pyridine and acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (3.25 x 10^5 cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-
Figure 70. Silicic acid-Super Cel column chromatographic analysis of LiAlH₄ reduction products of sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with rat liver homogenate preparations in the presence of AV-9944 but with no added cofactors. o-o, Tritium radioactivity. Δ-Δ, Cholesterol measured colorimetrically.
Figure 71. Thin-layer radiochromatographic analysis of the contents of fractions 125-142 recovered from the silicic acid-Super Cel column shown in Figure 70. The Silica Gel G plate was developed twice in chloroform-acetone (95:5, v/v). Authentic 14α-hydroxymethyl-5α-cholest-7-en-3β-ol and cholesterol are shown at 4.5 cm and 12.5 cm, respectively.
Figure 72. Chromatographic behaviour of Unknown D (fractions 48-56) on a column (1 x 100 cm) of silicic acid-Super Cel. The column was eluted with benzene-ether (9:1, v/v). o-o, Tritium radioactivity. △-△, Cholesterol measured colorimetrically.
Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-$\Delta^8,14$-cholestadiene (4 mg), 3β-acetoxy-$\Delta^5,7$-cholestadiene (7 mg), and cholesteryl acetate (7 mg). Using a mixture of hexane and benzene (7:3, v/v) as the eluting solvent (changed to hexane-benzene (5:5, v/v) at fraction 222), fractions 5.5 ml in volume (20 minutes per fraction; 0.275 ml/min) were collected. The result of this column chromatography is shown in Figure 73. Approximately 40% of the recovered radioactivity corresponded chromatographically to the mobility of authentic 3β-acetoxy-$\Delta^8,14$-cholestadiene (fractions 100–140), and another 15% of the applied radioactivity was eluted prior to 3β-acetoxy-$\Delta^8,14$-cholestadiene, corresponding to the mobility of 3β-acetoxy-$\Delta^7,14$-cholestadiene (peak at fraction 90). A small portion (2%) of the applied radioactivity was eluted in the region of 3β-acetoxy-$\Delta^5,7$-cholestadiene (peak at fraction 228).

Another sample of Unknown D was pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (2.15 x 10$^5$ cpm) were applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-$\Delta^8,14$-cholestadiene (8 mg), 3β-acetoxy-$\Delta^5,7$-cholestadiene (8 mg), and cholesteryl acetate (5 mg). Using hexane-benzene (7:3, v/v) as the eluting solvent (changed to hexane-benzene (5:5, v/v) at fraction 222), fractions 5.6 ml in volume (20 minutes per fraction; 0.28 ml/min) were collected. The results are shown in Figure 74. Approximately 47% of the recovered radioactivity cochromatographed with 3β-acetoxy-$\Delta^8,14$-cholestadiene (peak at fraction 126) and another 19% was associated with 3β-acetoxy-$\Delta^5,7$-cholestadiene (peak at fraction 231). A significant portion (22%) of the
Figure 73. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of Unknown D. The column was eluted with a mixture of hexane and benzene (7:3, v/v) and fractions 5.5 ml in volume were collected. o-o, Tritium radioactivity. A-A, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ\(^{8}\),14-cholestadiene, and the third is due to 3β-acetoxy-Δ\(^{5}\),7-cholestadiene.
Figure 74. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of Unknown D. The column was eluted with a mixture of hexane and benzene (7:3, v/v) and fractions 5.6 ml in volume were collected. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ^{8,14}-cholestadiene, and the third is due to 3β-acetoxy-Δ^5,7-cholestadiene.
applied radioactivity was eluted just before 3β-acetoxy-Δ^8,14-cholesta-
diene, corresponding to the mobility of 3β-acetoxy-Δ^7,14-cholesta-
diene (fractions 87-110).

The material corresponding chromatographically to the supposed
mobility of 3β-acetoxy-Δ^7,14-cholestdiene from above columns (frac-
tions 80-98 from the first column and fractions 87-110 from the second
column) was combined and subjected to repetitive cocrystallization
with 15 mg of authentic unlabeled 3β-acetoxy-Δ^7,14-cholestdiene from
methanol and from acetone-water. The results, given in Table 20, in-
dicated essentially a single component cocrystallizing with the added
carrier.

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>Once from methanol</td>
<td>860</td>
</tr>
<tr>
<td>Twice from methanol</td>
<td>820</td>
</tr>
<tr>
<td>Once from acetone-water</td>
<td>870</td>
</tr>
<tr>
<td>Twice from acetone-water</td>
<td>800</td>
</tr>
</tbody>
</table>

The material corresponding to the chromatographic mobility of 3β-
acetoxy-Δ^8,14-cholestdiene from previous columns (fractions 102-138
from the first column and fractions 112-165 from the second column)
was combined and subjected to repetitive cocrystallization with authen-
tic unlabeled 3β-acetoxy-Δ^8,14-cholestdiene (20 mg) from methanol and
from acetone-water. The results (Table 21) showed that the specific activities remained essentially constant after the first recrystal-
lization from methanol.

Table 21

<table>
<thead>
<tr>
<th>Sample</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
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<tr>
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<td>1,280</td>
</tr>
<tr>
<td>Twice from acetone-water</td>
<td>1,250</td>
</tr>
</tbody>
</table>

Fractions 225-265 from the second column were pooled and subject-
ed to repetitive cocrystallization with authentic unlabeled 3β-acetoxy-

Table 22

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>910</td>
</tr>
<tr>
<td>Once from methanol</td>
<td>760</td>
</tr>
<tr>
<td>Twice from methanol</td>
<td>770</td>
</tr>
<tr>
<td>Once from acetone-water</td>
<td>790</td>
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<tr>
<td>Twice from acetone-water</td>
<td>780</td>
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</table>
$\Delta^{5,7}$-cholestadiene (20 mg) from methanol and from acetone-water. The results are given in Table 22. The specific activities of the crystals remained essentially constant after the first crystallization from methanol.
IV. AEROBIC INCUBATION OF [3α−3H]−14α−HYDROXYMETHYL-5α-CHOLEST-7-EN-3β-OL WITH WASHED RAT LIVER MICROSOMES

INTRODUCTION AND DISCUSSION

The status of cholest-8(14)−en-3β-ol in the biosynthesis of cholesterol, as described previously in Chapter I, has been a long and controversial one. The evidence presently available in support of its possible intermediary role has been primarily twofold: 1. the isolation of cholest-8(14)−en-3β-ol from rat skin (Lee et al., 1969), and moreover several other 8Δ sterols have also been isolated from plant sources, i.e., stigmasta-8(14),22-dien-3β-ol from 'rayless goldenrod' (Aplopappus heterophyllus; Zalkow et al., 1968) and 4α-methylcholesta-8(14),24-dien-3β-ol from dried bakers yeast (Saccharomyces cerevisiae; Barton et al., 1970); 2. the enzymatic conversion of [3α−3H]−cholest-8(14)−en-3β-ol to cholesterol, cholest-7-en-3β-ol, and cholesta-8,14-dien-3β-ol, both in vivo and in rat liver homogenate preparations (Lee and Schroepfer, 1968; Lee et al., 1969; Schroepfer et al., 1971), and the demonstration of the conversion of 4,4-dimethyl-cholesta-8(14)−en-3β-ol to cholesterol in rat liver homogenates (Fried et al., 1968).

Although Fried et al. (Fried et al., 1968; Dudowitz and Fried, 1969) had reported early in 1968 the formation of 4,4-dimethyl-5α-cholesta-8(14)−en-3β-ol upon incubation of [3α−3H]−lanost-7-en-3β,32-diol and [3α−3H]−lanost-7-en-3β-ol−32-al with rat liver microsomes, the methodology (gas-liquid radiochromatographic analysis of acetylated
nonsaponifiable fraction of the incubation mixture) used to identify the \( \Delta^{8(14)} \)-monoene compound was not unambiguous, i.e., many other steryl acetates would have the same or very similar chromatographic mobility in the system employed. Later studies by Alexander et al. (1972), using \([3\alpha-^3H]\)- and \([32-^3H]\)-lanost-7-en-3\beta,32-diol as substrates, showed quite contrasting results. They reported the formation of 4,4-dimethyl-cholesta-7,14-dien-3\beta-ol (instead of 4,4-dimethyl-cholest-8(14)-en-3\beta-ol) concurrently with the release of formic acid upon incubation of the substrates with washed rat liver microsomes in the presence of added DPN and TPNH generator. The removal of the C-14 methyl group as formic acid has since been confirmed by Trowbridge et al. (1975). This placed the status of cholest-8(14)-en-3\beta-ol in an even more uncertain position.

One particular weak point for the conclusive establishment of the intermediary role of cholest-8(14)-en-3\beta-ol in cholesterol biosynthesis is therefore the lack of unambiguous experimental data concerning the formation of cholest-8(14)-en-3\beta-ol from a known cholesterol precursor. These experimental data will constitute the major theme of this chapter. In addition, preliminary investigations regarding its cofactor requirements will also be described.

The substrate used in these studies is \([3\alpha-^3H]\)-14\alpha-hydroxymethyl-5\alpha-cholest-7-en-3\beta-ol, which has been shown to be convertible to cholesterol (Trowbridge et al., 1975). Six major sets of experiments were carried out:

A. Control

The substrate was incubated aerobically for 2 hours with a heat-inactivated rat liver microsomal preparation in the pre-
sence of added TPNH generating system. Only unreacted substrate could be recovered. This assures us that all subsequent experiments described are enzymatic in nature. It eliminates the possibility that cholest-8(14)-en-3β-ol could be formed from the added substrate by a nonenzymatic process.

B. In the presence of added DPN and TPNH

Experiment 1. The substrate was incubated aerobically for 2 hours with washed rat liver microsomes in the presence of added DPN and TPNH generator. Labeled cholest-8(14)-en-3β-ol, cholest-7-en-3β-ol, and cholesterol were isolated. Contrary to the results reported by Alexander et al. (1972), no significant accumulation of cholesta-7,14-dien-3β-ol could be detected.

Experiment 2. Alexander et al. (1972) had noted in their communication that the amount of cholesta-7,14-dien-3β-ol formed was inversely proportional to the duration of the incubation period. Consequently, an experiment in which the substrate was incubated aerobically for 30 minutes with washed rat liver microsomes in the presence of added DPN and TPNH generator was carried out. The results were similar to those of Experiment 1, i.e., no significant incorporation of label into cholesta-7,14-dien-3β-ol could be observed.

C. In the presence of only TPNH

The substrate was incubated aerobically for 2 hours with washed rat liver microsomes in the presence of added TPNH generator. Labeled cholest-8(14)-en-3β-ol, cholest-7-en-3β-ol, and cholesterol were isolated.

D. In the presence of only DPN

Experiments 1 and 2. The substrate was incubated aerobically
for 2 hours with washed rat liver microsomes supplemented with DPN. The resulting labeled product was almost exclusively cholest-8(14)-en-3β-ol. No labeled cholest-7-en-3β-ol or cholesterol could be detected. A small portion of the recovered radioactivity (< 2%) corresponded very closely to the chromatographic mobility of cholest-8(9)-en-3β-ol on alumina-Super Cel-silver nitrate column, but was shown by gas-liquid radiochromatographic analysis to be much less polar than the $\Delta^{8(9)}$ sterol. Experiment 3. To show that the method of sterol extraction did not affect the incubation results, the substrate was incubated aerobically for 2 hours with washed rat liver microsomes in the presence of added DPN. But instead of extracting the sterols directly with acetone as in all other incubations, the incubation mixture was first saponified for 3 hours with 15% ethanolic KOH and then extracted with petroleum ether. The results were identical with those obtained in Experiments 1 and 2. Experiment 4. A major portion of the substrate remained un-reacted in all of the foregoing incubations and this could be due to either limiting enzyme and/or cofactor concentration or inhibition of the demethylase enzyme by one of the products formed. The effect of cofactor concentration was explored here by incubating the substrate aerobically for 2 hours with washed rat liver microsomes in the presence of 'excess' DPN (5 mM as opposed to 1.5 mM in previous incubations). The results indicated that the amount of unreacted substrate did decrease dramatically (though not completely), yet the total amount of labeled cholest-8(14)-en-3β-ol isolated from the in-
cubation mixture remained essentially the same. Several other unidentified products, more polar than cholesterol in mobility, showed a corresponding increase.

Experiment 5. The chromatographic behaviour of cholesterol and cholest-8(14)-en-3β-ol on a column of silicic acid-Super Cel, when eluted with a mixture of benzene and ether (9:1, v/v), was studied. The results confirmed that the two sterols could be separated from one another under the conditions employed. This further supported my claim that the major labelled product isolated is cholest-8(14)-en-3β-ol and not some other compounds which then rearrange or decompose to yield the Δ8(14) sterol.

E. In the absence of any added cofactors

Experiments 1 and 2. The substrate was incubated aerobically for 2 hours with washed rat liver microsomes in the absence of any added cofactors. In two separate experiments, the results obtained were identical to those of incubations done in the presence of only added DPN, i.e., only labeled cholest-8(14)-en-3β-ol was isolated. This was quite surprising and these results were attributed to the presence (in washed rat liver microsomes) of some very tightly bound cofactors which could not readily be washed away. Due to the extremely small mass of the substrate used in these studies, even such limited amount of tightly bound cofactors would be sufficient to initiate the enzymatic reaction involved. In the following chapter, evidence in support of this explanation will be presented.
F. In the presence of AY-9944

AY-9944, as described previously, is a specific inhibitor of both the Δ⁷- and the Δ¹⁴-reductase. It then follows that if any Δ⁷,¹⁴ sterol were formed, as reported by Alexander et al. (1972), we would expect it to accumulate in the presence of AY-9944. The substrate was therefore incubated aerobically for 2 hours with washed rat liver microsomes in the presence of added TPNH generator and AY-9944 inhibitor solution. Only a very small portion (<2%) of the total recovered radioactivity corresponded in chromatographic mobility to that of Δ⁷,¹⁴ sterol. The major monohydroxy sterol isolated had chromatographic mobility intermediate between those of 3β-acetoxy-Δ⁷,¹⁴-cholestadiene and cholesteryl acetate on columns of Silica Gel G-Super Cel-silver nitrate.

Based on the results of these incubation studies, the following conclusions can be drawn:

1. Aerobic incubation of [3α-³H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with washed rat liver microsomes in the presence of added TPNH generator or both TPNH generator and DPN yielded cholest-8(14)-en-3β-ol, cholest-7-en-3β-ol, and cholesterol as the major labeled products.

2. Aerobic incubation of [3α-³H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with washed rat liver microsomes in the presence of added DPN yielded labeled cholest-8(14)-en-3β-ol as the major product.

3. Little or no incorporation of the label into cholesta-7,14-dien-3β-ol, cholesta-8,14-dien-3β-ol, and cholesta-5,7-dien-
38-ol was observed in all incubation studies, despite efforts to induce their accumulation by either shortening the incubation period or using specific Δ⁷- and Δ¹⁴-reductase inhibitor. Before jumping to a hasty conclusion that cholesta-7,14-dien-38-ol is not the immediate product of C-14 demethylation as Alexander et al. (1972) has claimed, it must be pointed out however the differences between their experiments and those of mine. First of all, they used a slightly different substrate, [3α-²H]-lanost-7-en-3β,32-diol (with two more methyl groups at C-4). Secondly, their substrate concentration per incubation is much greater than mine. They used 26.9 μM of substrate per incubation while I used only 4.4 μM of substrate per incubation. Thirdly, the cofactor concentrations used in incubation studies are different. For DPN, they used 0.4 mM in contrast to my 1.5 mM; and for TPNH (TPN concentration), they used 0.5 mM in contrast to my 1.2 mM. In spite of these differences, there is no obvious reason why any of them should seriously affect the results obtained in the two laboratories.

One puzzling observation, as noted previously, is the unexpected behaviour of [3α-³H]-14α-hydroxymethyl-5α-cholest-7-en-38-ol when incubated aerobically with washed rat liver microsomes in the absence of any added cofactors. The experiment yielded identical results as when incubated in the presence of only added DPN. It has been suggested that these results are due to the presence of some tightly bound cofactors in the washed rat liver microsomes, and in the next chapter, this possibility will be examined in more detail.
EXPERIMENTAL

The radiopurity of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol used in the following experiments was judged to be in excess of 98% on the basis of previous chromatographic analyses (see Chapter III).

A. CONTROL

The livers (43 g) of six male rats (Sprague-Dawley strain; 150-200 g each) were homogenized in potassium phosphate buffer (430 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by centrifugation for 30 minutes at 4°C. This was then centrifuged at 105,000 x g for 60 minutes in a Beckman L2-65B Ultracentrifuge, using a Type 35 (35,000 rpm) rotor and polycarbonate screw-capped tubes. The pellets were removed and resuspended in potassium phosphate buffer (215 ml; 0.1 M, pH 7.4), using a teflon-on-glass homogenizer operated manually. The suspension was recentrifuged at 105,000 x g for 60 minutes. The washed microsomal pellets were again removed and resuspended in 16 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride. The protein concentration, as determined by Lowry method (Lowry et al., 1951), was 18.4 mg/ml.

A test tube, containing 2 ml of the above microsomal preparation, was heated at 100°C for 20 minutes. A TPNH generating system, prepared as described in Methods, was then added to give a final concentration of 1.2 mM of TPN. [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol (1.72 x 10^6 cpm, 5.5 μg) in propylene glycol (0.05 ml) was added last. Aerobic incubation was carried out for 2 hours at 37°C with constant shaking.

The incubation was terminated by the addition of 40 ml of acetone and the mixture was then homogenized. The resulting coagulum was re-
moved by filtration; the filter paper and the coagulum were washed several times with fresh acetone. The combined filtrates were evaporated to dryness under reduced pressure, and the resulting residue \((1.70 \times 10^6 \text{ cpm}; 98.8\% \text{ recovery of incubated radioactivity})\) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 8 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 124), fractions 1.6 ml in volume (20 minutes per fractions, changed to 30 minutes per fraction at fraction 124; 0.08 ml/min) were collected. Appropriate aliquots were then removed for determination of radioactivity and mass. The resulting chromatogram, shown in Figure 75, indicated that essentially all (94.1%) of the applied radioactivity was recovered as the unreacted substrate (peak centered at fraction 196). Its identity was further supported by its chromatographic behaviour on Silica Gel G thin-layer plate, developed twice in chloroform-acetone (95:5, v/v; Figure 76).

B. IN THE PRESENCE OF ADDED DPN AND TPNH GENERATING SYSTEM

Experiment 1

The livers (43 g) of six male rats (Sprague-Dawley strain; 150-200 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (430 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by differential centrifugation. This was then centrifuged at 105,000 x g for 60 minutes. The pellets were removed and resuspended in 215 ml of potassium phosphate buffer (0.1 M, pH 7.4). The suspension was recentrifuged at 105,000 x g for 60 minutes. The washed microsomal pellets were again removed and resuspended in 16 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM
Figure 75. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with heat-inactivated washed rat liver microsomes. o-o, Tritium radioactivity. △-△, Cholesterol measured colorimetrically.
Figure 76. Thin-layer radiochromatographic analysis of pooled contents of fractions 180-210 from the silicic acid-Super Cel column shown in Figure 75. The Silica Gel G plate was developed twice in chloroform-acetone (95:5, v/v). Authentic 14α-hydroxymethyl-5α-cholest-7-en-3β-ol and cholesterol are shown at 4.5 cm and 9.5 cm, respectively.
magnesium chloride to give a final protein concentration of 18.4 mg/ml.

To a test tube containing 2 ml of this microsomal preparation, DPN (3 mg) and TPNH generator (0.9 ml) were added to give final concentrations of 1.5 mM of DPN and 1.2 mM of TPN. [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3α-ol (1.72 x 10^6 cpm, 5.5 μg) in propylene glycol (0.05 ml) was added last. Aerobic incubation was carried out for 2 hours at 37°C with constant shaking.

At the end of the incubation period, acetone (40 ml) was added and the mixture was homogenized. The resulting coagulum was removed by filtration; the filter paper and the coagulum were washed several times with fresh acetone. The combined filtrates were evaporated to dryness and the residue (1.73 x 10^6 cpm; 100.6% recovery of applied radioactivity) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 8 mg of unlabeled cholesterol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 134), fractions 1.6 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 134 and to 50 minutes per fraction at fraction 195; 0.08 ml/min) were collected. The results (Figure 77) indicated that approximately 53% of the recovered radioactivity corresponded chromatographically to the mobility of cholesterol or monohydroxy sterols (fractions 52-65). A portion (25%) of the recovered radioactivity corresponded in chromatographic mobility to that of unreacted substrate (peak centered at fraction 176).

The contents of fractions 52-77 were pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The resulting labeled steryl acetates (6.54 x 10^5 cpm) were applied
Figure 77. Silicic acid-Super Cel column chromatogram of sterols recovered from aerobic incubation of \([3\alpha^{-3}H]-14\alpha\)-hydroxymethyl-5\alpha-cholest-7-en-3\beta-ol\) with washed rat liver microsomes in the presence of added DPN and TPNH generating system. o-o, Tritium radioactivity. △-△, Cholesterol measured colorimetrically.
to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-\textit{Δ}^8\textit{14}-cholestadiene (6 mg), 3β-acetoxy-\textit{Δ}^5\textit{7}-cholestadiene (7 mg), and cholesteryl acetate (5 mg). Using hexane-benzene (7:3, v/v) as the eluting solvent, fractions 5.4 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 70; 0.27 ml/min) were collected. The resulting chromatogram is shown in Figure 78. Most (90%) of the recovered radioactivity cochromatographed with authentic cholesteryl acetate (fractions 12-22).

The contents of fractions 12-22 were pooled and applied (4.00 x 10\textsuperscript{5} cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-\textit{Δ}^8\textsuperscript{(14)}-cholestene (5 mg), 3β-acetoxy-\textit{Δ}^7-cholestene (6 mg), and cholesteryl acetate (5 mg). Using hexane-benzene (9:1, v/v) as the eluting solvent (changed to hexane-benzene (8:2, v/v) at fraction 130), fractions 2.3 ml in volume (20 minutes per fraction; 0.115 ml/min) were collected. The results (Figure 79) showed that approximately 18% of the eluted radioactivity corresponded chromatographically to 3β-acetoxy-\textit{Δ}^8\textsuperscript{(14)}-cholestene (fractions 49-62), and another 18% had the chromatographic mobility of 3β-acetoxy-\textit{Δ}^7-cholestene (fractions 79-100). A major portion (62%) of the recovered radioactivity showed the same chromatographic mobility as cholesteryl acetate (fractions 132-165).

The contents of fractions 49-62 were pooled and a portion of it was subjected to repetitive cocrystallization with unlabeled authentic 3β-acetoxy-\textit{Δ}^8\textsuperscript{(14)}-cholestene (20 mg) from methanol and from acetonewater. The results, given in Table 23, showed essentially a single
Figure 78. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesterol-7-en-3β-ol with washed rat liver microsomes in the presence of added DPN and TPNH generating system. ○-○, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ8,14-cholestadiene, and the third is due to 3β-acetoxy-Δ5,7-cholestadiene.
Figure 79. Alumina-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monounsaturated sterols recovered from aerobic incubation of \([3\alpha-3^H]-14\alpha\text{-hydroxymethyl-5\alpha\text{-cholesten-3\beta\text{-ol}}\) with washed rat liver microsomes in the presence of added DPN and TPNH generating system. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to 3β-acetoxy-Δ7(15)-cholesterol, the second is due to 3β-acetoxy-Δ4-cholesterol, and the third is due to cholesterol acetate.
Table 23

Cocrystallization of Acetate Derivative of [3α-3H]-Cholest-8(14)-en-3β-ol Recovered from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol in the Presence of DPN and TPNH with Authentic 3β-Acetoxy-Δ^8(14)-cholesten

<table>
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<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
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</tr>
<tr>
<td>Once from acetone-water</td>
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</tr>
<tr>
<td>Twice from acetone-water</td>
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</table>

component cocrystallizing with the added carrier. Another portion of it was subjected to gas-liquid radiochromatographic analysis on a column of 3% OV-17, along with unlabeled 3β-acetoxy-Δ^8(14)-cholestone. The radioactive material showed the same mobility as that of 3β-acetoxy-Δ^8(14)-cholesten (Figure 80).

The material, corresponding in chromatographic mobility to 3β-acetoxy-Δ^7-cholesten on the alumina column (fractions 70-100), was combined and subjected to cocrystallization with a sample of unlabeled 3β-acetoxy-Δ^7-cholesten (35 mg). The mixture was recrystallized twice from methanol and twice from acetone-water. The specific activity remained essentially unchanged (Table 24). Appropriate aliquot was also removed and subjected to gas-liquid radiochromatographic analysis on a 3% OV-17 column, along with unlabeled samples of 3β-acetoxy-Δ^8(14)-cholesten and 3β-acetoxy-Δ^7-cholesten. The radioactivity emerged in a single peak which corresponded chromatographically to authentic 3β-acetoxy-Δ^7-cholesten (Figure 81).
Figure 80. Gas-liquid radiochromatographic analysis of pooled contents of fractions 49-62 from the alumina column shown in Figure 79, corresponding chromatographically to authentic 3β-acetoxy-Δ8(14)-cholestene. The mass peak shown is due to unlabeled 3β-acetoxy-Δ8(14)-cholestene.
Figure 81. Gas-liquid radiochromatographic analysis of pooled contents of fractions 79-100 from the alumina column shown in Figure 79, corresponding to the chromatographic mobility of 3β-acetoxy-Δ7-cholestanol. The first mass peak is due to authentic 3β-acetoxy-Δ3(14)-cholestanol and the second is due to 3β-acetoxy-Δ7-cholestanol.
Table 24

Cocrystallization of Acetate Derivative of [3α-3H]-Cholest-7-en-3β-ol Recovered from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholesterol-7-en-3β-ol in the Presence of DPN and TPNH with Authentic 3β-Acetoxy-Δ7-cholestene

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>1,170</td>
</tr>
<tr>
<td>Once from methanol</td>
<td>1,150</td>
</tr>
<tr>
<td>Twice from methanol</td>
<td>1,160</td>
</tr>
<tr>
<td>Once from acetone-water</td>
<td>1,110</td>
</tr>
<tr>
<td>Twice from acetone-water</td>
<td>1,140</td>
</tr>
</tbody>
</table>

A portion of the pooled material (fractions 132-165) from the alumina column was subjected to cocrystallization with 20 mg of authentic cholesteryl acetate as described above. The results, given in Table 25, showed that the specific activity of the sample remained

Table 25

Cocrystallization of Acetate Derivative of [3α-3H]-Cholesterol Recovered from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholesterol-7-en-3β-ol in the Presence of DPN and TPNH with Authentic Cholesteryl Acetate

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>2,290</td>
</tr>
<tr>
<td>Once from methanol</td>
<td>2,240</td>
</tr>
<tr>
<td>Twice from methanol</td>
<td>2,260</td>
</tr>
<tr>
<td>Once from acetone-water</td>
<td>2,260</td>
</tr>
<tr>
<td>Twice from acetone-water</td>
<td>2,250</td>
</tr>
</tbody>
</table>
essentially unchanged. This material was also subjected to gas-liquid radiochromatographic analysis as before, along with unlabeled cholesteryl acetate. The radioactivity showed the same chromatographic mobility as that of authentic cholesteryl acetate (Figure 82). The remaining material was hydrolyzed to free sterol by alkaline hydrolysis (10 ml of 15% ethanolic KOH) and further purified via the dibromide purification procedure, as described in Methods. The results, given in Table 26, showed the specific activity before and after dibromide treatment remained essentially constant.

Table 26

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before dibromide purification</td>
<td>3,010</td>
</tr>
<tr>
<td>After dibromide purification</td>
<td>3,040</td>
</tr>
</tbody>
</table>

The recovered material from dibromide treatment was dissolved in 2 ml of redistilled dry pyridine, and the solution was chilled to \(4^\circ\O\). This was added to a solution of 40 mg of chromium trioxide in 2 ml of pyridine, previously chilled to \(4^\circ\O\). Oxidation was allowed to proceed at \(4^\circ\O\) for 24 hours, at the end of which time the mixture was diluted with 10 volumes of distilled water (40 ml). The product was extracted with four 50 ml portions of ether, the combined extracts were washed with three 200 ml portions of water, and dried over anhydrous sodium sulfate. The results, given in Table 27, indicated that
Figure 82. Gas-liquid radiochromatogram of the contents of fractions 132-165 from the alumina column shown in Figure 79, corresponding chromatographically to the mobility of cholesteryl acetate. The mass peak is due to authentic unlabeled cholesteryl acetate.
Table 27

Chromium Trioxide Oxidation of [3α-3H]-Cholesterol Recovered from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol in the Presence of DPN and TPNH

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before CrO₃ oxidation</td>
<td>2,740</td>
</tr>
<tr>
<td>After CrO₃ oxidation</td>
<td>60</td>
</tr>
</tbody>
</table>

CrO₃ treatment resulted in the loss of tritium label from the radioactive sample. Since CrO₃ treatment oxidized the 3-hydroxyl group to 3-ketone, this result showed that the tritium label of the isolated products remained at C-3.

Percent recovery of each of these isolated sterols, in relation to the total amount of radioactivity incubated, is given in Table 28.

Table 28

Percent Recovery of Labeled Sterols Isolated from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol with Rat Liver Microsomes in the Presence of DPN and TPNH

<table>
<thead>
<tr>
<th>Sterols Isolated</th>
<th>% Recovery of Total Incubated Substrate</th>
<th>% Recovery of Total Reacted Substrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-8(14)-en-3β-ol</td>
<td>8.6%</td>
<td>11.5%</td>
</tr>
<tr>
<td>Cholest-7-en-3β-ol</td>
<td>8.6%</td>
<td>11.5%</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>29.6%</td>
<td>39.4%</td>
</tr>
<tr>
<td>Total</td>
<td>46.8%</td>
<td>62.4%</td>
</tr>
</tbody>
</table>

*Total reacted substrate = total incubated substrate - unreacted substrate recovered from silicic acid-Super Cel column.
Experiment 2

The livers (44.4 g) of four male rats (Sprague-Dawley strain; 200-250 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (444 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by differential centrifugation. This was then centrifuged at 105,000 x g for 60 minutes. The pellets were resuspended in fresh buffer and centrifugation was carried out as before, at 105,000 x g for 60 minutes. The washed microsomal pellet was isolated, resuspended in 21 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride, and protein concentration was determined by Lowry method (Lowry et al., 1951) to be 18.7 mg/ml.

To a test tube containing 2 ml of the microsomal suspension prepared as described above, DPN (3 mg) and a TPNH generating system (0.9 ml) were added to give final concentrations of 1.5 mM of DPN and 1.2 mM of TPN. [3α-^3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol (1.72 x 10^6 cpm, 5.5 µg) in propylene glycol (0.05 ml) was added last. Aerobic incubation was carried out for 30 minutes at 37°C with constant shaking.

At the end of incubation, acetone (40 ml) was added and the mixture was homogenized. The resulting coagulum was removed by filtration and was washed several times with fresh acetone. The filtrates were evaporated to dryness and the resulting residue (1.74 x 10^6 cpm; 101% recovery of applied radioactivity) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 7 mg of unlabeled cholesterol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at
fraction 113), fractions 1.4 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 113; 0.07 ml/min) were collected. The results (Figure 83) showed that approximately 21% of the recovered radioactivity corresponded chromatographically to the mobility of cholesterol (fractions 58-64), while another 41% of the eluted tritium corresponded in chromatographic mobility to that of the substrate (peak at fraction 170).

The contents of fractions 58-74 were pooled and acetylated with acetic anhydride-pyridine (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (1.65 x 10^5 cpm) were applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8,14-cholestadiene (6 mg), 3β-acetoxy-Δ^5,7-cholestadiene (6 mg), and cholesteryl acetate (4 mg). The steryl acetates were eluted with hexane-benzene (7:3, v/v) and fractions of 4.2 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 70; 0.21 ml/min) were collected. The elution pattern (Figure 84) showed that most (88%) of the recovered radioactivity cochromatographed with cholesteryl acetate (fractions 16-25).

Fractions 16-25 were pooled and the steryl acetates (1.20 x 10^5 cpm) were applied to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8(14)-cholestene (6 mg), 3β-acetoxy-Δ^7-cholestene (5 mg), and cholesteryl acetate (3 mg). The column was eluted with hexane-benzene (9:1, v/v) and fractions 2.6 ml in volume (20 minutes per fraction; 0.13 ml/min) were collected. The resulting chromatogram is shown in Figure 85. Approximately 33% of the recovered radioactivity was associated with 3β-acetoxy-Δ^8(14)-cholestene (fractions 37-44), 45% cochromatographed with 3β-acetoxy-
Figure 83. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from the second aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesta-7-en-3β-ol with washed rat liver microsomes in the presence of added DPN and TPNH generating system. o-o, Tritium radioactivity. Δ-Δ, Cholesterol measured colorimetrically.
Figure 84. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from the second aerobic incubation of \([3\alpha-{\text{H}}]-14\alpha\text{-hydroxymethyl-5\alpha-cholestan-7-en-3\beta-ol}\) with washed rat liver microsomes in the presence of added DPN and TPNH generating system. ◦-◦, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-\(\Delta^8,14\)-cholestadiene, and the third is due to 3β-acetoxy-\(\Delta^5,7\)-cholestadiene.
Figure 85. Alumina-Super Cel-silver nitrate column chromatogram of the acetate derivatives of monounsaturated sterols recovered from the second aerobic incubation of \([3\alpha-\text{H}]-14\alpha\text{-hydroxymethyl-5α-cholest-7-en-3β-ol}\) with washed rat liver microsomes in the presence of added DPN and TPNH generating system. o-o, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to 3β-acetoxy-\(\Delta^7\)-cholestene, the second is due to 3β-acetoxy-\(\Delta^7\)-cholestene, and the third is due to cholesteryl acetate.
$\Delta^7$-cholestene (fractions 56-77), and 21% corresponded in chromatographic mobility to that of cholesteryl acetate (fractions 106-134).

The contents of fractions 37-44 were pooled and subjected to repetitive cocrystallization with authentic unlabeled $3\beta$-acetoxy-$\Delta^{8(14)}$-cholestene (25 mg) from methanol and from acetone-water. The results, given in Table 29, showed essentially a single component cocrystallizing with the added carrier. Another portion of fractions 37-44 was subjected to gas-liquid radiocchromatographic analysis on a 3% OV-17 column, along with unlabeled $3\beta$-acetoxy-$\Delta^{8(14)}$-cholestene and $3\beta$-acetoxy-$\Delta^7$-cholestene. The radioactive material showed chromatographic mobility similar to that of $3\beta$-acetoxy-$\Delta^{8(14)}$-cholestene (Figure 86).

Table 29

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>930</td>
</tr>
<tr>
<td>Once from methanol</td>
<td>920</td>
</tr>
<tr>
<td>Twice from methanol</td>
<td>920</td>
</tr>
<tr>
<td>Once from acetone-water</td>
<td>930</td>
</tr>
<tr>
<td>Twice from acetone-water</td>
<td>910</td>
</tr>
</tbody>
</table>

The pooled material from fractions 56-77 was subjected to repetitive cocrystallization with 30 mg of authentic unlabeled $3\beta$-acetoxy-$\Delta^7$-cholestene as described previously. The results are given in Table 30. The specific activity after each recrystallizations remained es-
Figure 86. Gas-liquid radiochromatographic analysis of pooled contents of fractions 37-44 from the alumina column shown in Figure 85. The first mass peak is due to authentic 3β-acetoxy-Δ8(14)-cholestene and the second is due to 3β-acetoxy-Δ7-cholestene.
Table 30

Cocrystallization of Acetate Derivative of [3α-3H]-Cholest-7-en-3β-ol Recovered from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol in the Presence of DPN and TPNH with Authentic 3β-Acetoxy-Δ7-cholestene

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>1,460</td>
</tr>
<tr>
<td>Once from methanol</td>
<td>1,420</td>
</tr>
<tr>
<td>Twice from methanol</td>
<td>1,450</td>
</tr>
<tr>
<td>Once from acetone-water</td>
<td>1,430</td>
</tr>
<tr>
<td>Twice from acetone-water</td>
<td>1,420</td>
</tr>
</tbody>
</table>

essentially constant. Gas-liquid radiochromatographic analysis of this tritiated material showed (Figure 87) evidence for the presence of radioactivity with mobility similar to that of 3β-acetoxy-Δ7-cholesterol.

The contents of fractions 106-134, corresponding to cholesteryl acetate on the alumina column, were pooled and subjected to gas-liquid radiochromatographic analysis, along with authentic cholesteryl acetate. The radioactivity emerged in a single peak which corresponded to the chromatographic mobility of cholesteryl acetate (Figure 88). Another portion of this material was hydrolyzed to free sterol by alkaline hydrolysis (10 ml of 15% ethanolic KOH) and the cholesterol was purified via formation of the dibromide and subsequent regeneration of cholesterol. The results (Table 31) showed that the specific activity of cholesterol before and after dibromide treatment remained essentially constant.

The percent recovery of each of these isolated sterols, in rela-
Figure 87. Gas-liquid radiochromatographic analysis of pooled contents of fractions 56-77 from the alumina column shown in Figure 85. The mass peak is due to authentic unlabeled 3β-acetoxy-Δ⁷-cholestene.
Figure 88. Gas-liquid radiochromatographic analysis of pooled contents of fractions 106-134 from the alumina column shown in Figure 85, corresponding chromatographically to the mobility of cholesteryl acetate. The mass peak is due to authentic unlabeled cholesteryl acetate.
Table 31

Dibromide Purification Results of $[3\alpha-^{3}H]-\text{Cholesterol Recovered from Aerobic Incubation of } [3\alpha-^{3}H]-14\alpha-\text{Hydroxymethyl-5a-cholest-7-en-3beta-ol in the Presence of Added DPN and TPNH for 30 Minutes}$

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before dibromide purification</td>
<td>1,340</td>
</tr>
<tr>
<td>After dibromide purification</td>
<td>1,330</td>
</tr>
</tbody>
</table>

tion to the total amount of incubated radioactivity, is tabulated in Table 32.

Table 32

Percent Recovery of Labeled Sterols Isolated from Aerobic Incubation of $[3\alpha-^{3}H]-14\alpha-\text{Hydroxymethyl-5a-cholest-7-en-3beta-ol}$ with Rat Liver Microsomes for 30 Minutes in the Presence of DPN and TPNH

<table>
<thead>
<tr>
<th>Sterols, Isolated</th>
<th>% Recovery of Total Incubated Substrate</th>
<th>% Recovery of Total Reacted Substrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-8(14)-en-3beta-ol</td>
<td>6.1%</td>
<td>10.3%</td>
</tr>
<tr>
<td>Cholest-7-en-3beta-ol</td>
<td>8.4%</td>
<td>14.1%</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>3.9%</td>
<td>6.6%</td>
</tr>
<tr>
<td>Total</td>
<td>18.4%</td>
<td>31.0%</td>
</tr>
</tbody>
</table>

*Total reacted substrate = total incubated substrate - unreacted substrate recovered from silicic acid-Super Cel column.

C. IN THE PRESENCE OF ONLY TPNH GENERATING SYSTEM

Experiment 1

The livers (38.6 g) of four male rats (Sprague-Dawley strain; 150-200 g each) were homogenized in a medium of ice-cold potassium phosphate
buffer (386 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by differential centrifugation. This was then centrifuged at 105,000 x g for 60 minutes. The pellets were resuspended in 190 ml of fresh buffer (0.1 M, pH 7.4) and centrifuged again at 105,000 x g for 60 minutes. The washed microsomal pellet was resuspended in 15 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride, giving a final protein concentration of 15.2 mg/ml.

To a test tube containing 2 ml of the above microsomal suspension, TPNH generator (0.9 ml) was added to give a final concentration of 1.2 mM of TPN. [3α−3H]-14α-Hydroxymethyl-5α-cholestan-7-en-3β-ol (1.72 x 10⁶ cpm, 5.5 µg) in propylene glycol (0.05 ml) was added last. Aerobic incubation was carried out for 2 hours at 37° with constant shaking.

At the end of incubation, acetone (40 ml) was added and the mixture was homogenized. The resulting coagulum was removed by filtration; the filter paper and the coagulum were washed several times with fresh acetone. The filtrates were evaporated to dryness under reduced pressure, and the resulting residue (1.76 x 10⁶ cpm; 102.3% recovery of incubated radioactivity) was chromatographed on a column (1 x 100 cm) of silicic acid–Super Cel, along with 6 mg of unlabeled cholesterol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent (changed to benzene–ether (8:2, v/v) at fraction 130), fractions 1.6 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 130; 0.08 ml/min) were collected. The results are shown in Figure 89. Approximately 96% of the recovered radioactivity corresponded chromatographically to the mobility of
Figure 89. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5a-cholest-7-en-3β-ol with washed rat liver microsomes in the presence of added TPNH generating system. o-o, Tritium radioactivity. D-D, Cholesterol measured colorimetrically.
cholesterol (fractions 55-70).

The contents of fractions 55-70 were pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The resulting steryl acetates (1.02 x 10^6 cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8,14-cholestadiene (6 mg), 3β-acetoxy-Δ^5,7-cholestadiene (6 mg), and cholesteryl acetate (5 mg). Using hexane-benzene (7:3, v/v) as the eluting solvent, fractions 5.4 ml in volume (20 minutes per fraction; 0.27 ml/min) were collected. The resulting chromatogram (Figure 90) showed that approximately 83% of the recovered radioactivity corresponded in chromatographic mobility to cholesteryl acetate or monoene steryl acetates (peak at fraction 27).

The contents of fractions 22-38 were pooled and applied (5.21 x 10^5 cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8(14)-cholestene (6 mg), 3β-acetoxy-Δ^7-cholestene (5 mg), and cholesteryl acetate (5 mg). The column was eluted with hexane-benzene (9:1, v/v) and fractions 3.2 ml in volume (20 minutes per fraction; 0.16 ml/min) were collected. The elution pattern (Figure 91) showed three regions of radioactivity. The first peak (1.9%) corresponded chromatographically to the mobility of 3β-acetoxy-Δ^8(14)-cholestene (fractions 31-38), the second (50.9%) corresponded to 3β-acetoxy-Δ^7-cholestene (fractions 55-78), and the third (47.3%) cochromatographed with cholesteryl acetate (fractions 101-137).

The pooled material, corresponding to the chromatographic mobility of authentic 3β-acetoxy-Δ^8(14)-cholestene on the alumina column
Figure 90. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from aerobic incubation of [3α-3H]14α-hydroxymethyl-5α-cholest-7-en-3β-ol with washed rat liver microsomes in the presence of added TPNH generating system. o-o, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ6,14-cholestanol, and the third is due to 3β-acetoxy-Δ5,7-cholestadiene.
Figure 91. Alumina-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monounsaturated sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with washed rat liver microsomes in the presence of added TPNH generating system. o-o, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to 3β-acetoxy-Δ8(14)-cholestenone, the second is due to 3β-acetoxy-Δ7-cholestenone, and the third is due to cholesteryl acetate.
(fractions 31-38), did not contain enough radioactivity for further characterization.

The contents of fractions 55-78 were pooled and subjected to repetitive cocrystallization with authentic unlabeled 3β-acetoxy-Δ⁷-cholestene (30 mg) from methanol and from acetone-water. The results, given in Table 33, showed essentially a single component cocrystallization with the added carrier. Appropriate aliquot was also removed and subjected to gas-liquid radiochromatographic analysis on a 3% OV-17 column, along with unlabeled 3β-acetoxy-Δ⁷-cholestene. The radioactive material showed chromatographic mobility similar to that of authentic 3β-acetoxy-Δ⁷-cholestene (Figure 92).

The pooled material from fractions 101-137 was subjected to gas-liquid radiochromatographic analysis, along with authentic cholesteryl acetate. The radioactivity emerged in a single peak which corresponded to the chromatographic mobility of cholesteryl acetate (Figure 93).
Figure 92. Gas-liquid radiochromatographic analysis of the contents of fractions 55-78 from the alumina column shown in Figure 91, corresponding to the chromatographic mobility of authentic 3β-acetoxy-Δ⁷-cholestene. The mass peak is due to unlabeled authentic 3β-acetoxy-Δ⁷-cholestene.
Figure 93. Gas-liquid radiochromatogram of the contents of fractions 101-137 from the alumina column shown in Figure 91, corresponding chromatographically to the mobility of cholesteryl acetate. The mass peak is due to authentic cholesteryl acetate.
A portion of fractions 101-137 was hydrolyzed to free sterol by alkaline hydrolysis (10 ml of 15% ethanolic KOH) and the resulting sterol was purified via formation of the dibromide. The results (Table 34) showed the specific activity before and after dibromide treatment remained essentially unchanged.

Table 34

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before dibromide purification</td>
<td>5,520</td>
</tr>
<tr>
<td>After dibromide purification</td>
<td>5,590</td>
</tr>
</tbody>
</table>

The percent recovery of each of these sterols is tabulated in Table 35.

Table 35

<table>
<thead>
<tr>
<th>Sterols Isolated</th>
<th>% Recovery of Total Incubated Substrate</th>
<th>% Recovery of Total Reacted Substrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-8(14)-en-3β-ol</td>
<td>1.5%</td>
<td>1.5%</td>
</tr>
<tr>
<td>Cholest-7-en-3β-ol</td>
<td>39.3%</td>
<td>40.6%</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>36.5%</td>
<td>37.7%</td>
</tr>
<tr>
<td>Total</td>
<td>77.3%</td>
<td>79.8%</td>
</tr>
</tbody>
</table>

*Total reacted substrate = total incubated substrate− unreacted substrate recovered from silicic acid-Super Cel column.
Experiment 2

The livers (43 g) of six male rats (Sprague-Dawley strain; 150-200 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (430 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by differential centrifugation. This was then centrifuged at 105,000 x g for 60 minutes in a Beckman L2-65B Ultracentrifuge as described previously. The pellets were removed and re-suspended in 215 ml of fresh buffer (0.1 M, pH 7.4) using a teflon-on-glass homogenizer operated manually. The suspension was recentrifuged at 105,000 x g for 60 minutes. The washed microsomal pellets were finally resuspended in 16 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride to give a final protein concentration of 18.4 mg/ml.

To a test tube containing 2 ml of this microsomal preparation, TPNH generating system (0.9 ml) was added to give a final concentration of 1.2 mM of TPN. [3α-^3H]-14α-Hydroxymethyl-5α-cholest-7-en-3α-ol (1.72 x 10^6 cpm, 5.5 μg) in propylene glycol (0.05 ml) was added last. Aerobic incubation was carried out for 2 hours at 37°C with constant shaking.

At the end of incubation, acetone (40 ml) was added and the mixture was homogenized. The resulting coagulum was removed by filtration; the filter paper and the coagulum were washed several times with fresh acetone. The combined filtrates were evaporated to dryness and the resulting residue (1.75 x 10^6 cpm; 101.7% recovery of incubated radioactivity) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 8 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to
benzene-ether (8:2, v/v) at fraction 134), fractions 1.8 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 134; 0.09 ml/min) were collected. The results of this column chromatography are shown in Figure 94. A major portion (51%) of the eluted radioactivity cochromatographed with cholesterol (fractions 52-65), and another 32% of the eluted radioactivity showed the chromatographic mobility of the unreacted substrate (peak centered at fraction 167).

The contents of fractions 52-65 were pooled and acetylated with dry pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (5.05 x 10^5 cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled 3β-acetoxy-Δ^{8,14}-cholestadiene (6 mg), 3β-acetoxy-Δ^{5,7}-cholestadiene (7 mg), and cholesteryl acetate (6 mg). The column was eluted with hexane-benzene (7:3, v/v) and fractions 4.0 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 69; 0.2 ml/min) were collected. The results, shown in Figure 95, indicated that most (83.2%) of the recovered radioactivity corresponded chromatographically to the mobility of cholesteryl acetate (fractions 14-28).

Fractions 14-28 were pooled and applied (2.64 x 10^5 cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^{8(14)}-cholestene (6 mg), 3β-acetoxy-Δ^{7}-cholestene (6 mg), and cholesteryl acetate (6 mg). Using hexane-benzene (9:1, v/v) as the eluting solvent (changed to hexane-benzene (8:2, v/v) at fraction 103), fractions 2.6 ml in volume (20 minutes per fraction; 0.13 ml/min) were collected. The elution profile is
Figure 94. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from the second aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesten-7-en-3β-ol with washed rat liver microsomes in the presence of added TPNH generating system. ••, Tritium radioactivity. ••, Cholesterol measured colorimetrically.
Figure 95. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from the second aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with washed rat liver microsomes in the presence of added TPNH generating system. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxo-Δ6,14Δ, the third is due to 3β-acetoxo-Δ5,7-cholestadiene.
shown in Figure 96. Approximately 25% of the recovered radioactivity cochromatographed with authentic 3β-acetoxy-Δ8(14)-cholestene (peak at fraction 48), another 23% was associated with 3β-acetoxy-Δ7-cholesterol (peak at fraction 71), and 52.5% corresponded chromatographically to the mobility of cholesteryl acetate (peak at fraction 127).

The contents of fractions 46-56 were pooled and subjected to repetitive cocrystallization with 20 mg of authentic unlabeled 3β-acetoxy-Δ8(14)-cholestene from two different solvent systems. The results are given in Table 36, and they showed essentially a single component cocrystallizing with the added carrier. Appropriate aliquot was then removed and subjected to gas-liquid radiochromatographic analysis on a 3% OV-17 column, along with unlabeled 3β-acetoxy-Δ8(14)-cholestene and 3β-acetoxy-Δ7-cholesterol. The radioactive material showed chromatographic mobility similar to that of authentic 3β-acetoxy-Δ8(14)-cholestene (Figure 97).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>1,310</td>
</tr>
<tr>
<td>Once from methanol</td>
<td>1,270</td>
</tr>
<tr>
<td>Twice from methanol</td>
<td>1,340</td>
</tr>
<tr>
<td>Once from acetone-water</td>
<td>1,290</td>
</tr>
<tr>
<td>Twice from acetone-water</td>
<td>1,290</td>
</tr>
</tbody>
</table>
Figure 96. Alumina-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monounsaturated sterols recovered from the second aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with washed rat liver microsomes in the presence of added TPNI generating system. o-o, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to 3β-acetoxy-Δ7(14)-cholestene, the second is due to 3β-acetoxy-Δ7-cholestene, and the third is due to cholesteryl acetate.
Figure 97. Gas-liquid radiochromatogram of the contents of fractions 46-56 from the alumina column shown in Figure 96, corresponding chromatographically to the mobility of 3β-acetoxy-Δ9(14)-cholestan. The first mass peak is due to 3β-acetoxy-Δ9(14)-cholestane and the second is due to 3β-acetoxy-Δ7'-cholestene.
The material corresponding chromatographically to 3β-acetoxy-Δ⁷-cholestene on the alumina column (fractions 73-92) was combined and subjected to cocrystallization with 25 mg of unlabeled 3β-acetoxy-Δ⁷-cholestene. The mixture was recrystallized twice from methanol and twice from acetone-water. The specific activity of the crystals remained essentially constant, as shown in Table 37. This material was also subjected to gas-liquid radiochromatographic analysis on a 3% OV-17 column, along with unlabeled 3β-acetoxy-Δ⁷-cholestene. The radioactivity emerged in a single peak which corresponded in chromatographic mobility to authentic 3β-acetoxy-Δ⁷-cholestene (Figure 98).

A portion of the pooled material (fractions 124-140) from the alumina column was subjected to cocrystallization with 30 mg of authentic unlabeled cholesteryl acetate from methanol and from acetone-water. The results (Table 38) showed no significant change in specific activity. This material was also subjected to gas-liquid radiochromato-

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>1,060</td>
</tr>
<tr>
<td>Once from methanol</td>
<td>1,050</td>
</tr>
<tr>
<td>Twice from methanol</td>
<td>1,070</td>
</tr>
<tr>
<td>Once from acetone-water</td>
<td>1,100</td>
</tr>
<tr>
<td>Twice from acetone-water</td>
<td>1,050</td>
</tr>
</tbody>
</table>
Figure 98. Gas-liquid radiochromatogram of the contents of fractions 73-92 from the alumina column shown in Figure 96, corresponding in chromatographic mobility to authentic 3β-acetoxy-Δ⁴'-cholestene. The mass peak is due to authentic unlabeled 3β-acetoxy-Δ⁴'-cholestene.
Table 38
Cocrystallization of \(3\alpha^-{\text{H}}\)-Cholesteryl Acetate Recovered from Aerobic Incubation of \(3\alpha^-{\text{H}}\)-14\(\alpha\)-Hydroxymethyl-5\(\alpha\)-cholest-7-en-3\(\beta\)-ol in the Presence of TPNH with Authentic Unlabeled Cholesteryl Acetate

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>3,900</td>
</tr>
<tr>
<td>Once from methanol</td>
<td>3,810</td>
</tr>
<tr>
<td>Twice from methanol</td>
<td>3,880</td>
</tr>
<tr>
<td>Once from acetone-water</td>
<td>3,810</td>
</tr>
<tr>
<td>Twice from acetone-water</td>
<td>3,870</td>
</tr>
</tbody>
</table>

graphic analysis as described before, along with unlabeled cholesteryl acetate. The resulting chromatogram is shown in Figure 99. The recovered radioactivity showed chromatographic mobility similar to that of authentic cholesteryl acetate. The remaining material was hydrolyzed to free sterol by alkaline hydrolysis (10 ml of 15% ethanolic KOH) and further purified via the dibromide purification procedure, as described previously in Methods. The results, given in Table 39, showed that the specific activity before and after dibromide formation

Table 39
Dibromide Purification Results of \(3\alpha^-{\text{H}}\)-Cholesterol Recovered from Aerobic Incubation of \(3\alpha^-{\text{H}}\)-14\(\alpha\)-Hydroxymethyl-5\(\alpha\)-cholest-7-en-3\(\beta\)-ol in the Presence of TPNH

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before dibromide purification</td>
<td>2,230</td>
</tr>
<tr>
<td>After dibromide purification</td>
<td>2,230</td>
</tr>
</tbody>
</table>
Figure 99. Gas-liquid radiochromatogram of the contents of fractions 124-130 from the alumina column shown in Figure 96, corresponding to the expected mobility of cholesteryl acetate. The mass peak is due to authentic unlabeled cholesteryl acetate.
remained constant.

The percent recovery of each of these isolated sterols is tabulated in Table 40.

Table 40

Percent Recovery of Labeled Sterols Isolated from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-38-ol with Washed Rat Liver Microsomes in the Presence of TPNH Generating System

<table>
<thead>
<tr>
<th>Sterols</th>
<th>% Recovery of Isolated</th>
<th>% Recovery of Total Incubated Substrate</th>
<th>% Recovery of Total Reacted Substrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-8(14)-en-38-ol</td>
<td>10.6%</td>
<td>15.5%</td>
<td></td>
</tr>
<tr>
<td>Cholest-7-en-38-ol</td>
<td>9.5%</td>
<td>14.0%</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>22.2%</td>
<td>32.7%</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>42.3%</td>
<td>62.2%</td>
<td></td>
</tr>
</tbody>
</table>

*Total reacted substrate = total incubated substrate - unreacted substrate recovered from silicic acid-Super Cel column.

D. IN THE PRESENCE OF ONLY ADDED DPN

Experiment 1

The livers (38.6 g) of four male rats (Sprague-Dawley strain; 150-200 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (386 ml; 0.1 M, pH 7.4). The resulting homogenate was then centrifuged at 10,000 x g for 30 minutes. The 10,000 x g supernatant fraction was centrifuged at 105,000 x g for 60 minutes and the resulting pellets were resuspended in fresh buffer (190 ml; 0.1 M, pH 7.4). The suspension was recentrifuged at 105,000 x g for 60 minutes and the washed rat liver microsomal pellet was suspended in 15 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM mag-
nesium chloride to yield a final protein concentration of 15.2 mg/ml.

To a test tube containing 2 ml of the rat liver microsomal preparation, DPN (3 mg) was added to give a final concentration of 1.5 mM of DPN. [3α-3H]-14α-Hydroxymethyl-5α-cholesterol-7-en-3β-ol (1.72 x 10⁶ cpm, 5.5 μg) in propylene glycol (0.05 ml) was added last. Aerobic incubation was carried out for 2 hours at 37°C with constant shaking.

At the end of incubation, 40 ml of acetone was added and the mixture was homogenized. The resulting coagulum was removed by filtration; the filter paper and the coagulum were washed several times with fresh acetone. The filtrates were evaporated to dryness and the residue (1.72 x 10⁶ cpm; 100% recovery of incubated radioactivity) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 6 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 130), fractions 1.2 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 130; 0.06 ml/min) were collected. The results are shown in Figure 100. Approximately 30% of the eluted radioactivity resided in a peak slightly more polar than cholesterol (fractions 58-70), while another 45% of the recovered radioactivity corresponded chromatographically to the unreacted substrate (peak centered at fraction 190).

The contents of fractions 58-70 were pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled sterol acetates (2.46 x 10⁵ cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ⁸,14-cholestadiene (6 mg), 3β-acetoxy-Δ₅,₇-cholestadiene (6 mg), and cholesteryl acetate (5 mg).
Figure 100. Silicic acid-Super Cel column chromatographic analysis of steroids recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with washed rat liver microsomes in the presence of added DPN. △-△, Tritium radioactivity. ○-○, Cholesterol measured colorimetrically.
The column was eluted with hexane-benzene (7:3, v/v) and fractions 5.6 ml in volume (20 minutes per fraction; 0.28 ml/min) were collected. The resulting chromatogram (Figure 101) indicated that essentially all (99.5%) of the recovered radioactivity was eluted a little before cholesteryl acetate (fractions 16-23).

The contents of fractions 16-23 were pooled and applied (1.83 x 10⁵ cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ⁸(14)-cholestene (5 mg), 3β-acetoxy-Δ⁷-cholestene (5 mg), and cholesteryl acetate (5 mg). Using a mixture of hexane and benzene (9:1, v/v) as the eluting solvent, fractions 4.2 ml in volume (20 minutes per fraction; 0.21 ml/min) were collected. The results of this column chromatography are shown in Figure 102. Most (91.6%) of the recovered radioactivity corresponded chromatographically to the mobility of 3β-acetoxy-Δ⁸(14)-cholestene (peak at fraction 30). Approximately 8.2% of the radioactivity was eluted just before 3β-acetoxy-Δ⁷-cholestene (peak at fraction 46), corresponding to the chromatographic mobility of 3β-acetoxy-Δ⁸(9)-cholestene.

Fractions 25-33, corresponding chromatographically to authentic 3β-acetoxy-Δ⁸(14)-cholestene on the alumina column, were pooled and subjected to repetitive cocrystallization with 20 mg of authentic unlabeled 3β-acetoxy-Δ⁸(14)-cholestene from methanol and from acetone-water. The results, given in Table 41, showed essentially a single component cocrystallizing with the added carrier.

Appropriate aliquot of the above material was then subjected to gas-liquid radiochromatographic analysis on a 3% OV-17 column, along with unlabeled 3β-acetoxy-Δ⁸(14)-cholestene and 3β-acetoxy-Δ⁷-cholestene. The radioactive material showed chromatographic mobility simi-
Figure 101. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with washed rat liver microsomes in the presence of added DPN. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ6,14-cholesta-5,7-diene, and the third is due to 3β-acetoxy-Δ5,7-cholesta-5,7-diene.
Figure 102. Alumina-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monounsaturated sterols recovered from aerobic incubation of [3α-3H]-1α-hydroxymethyl-5α-cholest-7-en-3β-ol with washed rat liver microsomes in the presence of added DPN. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to 3β-acetoxy-Δ7(14)-cholestenone, the second is due to 3β-acetoxy-Δ5'-cholestenone, and the third is due to cholesteryl acetate.
Table 41

Coocrystallization of Acetate Derivative of \([3\alpha-^3H]\)-Cholest-8(14)-en-3β-ol Recovered from Aerobic Incubation of \([3\alpha-^3H]\)-14α-Hydroxymethyl-5α-cholester-7-en-3β-ol in the Presence of Added DPN with Authentic 3β-Acetoxy-Δ8(14)-cholestan

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>3,750</td>
</tr>
<tr>
<td>Once from methanol</td>
<td>3,740</td>
</tr>
<tr>
<td>Twice from methanol</td>
<td>3,740</td>
</tr>
<tr>
<td>Once from acetone-water</td>
<td>3,760</td>
</tr>
<tr>
<td>Twice from acetone-water</td>
<td>3,750</td>
</tr>
</tbody>
</table>

lar to that of authentic 3β-acetoxy-Δ8(14)-cholestan (Figure 103).

The percent recovery of isolated sterols from this incubation is tabulated in Table 42.

Table 42

Percent Recovery of Labeled Sterols Isolated from Aerobic Incubation of \([3\alpha-^3H]\)-14α-Hydroxymethyl-5α-cholester-7-en-3β-ol with Washed Rat Liver Microsomes in the Presence of Added DPN

<table>
<thead>
<tr>
<th>Sterol</th>
<th>% Recovery of Isolated</th>
<th>% Recovery of Total Incubated Substrate</th>
<th>% Recovery of Total Reacted Substrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-8(14)-en-3β-ol</td>
<td>27.3%</td>
<td>49.7%</td>
<td></td>
</tr>
</tbody>
</table>

*Total reacted substrate = total incubated substrate - unreacted substrate recovered from silicic acid-Super Cel column.

Experiment 2

The livers (43 g) of six male rats (Sprague-Dawley strain; 150-200 g each) were homogenized in a medium of ice-cold potassium phos-
Figure 103. Gas-liquid radiochromatogram of the contents of fractions 25-33 from the alumina column shown in Figure 102, corresponding chromatographically to the mobility of authentic 3β-acetoxy-Δ\(^8\)(14)-cholestene. The first mass peak is due to 3β-acetoxy-Δ\(^8\)(14)-cholestene and the second is due to 3β-acetoxy-Δ\(^7\)-cholestene.
phate buffer (430 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by differential centrifugation. This was then centrifuged at 105,000 x g for 60 minutes as described previously. The pellets were removed and resuspended in 215 ml of potassium phosphate buffer (0.1 M, pH 7.4). The suspension was recentrifuged at 105,000 x g for 60 minutes and the washed rat liver microsomal pellet was again resuspended in 16 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride to give a final protein concentration of 18.4 mg/ml.

To a test tube containing 2 ml of the rat liver microsomal suspension prepared as described above, DPN was added to give a final concentration of 1.5 mM of DPN. [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol (1.72 x 10⁶ cpm, 5.5 μg) in propylene glycol (0.05 ml) was added last. Aerobic incubation was carried out for 2 hours at 37° with constant shaking.

At the end of incubation, acetone (40 ml) was added and the mixture was homogenized. The resulting coagulum was removed by filtration; the filter paper and the coagulum were washed several times with fresh acetone. The combined filtrates were evaporated to dryness and the residue (1.70 x 10⁶ cpm; 98.8% recovery of incubated radioactivity) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 8 mg of unlabeled cholesterol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 134), fractions 1.7 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 134; 0.085 ml/min) were collected. The results of this column chromatography (Figure 104) indicated that approximately 23%
Figure 104. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from the second aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholester-7-en-3β-ol with washed rat liver microsomes in the presence of added DPN. ○-○, Tritium radioactivity. △-△, Cholesterol measured colorimetrically.
of the recovered radioactivity was eluted right after cholesterol (fractions 53-63), while the major region of radioactivity (75%) had the chromatographic mobility of the unreacted substrate (peak centered at fraction 167).

Fractions 53-63 were pooled and acetylated with dry pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (3.02 x 10^5 cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8,14-cholestadiene (6 mg), 3β-acetoxy-Δ^5,7-cholestadiene (7 mg), and cholesteryl acetate (6 mg). Using hexane-benzene (7:3, v/v) as the eluting solvent, fractions 4.6 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 69; 0.23 ml/min) were collected. The resulting elution profile is shown in Figure 105. Almost all (99.3%) of the recovered radioactivity was eluted right before cholesteryl acetate (fractions 13-20).

The contents of fractions 13-20 were pooled and applied (1.92 x 10^5 cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8(14)-cholestene (6 mg), 3β-acetoxy-Δ^8(9)-cholestene (4 mg), 3β-acetoxy-Δ^7-cholestene (5 mg), and cholesteryl acetate (5 mg). The column was eluted with hexane-benzene (9:1, v/v) and fractions 2.5 ml in volume (20 minutes per fraction; 0.125 ml/min) were collected. The results (Figure 106) indicated that most (96%) of the recovered radioactivity corresponded chromatographically to the mobility of authentic 3β-acetoxy-Δ^8(14)-cholestene (fractions 43-56). A small portion (4.2%) of the eluted radioactivity corresponded very closely to the chromatographic mobility of 3β-acetoxy-Δ^8(9)-cholestene (peak centered at fraction 73).
Figure 105. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from the second aerobic incubation of \([3\alpha-\text{H}]-14\alpha\text{-hydroxymethyl}-5\alpha\text{-cholest-7-en-3\beta-ol} with washed rat liver microsomes in the presence of added DPN. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ\(^{6,14}\)-cholestadiene, and the third is due to 3β-acetoxy-Δ\(^{5,7}\)-cholestadiene.
Figure 106. Alumina-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monguunsaturated sterols recovered from the second aerobic incubation of \([3\alpha-^3H]\)-1\(\alpha\)-hydroxymethyl-5\(\alpha\)-cholest-7-en-3\(\beta\)-ol with washed rat liver microsomes in the presence of added DPN. \(\triangle\triangle\triangle\), Tritium radioactivity. \(\bigtriangleup\bigtriangleup\bigtriangleup\), Steryl acetate measured colorimetrically. The first peak is due to 3\(\beta\)-acetoxy-\(\Delta(14)\)-cholestenone, the second is due to 3\(\beta\)-acetoxy-\(\Delta(9)\)-cholestenone, the third is due to 3\(\beta\)-acetoxy-\(\Delta(1)\)-cholestenone, and the fourth is due to cholesteryl acetate.
The contents of fractions 43-56 were pooled and subjected to repetitive cocrystallization with authentic unlabeled 3β-acetoxy-Δ8(14)-cholestene (20 mg) from methanol and from acetone-water. The results, given in Table 43, showed essentially a single component cocrystallizing with the added carrier. A portion of this material was also

Table 43

Cocrystallization of Acetate Derivative of [3α-3H]-Cholest-8(14)-en-3β-ol Recovered from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol in the Presence of Added DPN with Authentic 3β-Acetoxy-Δ8(14)-cholestene

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>4,650</td>
</tr>
<tr>
<td>Once from methanol</td>
<td>4,520</td>
</tr>
<tr>
<td>Twice from methanol</td>
<td>4,560</td>
</tr>
<tr>
<td>Once from acetone-water</td>
<td>4,590</td>
</tr>
<tr>
<td>Twice from acetone-water</td>
<td>4,510</td>
</tr>
</tbody>
</table>

subjected to gas-liquid radiochromatographic analysis on a column of 3% OV-17, along with unlabeled samples of 3β-acetoxy-Δ8(14)-cholestene and 3β-acetoxy-Δ7-cholestene. The radioactive material emerged in a single peak which corresponded to the chromatographic mobility of 3β-acetoxy-Δ8(14)-cholestene (Figure 107).

The contents of fractions 72-75 were pooled and subjected to gas-liquid radiochromatographic analysis on a column of 3% OV-17, along with unlabeled 3β-acetoxy-Δ8(9)-cholestene and 3β-acetoxy-Δ7-cholestene. The radioactive material did not correspond in chromatographic mobility to any of the added samples (Figure 108).
Figure 107. Gas-liquid radiochromatogram of the contents of fractions 43-56 from the alumina column shown in Figure 106, corresponding in chromatographic mobility to authentic 3β-acetoxy-Δ8(14)-cholestene. The first mass peak is due to 3β-acetoxy-Δ8(14)-cholestene and the second is due to 3β-acetoxy-Δ′-cholestene.
Figure 108. Gas-liquid radiochromatogram of the contents of fractions 72-75 from the alumina column shown in Figure 106, corresponding very closely to the chromatographic mobility of $\text{3g}$-acetoxy-$\Delta^\gamma(9)$-cholesten. The first mass peak is due to $\text{3g}$-acetoxy-$\Delta^\gamma(9)$-cholestene and the second is due to $\text{3g}$-acetoxy-$\Delta^\gamma'$-cholestene.
The percent recovery of isolated sterols from this incubation is given in Table 44.

Table 44

<table>
<thead>
<tr>
<th>Sterol</th>
<th>% Recovery of Isolated</th>
<th>% Recovery of Total Incubated Substrate</th>
<th>% Recovery of Total Reacted Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-8(14)-en-3β-ol</td>
<td>21.9%</td>
<td>87.7%</td>
<td></td>
</tr>
</tbody>
</table>

*Total reacted substrate = total incubated substrate - unreacted substrate recovered from silicic acid-Super Cel column.

Experiment 3

The rat liver microsomes used in this experiment were prepared in identical manner as described in Experiment 2. The final protein concentration was, as in Experiment 2, 18.4 mg/ml.

To a test tube containing 2 ml of this microsomal preparation, DPN was added to give a final concentration of 1.5 mM of DPN in the incubation sample. [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol (1.72 x 10^5 cpm, 5.5 µg) in propylene glycol (0.05 ml) was added last. Aerobic incubation was carried out as before for 2 hours at 37° with constant shaking.

At the end of incubation, 15 ml of freshly-prepared 15% ethanolic KOH was added and the mixture was refluxed for 3 hours. After cooling to room temperature, the solution was extracted with four 40 ml portions of petroleum ether, the combined petroleum ether extracts were washed with three 160 ml portions of water, and dried over anhy-
drous sodium sulfate. Appropriate aliquots were removed from each solutions for assay of radioactivity. Percent recovery of radioactivity of the various solutions is given in Table 45.

Table 45

Percent Recovery of Radioactivity of Various Solutions from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol with Washed Rat Liver Microsomes in the Presence of Added DPN

<table>
<thead>
<tr>
<th>Incubation Sample</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous phase</td>
<td>8.4%</td>
</tr>
<tr>
<td>Petroleum ether extract</td>
<td>91.8%</td>
</tr>
<tr>
<td>Total recovery</td>
<td>100.2%</td>
</tr>
</tbody>
</table>

The recovered nonsaponifiable radioactive material (1.58 x 10^6 cpm) was applied to a column (1 x 100 cm) of silicic acid-Super Cel, along with 8 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 134), fractions 1.7 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 134; 0.085 ml/min) were collected. The resulting chromatogram is shown in Figure 109. The recovered radioactivity was evenly distributed among two peaks. The first peak, eluting slightly after cholesterol, contained 45.4% of the recovered tritium. The second peak, corresponding chromatographically to the unreacted substrate, contained 44.5% of the eluted radioactivity (peak at fraction 173).

The pooled material from the first peak (fractions 55-68) was acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (4.80 x 10^5 cpm) were
Figure 109. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from the third aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with washed rat liver microsomes in the presence of added DPN. o-o, Tritium radioactivity. Δ-Δ, Cholesterol measured colorimetrically.
then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of $3\beta$-acetoxy-$\Delta^8(14)$-cholestadiene (6 mg), $3\beta$-acetoxy-$\Delta^5,7$-cholestadiene (7 mg), and cholesteryl acetate (7 mg). Using hexane-benzene (7:3, v/v) as the eluting solvent, fractions 3.6 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 69; 0.18 ml/min) were collected. The resulting chromatogram (Figure 110) indicated that essentially all (99.8%) of the recovered radioactivity was eluted prior to cholesteryl acetate (fractions 16-21).

The contents of fractions 16-21 were pooled and applied (2.50 x $10^5$ cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of $3\beta$-acetoxy-$\Delta^8(14)$-cholestene (6 mg), $3\beta$-acetoxy-$\Delta^8(9)$-cholestene (5 mg), $3\beta$-acetoxy-$\Delta^7$-cholestene (4 mg), and cholesteryl acetate (3 mg). The column was eluted with hexane-benzene (9:1, v/v) and fractions 2.7 ml in volume (20 minutes per fraction; 0.135 ml/min) were collected. As shown by the elution profile in Figure 111, most (96.4%) of the recovered radioactivity corresponded in chromatographic mobility to $3\beta$-acetoxy-$\Delta^8(14)$-cholestene (fractions 49-59). A minor portion (3.6%) of the eluted radioactivity corresponded very closely to the mobility of $3\beta$-acetoxy-$\Delta^8(9)$-cholestene (fractions 73-78).

The pooled material from fractions 49-59, corresponding chromatographically to authentic $3\beta$-acetoxy-$\Delta^8(14)$-cholestene on the alumina column, was subjected to cocrystallization studies with 20 mg of unlabeled $3\beta$-acetoxy-$\Delta^8(14)$-cholestene. The results, given in Table 46, showed essentially a single component cocrystallizing with the added carrier. A portion of this material was also subjected to gas-
Figure 110. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from the third aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with washed rat liver microsomes in the presence of added DPN. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ5,14-cholestadiene, and the third is due to 3β-acetoxy-Δ5,7-cholestadiene.
Figure 111. Alumina-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of unsaturated sterols recovered from the third aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with washed rat liver microsomes in the presence of added DPN. o-o, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to 3β-acetoxy-Δ8(14)-cholestene, the second is due to 3β-acetoxy-Δ8(9)-cholestene, the third is due to 3β-acetoxy-Δ7-cholestene, and the fourth is due to cholesteryl acetate.
Table 46

Cocrystallization of Acetate Derivative of $[3\alpha-^3H]$-Cholest-8(14)-en-3β-ol Recovered from Aerobic Incubation of $[3\alpha-^3H]$-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol in the Presence of Added DPN with Authentic 3β-Acetoxy-$\Delta^{8(14)}$-cholestene

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>3,420</td>
</tr>
<tr>
<td>Once from methanol</td>
<td>3,380</td>
</tr>
<tr>
<td>Twice from methanol</td>
<td>3,350</td>
</tr>
<tr>
<td>Once from acetone-water</td>
<td>3,350</td>
</tr>
<tr>
<td>Twice from acetone-water</td>
<td>3,370</td>
</tr>
</tbody>
</table>

Liquid radiochromatographic analysis on a column of 3% OV-17, along with unlabeled 3β-acetoxy-$\Delta^{8(14)}$-cholestene. The radioactivity emerged in a single peak which corresponded to the chromatographic mobility of 3β-acetoxy-$\Delta^{8(14)}$-cholestene (Figure 112).

The pooled material from fractions 73-78 was subjected to gas-liquid radiochromatographic analysis on a 3% OV-17 column, along with unlabeled 3β-acetoxy-$\Delta^{8(9)}$-cholestene. As shown in Figure 113, the radioactive peak did not correspond to the chromatographic mobility of the added carrier.

The percent recovery of isolated sterols from this incubation is given in Table 47.

Experiment 4

The livers (43 g) of six male rats (Sprague-Dawley strain; 150-200 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (430 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by differential centrifugation. This was then
Figure 112. Gas-liquid radiochromatogram of the contents of fractions 49-59 from the alumina column shown in Figure 111. The mass peak is due to authentic unlabeled 3ß-acetoxy-Δ5(14)-cholestene.
Figure 113. Gas-liquid radiochromatogram of the contents of fractions 73-78 from the alumina column shown in Figure 111. The major peak is due to authentic unlabeled 3β-acetoxy-Δ9(9)-cholestene.
Percent Recovery of Labeled Sterols Isolated from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholesten-3β-ol with Washed Rat Liver Microsomes in the Presence of Added DPN

<table>
<thead>
<tr>
<th>Sterol Isolated</th>
<th>% Recovery of Total Incubated Substrate</th>
<th>% Recovery of Total Reacted Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-8(14)-en-3β-ol</td>
<td>40.1%</td>
<td>72.2%</td>
</tr>
</tbody>
</table>

centrifuged at 105,000 x g for 60 minutes in a Beckman L2-65B Ultracentrifuge. The pellets were removed and resuspended in potassium phosphate buffer (215 ml; 0.1 M, pH 7.4). The suspension was re-centrifuged at 105,000 x g for 60 minutes and the washed microsomal pellets were again resuspended in 16 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride to give a final concentration of 18.4 mg of protein per ml of microsomal suspension.

To a test tube containing 2 ml of the above microsomal preparation, DPN was added to give a final concentration of 5 mM of DPN. [3α-3H]-14α-Hydroxymethyl-5α-cholesten-3β-ol (1.72 x 10^6 cpm, 5.5 μg) in propylene glycol (0.05 ml) was added last. Aerobic incubation was carried out for 2 hours at 37°C with constant shaking.

At the end of incubation, acetone (40 ml) was added and the mixture was homogenized. The resulting coagulum was removed by filtration; the filter paper and the coagulum were washed several times with fresh acetone. The combined filtrates were evaporated to dryness and the residue (1.68 x 10^6 cpm; 97.7% recovery of incubated radioactivity) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 8 mg of unlabeled cholesterol. Using a
mixture of benzene and ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 104), fractions 1.5 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 104; 0.075 ml/min) were collected. The resulting chromatogram is shown in Figure 114. Approximately 19.5% of the recovered radioactivity corresponded chromatographically to the mobility of cholesterol (peak at fraction 57), another 10.5% cochromatographed with the unreacted substrate (peak at fraction 161). The major portion (40.5%) of the recovered radioactivity was eluted right after the cholesterol sample (peak at fraction 60).

Fractions 51-65 were pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The resulting labeled steryl acetates (2.46 x 10^5 cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8,14-cholestenediene (6 mg), 3β-acetoxy-Δ^5,7-cholestenediene (7 mg), and cholesteryl acetate (6 mg). The column was eluted with hexane-benzene (7:3, v/v) and fractions 4.6 ml in volume (20 minutes per fraction; 0.23 ml/min) were collected. The results, shown in Figure 115, indicated that most (74%) of the eluted radioactivity was associated with authentic cholesteryl acetate (fractions 13-20).

The contents of fractions 13-20 were pooled and applied (1.02 x 10^5 cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8(14)-cholestenene (5 mg), 3β-acetoxy-Δ^7-cholestenene (4 mg), and cholesteryl acetate (4 mg). The column was eluted with hexane-benzene (9:1, v/v) and fractions 5.8 ml in volume (20 minutes per fraction; 0.29 ml/min) were collected.
Figure 114. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from the fourth aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesterol-7-en-3β-ol with washed rat liver microsomes in the presence of 5 mM DPN. △-△, Tritium radioactivity. ○-○, Cholesterol measured colorimetrically.
Figure 115. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from the fourth aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholester-7-en-3β-ol with washed rat liver microsomes in the presence of 5 mM DPN. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ6,11-cholestadiene, and the third is due to 3β-acetoxy-Δ5,7-cholestadiene.
resulting chromatogram (Figure 116) showed that almost all (98%) of the eluted radioactivity cochromatographed with authentic 3β-acetoxyl-Δ^8(14)-cholestene (fractions 22-30). The other 2% of the recovered radioactivity was eluted just before 3β-acetoxy-Δ^7-cholestene (fractions 40-48).

Fractions 22-30 from the alumina column were pooled and subjected to repetitive recrystallization with authentic unlabeled 3β-acetoxyl-Δ^8(14)-cholestene (20 mg) from methanol and from acetone-water. The results, given in Table 48, showed essentially a single component cocrystallizing with the added carrier.

Table 48

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>1,660</td>
</tr>
<tr>
<td>Once from methanol</td>
<td>1,620</td>
</tr>
<tr>
<td>Twice from methanol</td>
<td>1,630</td>
</tr>
<tr>
<td>Once from acetone-water</td>
<td>1,590</td>
</tr>
<tr>
<td>Twice from acetone-water</td>
<td>1,600</td>
</tr>
</tbody>
</table>

A portion of this material was subjected to gas-liquid radiochromatographic analysis on a column of 3% OV-17, along with unlabeled 3β-acetoxy-Δ^8(14)-cholestene. The radioactive material emerged in a single peak which corresponded to the chromatographic mobility of authentic 3β-acetoxyl-Δ^8(14)-cholestene (Figure 117).
Figure 116. Alumina-Super Cel-silver nitrate column chromatogram of the acetate derivatives of monounsaturated sterols recovered from the fourth aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholestan-7-en-3β-ol with washed rat liver microsomes in the presence of 5 mM DPN. •••, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to 3β-acetoxy-Δ5(14)-cholestan, the second is due to 3β-acetoxy-Δ7-cholestan, and the third is due to cholesteryl acetate.
Figure 117. Gas-liquid radiochromatogram of the contents of fractions 22-30 from the alumina column shown in Figure 116. The mass peak is due to unlabeled authentic 3β-acetoxy-Δ5(14)-cholesten.
The percent recovery of isolated sterols from this incubation is given in Table 49.

Table 49

Percent Recovery of Labeled Sterols Isolated from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol with Washed Rat Liver Microsomes in the Presence of 5 mM DPN

<table>
<thead>
<tr>
<th>Sterol</th>
<th>% Recovery of Isolated</th>
<th>% Recovery of Total Incubated Substrate</th>
<th>% Recovery of Total Reacted Substrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-8(14)-en-3β-ol</td>
<td>29.4%</td>
<td></td>
<td>32.8%</td>
</tr>
</tbody>
</table>

*Total reacted substrate = total incubated substrate - unreacted substrate recovered from silicic acid-Super Cel column.

Experiment 5

Although it is well-documented that cholesterol and cholest-8(14)-en-3β-ol can be separated on silicic acid-Super Cel columns eluted with benzene, it is not known whether such a separation is possible when a more polar eluting solvent, such as benzene-ether (9:1, v/v), is used. To further establish that the radioactive peak eluted right after cholesterol carrier on silicic acid-Super Cel columns is indeed cholest-8(14)-en-3β-ol (and not some other sterols which then rearrange or decompose to form the cholest-8(14)-en-3β-ol), the chromatographic behaviour of authentic cholest-8(14)-en-3β-ol on silicic acid-Super Cel columns when eluted with benzene-ether (9:1, v/v) was studied.

[14C]-Cholesterol (7.76 x 10⁴ cpm) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 6 mg of authentic unlabeled cholest-8(14)-en-3β-ol. Using benzene as the eluting solvent, fractions 1.6 ml in volume (20 minutes per fraction; 0.08 ml/min)
were collected. The resulting chromatogram (Figure 118) indicated that under these conditions, cholesterol (peak at fraction 195) and cholest-8(14)-en-3β-ol (peak at fraction 213) did separate from each other as expected.

$[^{14}C]$-Cholesterol (7.01 x $10^4$ cpm) was next applied to a column (1 x 100 cm) of silicic acid-Super Cel, along with 5 mg of authentic unlabeled cholest-8(14)-en-3β-ol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent, fractions 1.6 ml in volume (20 minutes per fraction; 0.08 ml/min) were collected. The results of this column chromatography are shown in Figure 119. These results showed clearly the separation of cholesterol (peak at fraction 64) from cholest-8(14)-en-3β-ol (peak at fraction 69), though not to as great an extent as in the previous column.

E. IN THE ABSENCE OF ANY ADDED COFACTORS

Experiment 1

The livers (43 g) of six male rats (Sprague-Dawley strain; 150-
200 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (430 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by differential centrifugation. This was then centrifuged at 105,000 x g for 60 minutes and the resulting pellets were removed and resuspended in fresh potassium phosphate buffer (215 ml; 0.1 M, pH 7.4). The suspension was recentrifuged at 105,000 x g for 60 minutes. The washed microsomal pellets were again removed and resuspended in 16 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride to give a final protein concentration of 18.4 mg/ml.

To a test tube containing 2 ml of the above microsomal suspension,
Figure 118. Silicic acid-Super Cel column chromatogram of $^{14}$C-cholesterol and unlabeled cholest-8(14)-en-3\(\beta\)-ol. The column was packed with benzene and eluted with benzene. ○○, Carbon $^{14}$ radioactivity due to $^{14}$C-cholesterol. △△, Cholest-8(14)-en-3\(\beta\)-ol measured colorimetrically.
Figure 119. Silicic acid-Super Cel column chromatogram of \([^{14}C]\)-cholesterol and unlabeled cholest-\(\delta(14)\)-en-\(3\beta\)-ol. The column was packed with benzene and eluted with benzene-ether (9:1, v/v). \(\odot\)\(\odot\), Carbon 14 radioactivity due to \([^{14}C]\)-cholesterol. \(\Delta\)\(\Delta\), Cholest-\(\delta(14)\)-en-\(3\beta\)-ol measured colorimetrically.
\[3α^3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol (1.72 \times 10^6 \text{ cpm, 5.5} \ \mu \text{g}) \text{ in propylene glycol (0.05 ml) was added. Aerobic incubation was}
\text{carried out for 2 hours at 37°C with constant shaking.}

At the end of incubation, acetone (40 ml) was added and the mixture was homogenized. The resulting coagulum was removed by filtration; the filter paper and the coagulum were washed several times with fresh acetone. The combined filtrates were evaporated to dryness and the residue (1.74 \times 10^6 \text{ cpm; 101.2% recovery of incubated radioactivity}) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 8 mg of unlabeled cholesterol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 104), fractions 1.4 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 104; 0.07 ml/min) were collected. The resulting chromatogram, shown in Figure 120, indicated that a major portion (68%) of the recovered radioactivity was eluted right after cholesterol carrier (fractions 58-74), and another 32% of the eluted tritium corresponded chromatographically to the mobility of the unreacted substrate (fractions 161-182).

Fractions 58-74 from the previous column were pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (4.78 \times 10^5 \text{ cpm}) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of \(3β\text{-acetoxy-}Δ^8,14\)-cholestadiene (5 mg), \(3β\text{-acetoxy-}Δ^5,7\)-cholestadiene (7 mg), and cholesteryl acetate (7 mg). The column was eluted with hexane-benzene (7:3, v/v) and fractions 5.0 ml in volume (20 minutes per fraction; 0.25 ml/min) were collected. The
Figure 120. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with washed rat liver microsomes in the absence of any added cofactors. ○-○, Tritium radioactivity. △-△, Cholesterol measured colorimetrically.
The resulting elution pattern is shown in Figure 121. Almost all
(99%) of the recovered radioactivity cochromatographed with authen-
tic cholesteryl acetate (fractions 13-20).

The contents of fractions 13-20 were pooled and applied (3.56 x
10^5 cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate,
along with unlabeled samples of 3β-acetoxy-Δ^8(14)-cholestene (6 mg),
3β-acetoxy-Δ^8(9)-cholestene (5 mg), 3β-acetoxy-Δ^7-cholestene (4 mg),
and cholesteryl acetate (5 mg). Using hexane-benzene (9:1, v/v) as
the eluting solvent (changed to hexane-benzene (8:2, v/v) at fraction
115), fractions 2.5 ml in volume (20 minutes per fraction; 0.125
ml/min) were collected. The results (Figure 122) indicated that es-
sentially all (95%) of the eluted radioactivity corresponded chroma-
tographically to the mobility of 3β-acetoxy-Δ^8(14)-cholestene (peak
at fraction 49). A small portion (5%) of the recovered radioactivity
corresponded very closely to the chromatographic mobility of 3β-ac-
toxy-Δ^8(9)-cholestene (peak at fraction 74).

Fractions 46-56, corresponding to 3β-acetoxy-Δ^8(14)-cholestene
on the alumina column, were pooled and subjected to repetitive co-
crystallization with authentic unlabeled 3β-acetoxy-Δ^8(14)-cholestene
(20 mg) from methanol and from acetone-water. The results (Table 50)
showed essentially a single component cocrystallizing with the added
carrier. A portion of this material was also subjected to gas-liquid
radiochromatographic analysis on a column of 3% OV-17, along with un-
labeled 3β-acetoxy-Δ^8(14)-cholestene. The radioactive material emerged
in a single peak which corresponded to the chromatographic mobility of
3β-acetoxy-Δ^8(14)-cholestene (Figure 123).

The contents of fractions 73-77 were pooled and subjected to gas-
Figure 121. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from aerobic incubation of \([3\alpha-^3\text{H}]-14\alpha\text{-hydroxymethyl-}5\alpha\text{-cholest-7-en-3\beta-ol}\) with washed rat liver microsomes in the absence of added cofactors.

- o-o, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ\(5,14\)-cholestadiene, and the third is due to 3β-acetoxy-Δ\(5,7\)-cholestadiene.
Figure 122. Alumina-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monounsaturated sterols recovered from aerobic incubation of \([3\alpha-3^*H]-14\alpha\)-hydroxymethyl-5\(\alpha\)-cholest-7-en-3\(\beta\)-ol with washed rat liver microsomes in the absence of added cofactors. o-o, Tritium radioactivity. \(\Delta\)-\(\Delta\), Steryl acetate measured colorimetrically. The first peak is due to 3\(\beta\)-acetoxy-A\(8\)(14)-cholestene, the second is due to 3\(\beta\)-acetoxy-A\(8\)(9)-cholestene, the third is due to 3\(\beta\)-acetoxy-A\(7\)-cholestene, and the fourth is due to cholesteryl acetate.
Figure 123. Gas-liquid radiochromatogram of the contents of fractions 46-56 from the alumina column shown in Figure 122. The mass peak is due to authentic unlabeled 3β-acetoxy-5α(14)-cholestene.
Table 50

Cocrystallization of Acetate Derivative of [3α-3H]-Cholesterol-8(14)-en-3β-ol Recovered from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholestan-7-en-3β-ol in the Absence of Added Cofactors with Authentic 3β-Acetoxy-Δ8(14)-cholestan

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>2,170</td>
</tr>
<tr>
<td>Once from methanol</td>
<td>2,070</td>
</tr>
<tr>
<td>Twice from methanol</td>
<td>2,020</td>
</tr>
<tr>
<td>Once from acetone-water</td>
<td>2,050</td>
</tr>
<tr>
<td>Twice from acetone-water</td>
<td>2,030</td>
</tr>
</tbody>
</table>

Liquid radiochromatographic analysis on a column of 3% OV-17, along with unlabeled 3β-acetoxy-Δ8(9)-cholestan. The radioactive material did not correspond in chromatographic mobility to the added carrier (Figure 124).

The percent recovery of isolated sterols from this incubation is given in Table 51.

Table 51

Percent Recovery of Labeled Sterols Isolated from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholestan-7-en-3β-ol with Washed Rat Liver Microsomes in the Absence of Added Cofactors

<table>
<thead>
<tr>
<th>Sterol</th>
<th>% Recovery of Isolated</th>
<th>% Recovery of Total Incubated Substrate</th>
<th>% Recovery of Total Reacted Substrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol-8(14)-en-3β-ol</td>
<td>64.0%</td>
<td>94.1%</td>
<td></td>
</tr>
</tbody>
</table>

*Total reacted substrate = total incubated substrate - unreacted substrate recovered from silicic acid-Super Cel column.
Figure 124. Gas-liquid radiochromatogram of the contents of fractions 73-77 from the alumina column shown in Figure 122. The mass peak is due to authentic unlabeled 3β-acetoxy-Δ^7(9)-cholestene.
Experiment 2

The livers (44.4 g) of four male rats (Sprague-Dawley strain; 200-250 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (444 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by differential centrifugation. This was then centrifuged at 105,000 x g for 60 minutes. The resulting pellets were removed and resuspended in fresh potassium phosphate buffer (220 ml; 0.1 M, pH 7.4). Centrifugation was carried out as before, at 105,000 x g for 60 minutes. The washed rat liver microsomal pellets were isolated and resuspended in 21 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride to give a final protein concentration of 18.7 mg/ml.

To a test tube containing 2 ml of the above microsomal suspension, [3α-3H]-14α-hydroxymethyl-5α-cholesterol-7-en-3α-ol (1.72 x 10⁶ cpm, 5.5 μg) in propylene glycol (0.05 ml) was added. Aerobic incubation was carried out for 2 hours at 37°C with constant shaking.

At the end of incubation, acetone (40 ml) was added and the mixture was homogenized. The resulting coagulum was removed by filtration; the filter paper and the coagulum were washed several times with fresh acetone. The combined filtrates were evaporated to dryness and the residue (1.75 x 10⁶ cpm; 101.7% recovery of incubated radioactivity) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 6 mg of unlabeled cholesterol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 113), fractions 1.4 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 113; 0.07 ml/min) were collected. The results, shown in
Figure 125, indicated that approximately 30% of the recovered radioactivity was eluted right after cholesterol standard (fractions 65-80). Another 62% of the eluted radioactivity corresponded to the chromatographic mobility of the unreacted substrate (peak at fraction 164).

Fractions 65-80 were pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (2.53 x 10^5 cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8,14-cholestadiene (5 mg), 3β-acetoxy-Δ^5,7-cholestadiene (7 mg), and cholesteryl acetate (5 mg). The column was eluted with hexane-benzene (7:3, v/v) and fractions 4.1 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 60; 0.205 ml/min) were collected. The resulting chromatogram (Figure 126) showed that essentially all (99%) of the recovered radioactivity corresponded chromatographically to the mobility of cholesteryl acetate (fractions 15-22).

The contents of fractions 15-22 were pooled and applied (1.62 x 10^5 cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled 3β-acetoxy-Δ^8(14)-cholestenone (6 mg), 3β-acetoxy-Δ^7-cholestenone (5 mg), and cholesteryl acetate (3 mg). The steryl acetates were eluted with hexane-benzene (9:1, v/v) and fractions 3.5 ml in volume (20 minutes per fraction; 0.175 ml/min) were collected. The results (Figure 127) showed that approximately 98% of the eluted radioactivity corresponded in chromatographic mobility to that of 3β-acetoxy-Δ^8(14)-cholestenone (fractions 28-34).

The contents of fractions 28-34 from previous column were pooled and subjected to repetitive cocrystallization with authentic unlabeled
Figure 125. Stilbene acid-Super Cel column chromatographic analysis of sterols recovered from the second aerobic incubation of [3α-3H]-1α-hydroxycholesterol-7-en-3β-ol with washed rat liver microsomes in the absence of added cofactors. $\Delta 7$, Cholesterol measured colorimetrically.
Figure 126. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from the second aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with washed rat liver microsomes in the absence of added cofactors. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ5,14'-cholestadiene, and the third is due to 3β-acetoxy-Δ5,7'-cholestadiene.
Figure 127. Alumina-Super Cel-silver nitrate column chromatogram of the acetate derivatives of monounsaturated sterols recovered from the second aerobic incubation of $[3\alpha^{-3}H]$-14$\alpha$-hydroxymethyl-5$\alpha$-cholest-7-en-3$\beta$-ol with washed rat liver microsomes in the absence of added cofactors. o-o, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to 3$\beta$-acetoxy-$\Delta^{8}(14)$-cholesten, the second is due to 3$\beta$-acetoxy-$\Delta^{7}$-cholesten, and the third is due to cholesteryl acetate.
3β-acetoxy-Δ^8(14)-cholestene (20 mg) from methanol and from acetone-water. The results, given in Table 52, showed essentially a single component cocrystallizing with the added carrier.

Table 52

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (cpm per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>1,840</td>
</tr>
<tr>
<td>Once from methanol</td>
<td>1,790</td>
</tr>
<tr>
<td>Twice from methanol</td>
<td>1,810</td>
</tr>
<tr>
<td>Once from acetone-water</td>
<td>1,860</td>
</tr>
<tr>
<td>Twice from acetone-water</td>
<td>1,850</td>
</tr>
</tbody>
</table>

A portion of this material (fractions 28-34) was subjected to gas-liquid radiochromatographic analysis on a 3% OV-17 column, along with unlabeled 3β-acetoxy-Δ^8(14)-cholestene and 3β-acetoxy-Δ^7-cholestene. The radioactivity emerged in a single peak which corresponded to the chromatographic mobility of authentic 3β-acetoxy-Δ^8(14)-cholestene (Figure 128).

Percent recovery of isolated sterols from this experiment is given in Table 53.

F. IN THE PRESENCE OF AY-9944

The livers (44.4 g) of four male rats (Sprague-Dawley strain; 200-250 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (444 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction
Figure 128. Gas-liquid radiochromatogram of the contents of fractions 28-34 from the alumina column shown in Figure 127. The first mass peak is due to authentic 3β-acetoxy-Δ5(14)-cholestenole and the second is due to 3α-acetoxy-Δ'-cholestenole.
Table 53

Percent Recovery of Labeled Sterols Isolated from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol with Washed Rat Liver Microsomes in the Absence of Added Cofactors

<table>
<thead>
<tr>
<th>Sterol Isolated</th>
<th>% Recovery of Total Incubated Substrate</th>
<th>% Recovery of Total Reacted Substrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-8(14)-en-3β-ol</td>
<td>29.2%</td>
<td>76.6%</td>
</tr>
</tbody>
</table>

*Total reacted substrate = total incubated substrate - unreacted substrate recovered from silicic acid-Super Cel column.

was isolated by centrifugation for 30 minutes at 4°C. This was then centrifuged at 105,000 x g for 60 minutes and the resulting pellets were resuspended in fresh potassium phosphate buffer (220 ml; 0.1 M, pH 7.4). Centrifugation was carried out as before, at 105,000 x g for 60 minutes. The washed rat microsomal pellets thus obtained were resuspended in 10.5 ml of ice-cold potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride to give a final protein concentration of 36.8 mg/ml.

A solution of AY-9944 (3.0 x 10^-4 M) was prepared by dissolving 9.3 mg of the inhibitor in 75 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride.

To a test tube containing 1 ml of the microsomal suspension prepared as described above, a solution of AY-9944 (1 ml) was added to give a final concentration of 10^-4 M AY-9944 in the incubation sample. This was followed by the addition of TPNH generating system (0.9 ml) to give a final concentration of 1.2 mM of TPN. [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol (1.72 x 10^6 cpm, 5.5 μg) in propylene glycol (0.05 ml) was added last. Aerobic incubation was carried out
for 2 hours at 37° with constant shaking.

At the end of incubation, acetone (40 ml) was added and the mixture was homogenized. The resulting coagulum was removed by filtration; the filter paper and the coagulum were washed several times with fresh acetone. The combined filtrates were evaporated to dryness and the residue (1.51 x 10⁶ cpm; 88% recovery of incubated radioactivity) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 7 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 113), fractions 1.4 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 113; 0.07 ml/min) were collected. The resulting chromatogram is shown in Figure 129. Only about 60% of the applied radioactivity was recovered. A small portion (2.4%) of the eluted radioactivity corresponded to the chromatographic mobility of cholesterol (peak at fraction 66).

The contents of fractions 57-80 were pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled sterol acetates (3.78 x 10⁴ cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ⁸,14-cholestadiene (5 mg), 3β-acetoxy-Δ⁵,7-cholestadiene (6 mg), and cholesteryl acetate (4 mg). The column was eluted with hexane-benzene (7:3, v/v) and fractions 4.0 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 70; 0.2 ml/min) were collected. The elution profile (Figure 130) showed that approximately 76% of the recovered radioactivity was eluted in the region between carriers cholesteryl acetate and 3β-acetoxy-Δ⁸,14-cholestadiene (peak centered at fraction
Figure 129. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of $[3\alpha-^3\text{H}]$-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with washed rat liver microsomes in the presence of AY-9944 and added TPNH generating system. ○○, Tritium radioactivity. △△, Cholesterol measured colorimetrically.
Figure 130. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with washed rat liver microsomes in the presence of AY-9944 and TPNH generating system. o-o, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-△5,7'-cholestadiene, and the third is due to 3β-acetoxy-△5,7'-cholestadiene.
97). A significant portion (9.4%) of the radioactivity was eluted prior to 3β-acetoxy-Δ^8,14-cholestadiene (peak at fraction 126), corresponding to the chromatographic mobility of 3β-acetoxy-Δ^7,14-cholestadiene. Another 3% of the eluted radioactivity cochromatographed with authentic cholesteryl acetate (peak at fraction 18).
V. AEROBIC INCUBATION OF [3α-3H]-14α-HYDROXYMETHYL-5α-CHOLEST-7-EN-3β-OL WITH TRITON-TREATED RAT LIVER MICROSONES

INTRODUCTION AND DISCUSSION

The results presented above constitute the first conclusive demonstration of the enzymatic formation of cholest-8(14)-en-3β-ol from [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol.

Miller et al. (1967), while studying the process of C-4 demethylation, observed that the rate of C-4 demethylation in the absence of added DPN was about 30% of the rate with added DPN. They also found that treating the microsomes with Triton WR-1339 would further reduce the rate of demethylation to zero. In the presence of added DPN, Triton treatment produced only a slight loss of activity. Thus, treatment of rat liver microsomes with 10% Triton WR-1339 for 10 minutes produced an absolute requirement of the C-4 demethylase system for exogenous DPN. In addition, they observed that treatment of the rat liver microsomes with Triton WR-1339 resulted in a reduction of the nucleic acid content of the microsomes. They assayed spectrophotometrically the protein and nucleic acid content of the treated microsomes and found the ratio of protein to nucleic acid content for untreated microsomes was 6.5; for Triton-treated microsomes the ratio was 14.5. The specific effects of Triton treatment on microsomal enzymes were not studied. However, the important point is that such a treatment does produce an absolute requirement of the demethylase
system for exogenous DPN, which would prove to be very useful in answering some of our questions.

The incubations were carried out as before, except in this case, the 105,000 x g pellets were first incubated at 37° for 10 minutes with 10% (w/v) Triton WR-1339 in potassium phosphate buffer (0.1 M, pH 7.4). After this treatment, the pellets were again isolated by centrifugation and resuspended in fresh buffer in such a volume as to yield a final protein concentration of 15-20 mg/ml. This suspension then served as the enzyme source for all subsequent incubations. Using [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol as the substrate, the following four sets of experiments were carried out:

A. In the presence of added DPN and TPNH generating system

The substrate was incubated aerobically for 2 hours with Triton-treated rat liver microsomes in the presence of added DPN and TPNH generating system. Labeled cholest-8(14)-en-3β-ol, cholest-7-en-3β-ol, and cholesterol were isolated.

B. In the presence of only TPNH generating system

The substrate was incubated aerobically for 2 hours with Triton-treated rat liver microsomes in the presence of added TPNH generating system. Both labeled cholest-8(14)-en-3β-ol and cholest-7-en-3β-ol were isolated. No labeled cholesterol could be detected.

C. In the presence of only DPN

The substrate was incubated aerobically for 2 hours with Triton-treated rat liver microsomes in the presence of added DPN. The major labeled product isolated was cholest-8(14)-en-3β-ol.

D. In the absence of added cofactors
Experiment 1. The substrate was incubated aerobically for 2 hours with Triton-treated rat liver microsomes in the absence of added cofactors. Only unmetabolized substrate could be recovered.

Based on these results, the following two conclusions can be made:

1. The formation of cholest-8(14)-en-3β-ol upon aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with washed rat liver microsomes in the absence of added cofactors is due to the presence of tightly bound DPN and/or TPNH molecules in the microsomal preparation. These tightly bound cofactors can be removed by treating the microsomes with 10% Triton WR-1339.

2. The formation of labeled cholest-8(14)-en-3β-ol upon aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with washed rat liver microsomes has an absolute requirement for either DPN or TPNH.

While these experiments solved some of the problems, they also gave rise to new ones. The primary question being: why did aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with Triton-treated rat liver microsomes in the presence of either added DPN or added TPNH generating system yield cholest-8(14)-en-3β-ol as the major labeled product? Since DPN is the known cofactor for dehydrogenases and TPNH is the known cofactor for mixed function oxidases, the above results would then suggest that both a dehydrogenase and a mixed function oxidase could be involved in the formation of cholest-8(14)-en-3β-ol, or more specifically, the formation of the proposed immediate product of 14α-hydroxymethyl-5α-cholest-7-en-3β-ol — 14α-formyl-5α-cholest-7-en-3β-ol. Contradictory studies,
carried out by Fried et al. (1968) and Alexander et al. (1972) on anaerobic incubation of $[3\alpha-^3\text{H}]$-lanost-7-en-3β,32-diol with rat liver microsomes, further complicated the present dilemma. Fried et al. (1968) reported the formation of labeled $14\alpha$-formyl-5α-cholest-7-en-3β-ol upon anaerobic incubation of $[3\alpha-^3\text{H}]$-lanost-7-en-3β,32-diol with rat liver microsomes in the presence of added DPN. This would imply that C-14 demethylation involves a dehydrogenase type enzyme. On the other hand, Alexander et al. (1972) observed no significant formation of labeled $14\alpha$-formyl-5α-cholest-7-en-3β-ol from $[3\alpha-^3\text{H}]$-lanost-7-en-3β,32-diol when the latter was incubated anaerobically in the presence of either DPN or TPN; all the radioactivity being recovered as unmetabolized $[3\alpha-^3\text{H}]$-lanost-7-en-3β,32-diol. This result indicated that a mixed function oxidase type reaction is probably involved in the removal of the $14\alpha$-methyl group.

In all previous incubations involving TPNH, what was actually used is not TPNH per se, but rather a TPNH generating system consisting of TPN, isocitrate, and isocitrate dehydrogenase. A simpler explanation of the above results is therefore to assume that the C-14 demethylase could utilize both DPN and TPN. In other words, the results obtained upon aerobic incubation of $[3\alpha-^3\text{H}]$-$14\alpha$-hydroxymethyl-5α-cholest-7-en-3β-ol with rat liver microsomes in the presence of added TPNH generating system is not due to reaction of enzymes with TPNH, but rather with TPN which is readily available in a TPNH generating system. Although most of the pyridine-linked dehydrogenases are specific for either DPN or TPN, a few dehydrogenases, such as glutamate dehydrogenase, can react with either coenzyme. Miller et al. (1967) had also reported that in C-4 demethylation, TPN could be substituted for DPN,
although the rate of demethylation with TPN was considerably slower than with DPN.

To test out this possibility, the following experiments were then carried out:

D. In the absence of added cofactors

   Experiment 2. The substrate was incubated aerobically for 2 hours with Triton-treated rat liver microsomes in the absence of added cofactors. As before, only unmetabolized substrate could be recovered. This served as the control for the following two experiments.

E. In the presence of added TPN

   Aerobic incubation of substrate with Triton-treated rat liver microsomes for 2 hours in the presence of added TPN yielded labeled cholest-8(14)-en-3β-ol as the major product.

F. In the presence of preformed TPNH

   The substrate was incubated aerobically for 2 hours with Triton-treated rat liver microsomes in the presence of chemically reduced TPNH. Labeled cholest-8(14)-en-3β-ol was one of the products isolated.

The results from Experiment E showed that the C-14 demethylase is capable of utilizing TPN as well as DPN, and provided a tentative explanation for the puzzling behaviour of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol when incubated in the presence of a TPNH generating system. The results from Experiment F were quite unexpected and not as easily explained. These results indicated that somehow all the TPNH present initially in the incubation mixture were oxidized to TPN by certain microsomal enzymes. So instead of an incubation done in the
presence of TPNH, we were actually carrying out an incubation in the presence of only TPN. There is no question concerning the absence of TPNH in the incubation mixture, otherwise we would expect to see the formation of labeled cholest-7-en-3β-ol and/or cholesterol.

A significant portion of the incubated radioactivity was recovered as an unknown monohydroxy sterol, designated here as Unknown G. On a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, Unknown G was eluted in the region between carriers cholesteryl acetate and 3β-acetoxy-Δ^8,14-cholestadiene. Column chromatographic analyses indicated that Unknown G is neither cholesta-7,14-dien-3β-ol nor cholesta-7,9(11)-dien-3β-ol. However, reduction of Unknown G with Raney nickel yielded cholest-8(14)-en-3β-ol as the sole product.
EXPERIMENTAL

The radiopurity of [3α-^3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol used in the following experiments was judged to be in excess of 98% on the basis of previous chromatographic analyses (see Chapter III).

A. IN THE PRESENCE OF ADDED DPN AND TPNH GENERATING SYSTEM

The livers (55.0 g) of five male rats (Sprague-Dawley strain; 200-250 g each) were homogenized in a medium of potassium phosphate buffer (550 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by differential centrifugation. This was then centrifuged at 105,000 x g for 60 minutes in a Beckman L2-65B Ultracentrifuge as described previously. The pellets were removed and resuspended in fresh potassium phosphate buffer (100 ml; 0.1 M, pH 7.4) with the aid of a teflon-on-glass homogenizer operated manually. A portion (70 ml) of this suspension was added to an ice-cold solution (70 ml) of 20% (w/v) Triton WR-1339 in potassium phosphate buffer (0.1 M, pH 7.4). The mixture was incubated for 10 minutes at 37° with constant shaking. After incubation, it was cooled to 4° and an equal volume (140 ml) of potassium phosphate buffer (0.1 M, pH 7.4) was added. Centrifugation was carried out as before, at 105,000 x g for 60 minutes. The Triton-treated pellets were isolated and resuspended in 11 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride to give a final protein concentration of 17.3 mg/ml.

To a test tube containing 2 ml of the above microsomal suspension, DPN (3 mg) and TPNH generating system (0.9 ml) were added to give final concentrations of 1.5 mM of DPN and 1.2 mM of TPN. [3α-^3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol (1.72 x 10^6 cpm; 5.5 μCi) in propylene glycol (0.05 ml) was added last. Aerobic incubation was carried out
for 2 hours at 37° with constant shaking.

At the end of incubation, acetone (40 ml) was added and the mixture was homogenized. The resulting coagulum was removed by filtration; the filter paper and the coagulum were washed several times with fresh acetone. The combined filtrates were evaporated to dryness and the residue (1.78 x 10^6 cpm; 103.5% recovery of incubated radioactivity) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 5 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 110), fractions 1.4 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 110; 0.07 ml/min) were collected. The resulting chromatogram (Figure 131) indicated that the major portion (76.7%) of the recovered radioactivity corresponded chromatographically to the mobility of the unreacted substrate (peak centered at fraction 168), while only 11.2% of the eluted radioactivity cochromatographed with cholesterol carrier (fractions 52-64).

The contents of fractions 52-64 from previous column were pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (6.98 x 10^4 cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ8,14-cholestadiene (6 mg), 3β-acetoxy-Δ5,7-cholestadiene (5 mg), and cholesteryl acetate (3 mg). The column was eluted with hexane-benzene (7:3, v/v) and fractions 4.8 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 70; 0.24 ml/min) were collected. The resulting elution profile (Figure 132) showed that approximately 54% of the recovered radioactivity was eluted prior to cholesteryl
Figure 131. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of [3α-H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with Triton-treated rat liver microsomes in the presence of added DPN and TPNH generating system. o-o, Tritium radioactivity. △-△, Cholesterol measured colorimetrically.
Figure 132. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with Triton-treated rat liver microsomes in the presence of added DPN and TPNH generating system. ○-○, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ⁷,6-cholestadiene, and the third is due to 3β-acetoxy-Δ⁵,7-cholestadiene.
acetate (fractions 15-22), and another 44% of the radioactivity was eluted in the region between cholesteryl acetate and 3β-acetoxy-\(\Delta^8,14\)-cholestadiene (fractions 73-90). The latter steryl acetate (fractions 73-90) was designated as Unknown G and will be examined more closely later on.

Fractions 15-22 were pooled and applied (1.18 x 10\(^4\) cpn) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-\(\Delta^8(14)\)-cholestanol (5 mg), 3β-acetoxy-\(\Delta^7\)-cholestanol (5 mg), and cholesteryl acetate (4 mg). Using hexane-benzene (9:1, v/v) as the eluting solvent, fractions 5.4 ml in volume (30 minutes per fraction; 0.18 ml/min) were collected. The results, shown in Figure 133, indicated that approximately 51% of the recovered radioactivity corresponded chromatographically to the mobility of 3β-acetoxy-\(\Delta^8(14)\)-cholestanol (fractions 19-27), 45% cochromatographed with authentic 3β-acetoxy-\(\Delta^7\)-cholestanol (fractions 36-48), and 4% was associated with cholesteryl acetate (fractions 70-83).

The percent recovery of labeled sterols from this incubation is given in Table 54.

B. IN THE PRESENCE OF ADDED TPNH GENERATING SYSTEM

The livers (55.0 g) of five male rats (Sprague-Dawley strain; 200-250 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (550 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by differential centrifugation. This was then centrifuged at 105,000 x g for 60 minutes and the resulting pellets were resuspended in fresh buffer (100 ml; 0.1 M, pH 7.4). A portion (70 ml) of this suspension was added to a flask containing 70 ml of an ice-cold solution of 20% (w/v) Triton WR-1339 in potassium phosphate
Figure 133. Alumina-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monounsaturated sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with Triton-treated rat liver microsomes in the presence of added DPN and TPNH generating system. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to 3β-acetoxy-Δ8(14)-cholestene, the second is due to 3β-acetoxy-Δ7'-cholestene, and the third is due to cholesteryl acetate.
Table 54
Percent Recovery of Labeled Sterols Isolated from Aerobic Incubation of \([3\alpha^-\text{H}]-14\alpha\text{-Hydroxymethyl}-5\alpha\text{-cholest-7-en-3\beta-ol}\) with Triton-treated Rat Liver Microsomes in the Presence of DPN and TPNH

<table>
<thead>
<tr>
<th>Sterols Isolated</th>
<th>% Recovery of Total Incubated Substrate</th>
<th>% Recovery of Total Reacted Substrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-8(14)-en-3\beta-ol</td>
<td>3.1%</td>
<td>13.2%</td>
</tr>
<tr>
<td>Cholest-7-en-3\beta-ol</td>
<td>2.7%</td>
<td>11.7%</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.2%</td>
<td>1.0%</td>
</tr>
<tr>
<td>Total</td>
<td>6.0%</td>
<td>25.9%</td>
</tr>
</tbody>
</table>

*Total reacted substrate = total incubated substrate - unreacted substrate recovered from silicic acid-Super Cel column.

buffer (0.1 M, pH 7.4). The mixture was incubated for 10 minutes at 37\(^\circ\) with constant shaking. After incubation, it was cooled to 4\(^\circ\) and an equal volume (140 ml) of potassium phosphate buffer (0.1 M, pH 7.4) was added. Centrifugation was carried out as before, at 105,000 \(x g\) for 60 minutes. The Triton-treated pellets thus obtained were re-suspended in 11 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride to give a final protein concentration of 17.3 mg/ml.

To a test tube containing 2 ml of this microsomal preparation, TPNH generating system (0.9 ml) was added to give a final concentration of 1.2 mM of TPN. \([3\alpha^-\text{H}]-14\alpha\text{-Hydroxymethyl}-5\alpha\text{-cholest-7-en-3\beta-ol}\) (1.72 x 10\(^6\) cpm, 5.5 \(\mu\)g) in propylene glycol (0.05 ml) was added last. Aerobic incubation was carried out for 2 hours at 37\(^\circ\) with constant shaking.

At the end of incubation, acetone (40 ml) was added and the mix-
ture was homogenized. The resulting coagulum was removed by filtration; the filter paper and the coagulum were washed several times with fresh acetone. The combined filtrates were evaporated to dryness and the residue (1.70 x 10^6 cpm; 98.8% recovery of incubated radioactivity) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 5 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 110), fractions 1.2 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 110; 0.06 ml/min) were collected. The results (Figure 134) indicated that most (74%) of the eluted radioactivity corresponded chromatographically to the mobility of the unreacted substrate (peak at fraction 163). A small portion (8.1%) of the eluted radioactivity showed chromatographic mobility similar to that of cholesterol (peak at fraction 60).

Fractions 58-68 were pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (5.58 x 10^4 cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ8,14-cholestadiene (5 mg), 3β-acetoxy-Δ5,7-cholestadiene (6 mg), and cholesteryl acetate (4 mg). The column was eluted with hexane-benzene (7:3, v/v) and fractions 4.6 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 70; 0.23 ml/min) were collected. The resulting chromatogram (Figure 135) showed that approximately 39% of the recovered radioactivity cochromatographed with authentic cholesteryl acetate (fraction 16-20). Another 57% of the radioactivity was eluted between cholesteryl acetate and 3β-acetoxy-
Figure 134. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesterol-7-en-3β-ol with Triton-treated rat liver microsomes in the presence of added TPPII generating system. O-O, Tritium radioactivity. A-A, Cholesterol measured colorimetrically.
Figure 135. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with Triton-treated rat liver microsomes in the presence of added TPNH generating system. ○-○, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ5,14'-cholestadiene, and the third is due to 3β-acetoxy-Δ5,14'-cholestadiene.
\( \Delta^{8,14} \)-cholestadiene (fractions 67-104), corresponding to the chromatographic mobility of Unknown G.

The contents of fractions 16-20 were pooled and applied (8,20 x 10^3 cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3\( \beta \)-acetoxy-\( \Delta^{8(14)} \)-cholestone (5 mg), 3\( \beta \)-acetoxy-\( \Delta^{7} \)-cholestone (5 mg), and cholesteryl acetate (3 mg). The column was eluted with hexane-benzene (9:1, v/v) and fractions 5.4 ml in volume (30 minutes per fraction; 0.18 ml/min) were collected. The resulting chromatogram is shown in Figure 136. Most (93\%) of the eluted radioactivity corresponded in chromatographic mobility to 3\( \beta \)-acetoxy-\( \Delta^{8(14)} \)-cholestone (fractions 23-32). A significant portion (6.8\%) of the eluted radioactivity was associated in chromatographic mobility with authentic 3\( \beta \)-acetoxy-\( \Delta^{7} \)-cholestone (fractions 46-56).

Percent recovery of labeled sterols isolated from this experiment is given in Table 55.

Table 55
Percent Recovery of Labeled Sterols Isolated from Aerobic Incubation of [3\( \alpha \)-\( ^3 \)H]-14\( \alpha \)-Hydroxymethyl-5\( \alpha \)-cholesterol-7-en-3\( \beta \)-ol with Triton-treated Rat Liver Microsomes in the Presence of TPNH

<table>
<thead>
<tr>
<th>Sterols Isolated</th>
<th>% Recovery of Total Incubated Substrate</th>
<th>% Recovery of Total Reacted Substrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-8(14)-en-3( \beta )-ol</td>
<td>2.9%</td>
<td>11.3%</td>
</tr>
<tr>
<td>Cholest-7-en-3( \beta )-ol</td>
<td>0.2%</td>
<td>0.8%</td>
</tr>
<tr>
<td>Total</td>
<td>3.1%</td>
<td>12.1%</td>
</tr>
</tbody>
</table>

*Total reacted substrate = total incubated substrate - unreacted substrate recovered from silicic acid-Super Cel column.
Figure 136. Alumina-Super Cel-silver nitrate column chromatogram of the acetate derivatives of monounsaturated sterols recovered from aerobic incubation of $[\text{3a}^{3}H]^{-}\text{14a-hydroxymethyl-5a-cholesterol-7-en-38-ol}$ with Triton-treated rat liver microsomes in the presence of TPNH generating system. ○—○, Tritium radioactivity. △—△, Steryl acetate measured colorimetrically. The first peak is due to 38-acetoxy-Δ$^{5}$-cholestene, the second is due to 38-acetoxy-Δ$^{7}$-cholestene, and the third is due to cholesteryl acetate.
C. IN THE PRESENCE OF ADDED DPN

The livers (55.0 g) of five male rats (Sprague-Dawley strain; 200-250 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (550 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by differential centrifugation. This was then centrifuged at 105,000 x g for 60 minutes and the resulting pellets were resuspended in fresh potassium phosphate buffer (100 ml; 0.1 M, pH 7.4). A portion (70 ml) of this suspension was added to a flask containing 70 ml of an ice-cold solution of 20% (w/v) Triton WR-1339 in potassium phosphate buffer (0.1 M, pH 7.4). The mixture was incubated for 10 minutes at 37° with constant shaking. After incubation, the solution was cooled to 4° and an equal volume (140 ml) of potassium phosphate buffer (0.1 M, pH 7.4) was added. Centrifugation was carried out as before, at 105,000 x g for 60 minutes. The Triton-treated microsomal pellets thus obtained were resuspended in 11 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride to give a final protein concentration of 17.3 mg/ml.

To a test tube containing 2 ml of this microsomal preparation, DPN (3 mg) was added to give a final concentration of 1.5 mM of DPN. [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol (1.72 x 10^6 cpm, 5.5 μg) in propylene glycol (0.05 ml) was added last. Aerobic incubation was carried out for 2 hours at 37° with constant shaking.

At the end of incubation, 40 ml of acetone was added and the mixture was homogenized. The resulting coagulum was removed by filtration and was washed several times with fresh acetone. The combined filtrates were evaporated to dryness and the residue (1.70 x 10^6 cpm; 98.8% recovery of incubated radioactivity) was chromatographed on a
column (1 x 100 cm) of silicic acid-Super Cel, along with 5 mc of un-
labeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting
solvent (changed to benzene-ether (8:2, v/v) at fraction 110), frac-
tions 1.4 ml in volume (20 minutes per fraction, changed to 30 minutes
per fraction at fraction 110; 0.07 ml/min) were collected. The results,
shown in Figure 137, indicated that the major region (88.4%) of radio-
activity corresponded to the chromatographic mobility of the unreact-
ed substrate (peak at fraction 174). Approximately 8.5% of the recover-
ed radioactivity cochromatographed with authentic cholesterol (fractions
56-71).

Fractions 56-71 from previous column were pooled and acetylated
with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previous-
ly. The labeled steryl acetates (4.70 x 10^4 cpm) were then applied
to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along
with unlabeled samples of 3β-acetoxy-Δ^8,14-cholestadiene (5 mg), 3β-
acetoxy-Δ^5,7-cholestadiene (5 mg), and cholesteryl acetate (4 mg).
The column was eluted with hexane-benzene (7:3, v/v) and fractions
5.0 ml in volume (20 minutes per fraction, changed to 30 minutes per
fraction at fraction 70; 0.25 ml/min) were collected. The resulting
chromatogram (Figure 138) indicated that essentially all (99%) of the
recovered radioactivity was eluted prior to cholesteryl acetate (frac-
tions 19-23).

The contents of fractions 19-23 were pooled and applied (9.01 x
10^3 cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate,
along with unlabeled samples of 3β-acetoxy-Δ^8(14)-cholestene (5 mg),
3β-acetoxy-Δ^7-cholestene (5 mg), and cholesteryl acetate (3 mg). The
column was eluted with hexane-benzene (9:1, v/v) and fractions 5.4 ml
Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of [3α,5α-3H]-14α-hydroxyethyl-5α-cholestan-7-en-3β-ol with Triton-treated rat liver microsomes in the presence of added DNA. o-o, Tritium radioactivity. Δ-Δ, Cholesterol measured colorimetrically.
Figure 138. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholestan-7-en-3β-ol with Triton-treated rat liver microsomes in the presence of added DPN. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ8,14-cholestadiene, and the third is due to 3β-acetoxy-Δ5,7-cholestadiene.
in volume (30 minutes per fraction; 0.18 ml/min) were collected. The results are shown in Figure 139. Almost all (97.8%) of the recovered radioactivity corresponded chromatographically to the mobility of 3β-acetoxy-Δ^8(14)-cholestene (fractions 25-33).

Percent recovery of labeled sterols isolated from this incubation is tabulated in Table 56.

<table>
<thead>
<tr>
<th>Sterol Isolated</th>
<th>% Recovery of Total Incubated Substrate</th>
<th>% Recovery of Total Reacted Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-8(14)-en-3β-ol</td>
<td>8.2%</td>
<td>70.9%</td>
</tr>
</tbody>
</table>

*Total reacted substrate = total incubated substrate - unreacted substrate recovered from silicic acid-Super Cel column.

D. IN THE ABSENCE OF ADDED COFACTORS

**Experiment 1**

The livers (55.0 g) of five male rats (Sprague-Dawley strain; 200-250 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (550 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by differential centrifugation. This was then centrifuged at 105,000 x g for 60 minutes and the resulting pellets were resuspended in 100 ml of resh buffer. A portion (70 ml) of this suspension was added to a solution (70 ml) of 20% Triton WR-1339 in potassium phosphate buffer (0.1 M, pH 7.4), and the mixture was incubated at 37° for 10 minutes. After incubation, the solution was cooled
Figure 139. Alumina-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monounsaturated sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with Triton-treated rat liver microsomes in the presence of added DPN. o-o, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to 3β-acetoxy-△(14)’-cholestenone, the second is due to 3β-acetoxy-△’-cholestenone, and the third is due to cholesteryl acetate.
to 4°C and an equal volume (140 ml) of potassium phosphate buffer (0.1 M, pH 7.4) was added. Centrifugation was carried out as before, at 105,000 x g for 60 minutes. The Triton-treated microsomal pellets were finally resuspended in 11 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride to give a protein concentration of 17.3 mg/ml.

To a test tube containing 2 ml of the above microsomal preparation, [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3α-ol (1.72 x 10^6 cpm, 5.5 μg) in propylene glycol (0.05 ml) was added. Aerobic incubation was carried out for 2 hours at 37°C with constant shaking.

At the end of incubation, acetone (40 ml) was added and the mixture was homogenized. The resulting coagulum was removed by filtration; the filter paper and the coagulum were washed several times with fresh acetone. The combined filtrates were evaporated to dryness and the residue (1.73 x 10^6 cpm; 100.6% recovery of incubated radioactivity) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 6 mg of unlabeled cholesterol. Using a mixture of benzene and ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 110), fractions 1.3 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 110; 0.065 ml/min) were collected. The resulting chromatogram (Figure 140) showed that essentially all (99.5%) of the eluted radioactivity was recovered as the unreacted substrate (peak at fraction 174).

Experiment 2

The livers (11.2 g) of two male rats (Sprague-Dawley strain; 150-200 g each) were homogenized in a medium of ice-cold potassium phos-
Figure 140. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesterol-7-en-3β-ol with Triton-treated rat liver microsomes in the absence of added cofactors. □-□, Tritium radioactivity. △-△, Cholesterol measured colorimetrically.
phate buffer (112 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by differential centrifugation. This was then centrifuged at 105,000 x g for 60 minutes and the resulting pellets were resuspended in fresh potassium phosphate buffer (30 ml; 0.1 M, pH 7.4). A cooled solution (30 ml) of 20% Triton WR-1339 in potassium phosphate buffer (0.1 M, pH 7.4) was added to the suspension and the mixture was incubated at 37° for 10 minutes with constant shaking. After incubation, the solution was cooled to 4° and an equal volume (60 ml) of fresh buffer was added. Centrifugation was carried out as before, at 105,000 x g for 60 minutes. The Triton-treated microsomal pellets were finally resuspended in 6 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride to give a final protein concentration of 17.2 mg/ml.

To a test tube containing 2 ml of the above microsomal suspension, [3α-3H]-14α-hydroxymethyl-5α-cholesterol-7-en-3β-ol (1.45 x 10⁶ cpm, 4.64 µg) in propylene glycol (0.05 ml) was added. Aerobic incubation was carried out as before for 2 hours at 37° with constant shaking.

At the end of incubation, acetone (40 ml) was added and the mixture was homogenized. The resulting coagulum was removed by filtration; the filter paper and the coagulum were washed several times with fresh acetone. The combined filtrates were evaporated to dryness and the residue (1.38 x 10⁶ cpm; 95.2% recovery of incubated radioactivity) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 5 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 52), fractions 2.0 ml in volume (30 minutes per fraction; 0.067 ml/min) were collected. The resulting chromato-
gram is shown in Figure 141. Essentially all (99.7%) of the recovered radioactivity cochromatographed with the unreacted substrate (peak centered at fraction 114).

E. IN THE PRESENCE OF ADDED TPN

The livers (11.2 g) of two male rats (Sprague-Dawley strain; 150-200 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (112 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by differential centrifugation. This was then centrifuged at 105,000 x g for 60 minutes. The pellets were removed and resuspended in 30 ml of potassium phosphate buffer with a hand homogenizer. An ice-cold solution (30 ml) of 20% (w/v) Triton WR-1339 in potassium phosphate buffer (0.1 M, pH 7.4) was added and the mixture was incubated for 10 minutes at 37\(^\circ\) with constant shaking. After incubation, the solution was cooled to 4\(^\circ\) and an equal volume (60 ml) of fresh buffer (0.1 M, pH 7.4) was added. Centrifugation was carried out as before, at 105,000 x g for 60 minutes. The Triton-treated microsomal pellets were finally resuspended in 6 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride to give a protein concentration of 1.72 mg/ml.

To a test tube containing 2 ml of this microsomal preparation, TPN (2.68 mg) was added to give a final concentration of 1.2 mM of TPN in the incubation sample. [3\(^3\)H]-14\(\alpha\)-hydroxymethyl-5\(\alpha\)-cholest-7-en-3\(\beta\)-ol (2.94 x 10\(^6\) cpm, 9.3 \(\mu\)g) in propylene glycol (0.1 ml) was added last. Aerobic incubation was carried out for 2 hours at 37\(^\circ\) with constant shaking.

At the end of incubation, acetone (40 ml) was added and the mixture was homogenized. The resulting coagulum was removed by filtra-
Figure 141. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from the second aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholester-7-en-3α-ol with Triton-treated rat liver microsomes in the absence of added cofactors. o-o, Tritium radioactivity. △-△, Cholesterol measured colorimetrically.
tion; the filter paper and the coagulum were washed several times with fresh acetone. The combined filtrates were evaporated to dryness and the residue (2.98 \times 10^6 \text{ cpm}; 101.4\% \text{ recovery of incubated radioactivity}) was chromatographed on a column (1 x 100 cm) of silicic acid–Super Cel, along with unlabeled cholesterol (6 mg). Using benzene–ether (9:1, v/v) as the eluting solvent (changed to benzene–ether (8:2, v/v) at fraction 110), fractions 1.2 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 110; 0.06 ml/min) were collected. The results (Figure 142) indicated that most (84.3\%) of the recovered radioactivity corresponded in chromatographic mobility to that of unreacted substrate (peak at fraction 157). A small portion (12\%) of the applied radioactivity was eluted right after unlabeled cholesterol (peak at fraction 60).

Fractions 57-66 were pooled and acetylated with a mixture of pyridine and acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (1.26 \times 10^5 \text{ cpm}) were then applied to a column (1 x 50 cm) of Silica Gel G–Super Cel–silver nitrate, along with unlabeled samples of 3\beta-acetoxy-\Delta^8,14-cholestadiene (6 mg), 3\beta-acetoxy-\Delta^5,7-cholestadiene (6 mg), and cholesteryl acetate (3 mg). The column was eluted with hexane-benzene (7:3, v/v) and fractions 3.2 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 40; 0.16 ml/min) were collected. The resulting chromatogram (Figure 143) showed that approximately 58\% of the recovered radioactivity was eluted prior to cholesteryl acetate (fractions 15-23). Another 38\% of the applied radioactivity was eluted in the region between cholesteryl acetate and 3\beta-acetoxy-\Delta^8,14-cholesta-
diene, corresponding to the chromatographic mobility of Unknown G.
Figure 142. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesterol-7-en-3β-ol with Triton-treated rat liver microsomes in the presence of added THF. o-o, Tritium radioactivity. △-△, Cholesterol measured colorimetrically.
Figure 143. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with Triton-treated rat liver microsomes in the presence of added TPN. 0-0, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-Δ7,14-cholestadiene, and the third is due to 3β-acetoxy-Δ5,7-cholesta
diene.
(fractions 65-78).

Fractions 15-23 from the previous column were pooled and applied (3.70 x 10^4 cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8(14)-cholestene (5 mg), 3β-acetoxy-Δ^7-cholestene (4 mg), and cholesteryl acetate (5 mg). The column was eluted with hexane-benzene (9:1, v/v) and fractions 4.7 ml in volume (15 minutes per fraction; 0.313 ml/min) were collected. The results are shown in Figure 144. Most (95%) of the recovered radioactivity corresponded to the chromatographic mobility of authentic 3β-acetoxy-Δ^8(14)-cholestene (fractions 23-32).

Percent recovery of labeled sterols isolated from this experiment is given in Table 57.

Table 57

Percent Recovery of Labeled Sterols Isolated from Aerobic Incubation of [3α-^3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol with Triton-treated Rat Liver Microsomes in the Presence of Added TPN

<table>
<thead>
<tr>
<th>Sterol</th>
<th>% Recovery of Isolated Total Incubated Substrate</th>
<th>% Recovery of Total Reacted Substrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-8(14)-en-3β-ol</td>
<td>6.6%</td>
<td>42.1%</td>
</tr>
</tbody>
</table>

*Total reacted substrate = total incubated substrate - unreacted substrate recovered from silicic acid-Super Cel column.

F. IN THE PRESENCE OF CHEMICALLY REDUCED TPNH

The livers (11.2 g) of two male rats (Sprague-Dawley strain; 150-200 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (112 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by differential centrifugation. This was then
Alumina-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monounsaturated sterols recovered from aerobic incubation of $[3\alpha-^{3}H]^{-14\alpha}$-hydroxymethyl-5alpha-cholest-7-en-3beta-ol with Triton-treated rat liver microsomes in the presence of added TPN. $\bullet$-$\bullet$, Tritium radioactivity. $\Delta$-$\Delta$, Steryl acetate measured colorimetrically. The first peak is due to 3beta-acetoxy-$\Delta^{8(14)}$-cholestene, the second is due to 3beta-acetoxy-$\Delta^{7}$-cholestene, and the third is due to cholesteryl acetate.
centrifuged at 105,000 x g for 60 minutes. The pellets were removed and resuspended in fresh potassium phosphate buffer (30 ml; 0.1 M, pH 7.4). An ice-cold solution (30 ml) of 20% (w/v) Triton WR-1339 in potassium phosphate buffer (0.1 M, pH 7.4) was added and the mixture was incubated for 10 minutes at 37°C with constant shaking. After incubation, the solution was cooled to 4°C and an equal volume (60 ml) of fresh buffer was added. Centrifugation was carried out as before, at 105,000 x g for 60 minutes. The Triton-treated microsomal pellets were finally resuspended in 6 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride to give a final protein concentration of 17.2 mg/ml.

To a test tube containing 2 ml of the above microsomal preparation, chemically reduced TPNH (2.68 mg; Sigma Chemical Company, Lot 102C-7580) was added to yield a final concentration of 1.2 mM of TPNH. [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol (1.45 x 10⁶ cpm, 4.64 μg) in propylene glycol (0.05 ml) was added last. Aerobic incubation was carried out for 2 hours at 37°C with constant shaking.

At the end of incubation, acetone (40 ml) was added and the mixture was homogenized. The resulting coagulum was removed by filtration; the filter paper and the coagulum were washed several times with fresh acetone. The combined filtrates were evaporated to dryness and the residue (1.46 x 10⁶ cpm; 100.7% recovery of incubated radioactivity) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 6 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 110), fractions 1.4 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction
110; 0.07 ml/min) were collected. The results (Figure 145) showed that most (83%) of the recovered radioactivity cochromatographed with the unreacted substrate (peak at fraction 150). Approximately 14% of the applied radioactivity was eluted right after cholesterol (fractions 52-65).

The contents of fractions 52-65 were pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The resulting labeled steryl acetates (1.04 x 10^5 cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8,14-cholestanediene (5 mg), 3β-acetoxy-Δ^5,7-cholestenadiene (5 mg), and cholesteryl acetate (3 mg). The column was eluted with hexane-benzene (7:3, v/v) and fractions 3.4 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 40; 0.17 ml/min) were collected. The resulting chromatogram (Figure 146) showed that approximately 30% of the recovered radioactivity was eluted prior to cholesteryl acetate (fractions 15-24), and another 65% of the applied radioactivity was eluted in the region between cholesteryl acetate and 3β-acetoxy-Δ^8,14-cholestenadiene, corresponding to the chromatographic mobility of Unknown G (fractions 55-78).

Fractions 15-24 were pooled and applied (1.72 x 10^4 cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8(14)-cholesten (5 mg), 3β-acetoxy-Δ^7-cholesten (4 mg), and cholesteryl acetate (4 mg). The column was eluted with hexane-benzene (9:1, v/v) and fractions 4.2 ml in volume (15 minutes per fraction; 0.28 ml/min) were collected. The results, shown in Figure 147, indicated that most (96%) of the eluted radio-
Figure 145. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesterol-7-en-3β-ol with Triton-treated rat liver microsomes in the presence of chemically reduced TPNH. □-□, Tritium radioactivity. △-△, Cholesterol measured colorimetrically.
Figure 146. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesterol-7-en-3β-ol with Triton-treated rat liver microsomes in the presence of chemically reduced TPNH. O-O, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ5,14Δ-clesterol, and the third is due to 3β-acetoxy-Δ5,7Δ-clesterol.
Figure 147. Alumina-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monounsaturated sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesta-7-en-3β-ol with Triton-treated rat liver microsomes in the presence of chemically reduced TPNH. ○-○, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to 3β-acetoxy-Δ8(14)-cholestene, the second is due to 3β-acetoxy-Δ7-cholestene, and the third is due to cholesteryl acetate.
activity corresponded in chromatographic mobility to that of 3β-acetoxy-Δ8(14)-cholestene (peak at fraction 25).

Percent recovery of labeled sterols isolated from this experiment is given in Table 58.

Table 58

<table>
<thead>
<tr>
<th>Sterol Isolated</th>
<th>% Recovery of Total Incubated Substrate</th>
<th>% Recovery of Total Reacted Substrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-8(14)-en-3β-ol</td>
<td>4.0%</td>
<td>23.7%</td>
</tr>
</tbody>
</table>

*Total reacted substrate = total incubated substrate - unreacted substrate recovered from silicic acid-Super Cel column.

G. FURTHER ANALYSIS OF UNKNOWN G

The contents of fractions 55-78 from the Silica Gel G column shown in Figure 146, corresponding to the chromatographic mobility of Unknown G, were pooled and reapplied (2.84 x 10⁴ cpm) to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ7,14-cholestadiene (6 mg), 3β-acetoxy-Δ7,9(11)-cholestadiene (4 mg), and cholesteryl acetate (4 mg). The column was eluted with hexane-benzene (7:3, v/v) and fractions 5.7 ml in volume (30 minutes per fraction; 0.19 ml/min) were collected. The resulting chromatogram (Figure 148) indicated that Unknown G (fractions 42-54) did not correspond in chromatographic mobility to any of the added samples.

Fractions 42-54 from previous column were pooled and evaporated
Figure 148. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of Unknown G recovered from the Silica Gel G column shown in Figure 146. The column was eluted with hexane-benzene (7:3, v/v) and fractions 5.7 ml in volume were collected. o-o, Tritium radioactivity due to Unknown G. △-△, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ7,9(11)-cholestadiene, and the third is due to 3β-acetoxy-Δ7,14-cholestadiene.
to dryness under reduced pressure. The resulting residue was dissolved in 4 ml of benzene and transferred to a glass hydrogenation bottle. Freshly-prepared Raney nickel (4 scoops of nickel from a spatula) was added and the bottle was secured in a Parr Hydrogenation apparatus. Residual air was removed from the bottle by repeated flushings with hydrogen gas. The bottle was finally filled with hydrogen (50 psi) at room temperature and mechanically shaken for 24 hours. At the end of this time, the pressure was 45 psi.

The bottle was removed and its contents were vacuum filtered through Hyflo Super-Cel (Johns-Manville) in a fritted funnel, eluting four times with 20 ml portions of benzene. The combined filtrates were evaporated to dryness under reduced pressure and the resulting residue (1.25 x 10^4 cpm) was applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ7,14-cholestadiene (7 mg), 3β-acetoxy-Δ7,9(11)-cholestadiene (4 mg), and cholesterly acetate (4 mg). The column was eluted with hexane-benzene (7:3, v/v) and fractions 4.8 ml in volume (30 minutes per fraction; 0.16 ml/min) were collected. The results, shown in Figure 149, indicated that almost all (99%) of the eluted radioactivity corresponded chromatographically to the mobility of cholesterly acetate (fractions 12-19).

The contents of fractions 12-19 were pooled and applied (8.34 x 10^3 cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ8(14)-cholesten (5 mg), 3β-acetoxy-Δ7-cholesten (5 mg), and cholesteryl acetate (4 mg). The column was eluted with hexane-benzene (9:1, v/v) and fractions 3.0 ml in volume (20 minutes per fraction; 0.15 ml/min) were collected. The
Figure 149. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of Raney nickel reduction products of Unknown G. The column was eluted with hexane-benzene (7:3, v/v) and fractions 4.8 ml in volume were collected. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ7,9(11)-cholestadiene, and the third is due to 3β-acetoxy-Δ7,14-cholestadiene.
resulting chromatogram is shown in Figure 150. All (100%) of the recovered radioactivity corresponded in chromatographic mobility to that of authentic 3β-acetoxy-Δ^{8(14)}-cholestene (fractions 48-57).
Figure 150. Alumina-Super Cel-silver nitrate column chromatographic analysis of Raney nickel reduction products of Unknown G. The column was eluted with hexane-benzene (9:1, v/v) and fractions 3.0 ml in volume were collected. ○-○, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to 3β-acetoxy-Δ(II)-cholestene, the second is due to 3β-acetoxy-Δ1-cholestene, and the third is due to cholesteryl acetate.
VI. AEROBIC INCUBATION OF \([3\alpha^3H]-14\alpha\)-HYDROXYMETHYL-5\alpha-CHOLEST-7-EN-3\beta-OL WITH SNAKE VENOM-TREATED RAT LIVER MICROSONES

INTRODUCTION AND DISCUSSION

In the course of their studies on C-4 demethylation, Gaylor et al. reported that incubation of 50 mg of rat liver microsomal protein with either 25 \(\mu\)g of crude snake venom (Crotalus adamanteus) or 1 \(\mu\)g of purified phospholipase A resulted in essentially complete loss of DPN-dependent methyl sterol demethylase activity (Bechtold et al., 1972). However, the snake venom-treated microsomes contained all the known component enzymes of demethylase and microsomal electron carriers. Furthermore, methyl sterol demethylase activity was restored either by the addition of supernatant fraction from snake venom treatment or by the addition of reduced pyridine nucleotides.

These results were attributed to the existence of an endogenous, DPN-dependent cytochrome b\(_5\)-reducing system that supplied the required reducing equivalents. Snake venom treatment removed this endogenous reducing system from microsomes. Concomitant loss of the DPN-dependent methyl sterol demethylase and the reducing system was observed. Each enzymic activity could be restored by the addition of supernatant fraction, which is obtained from the snake venom treatment. The endogenous reducing system appeared to obtain its reducing equivalents via a microsomal alcohol dehydrogenase which was readily released by the
snake venom treatment. Both commercial horse liver alcohol dehydro-
genase and isolated rat liver alcohol dehydrogenase restored the DPN-
dependent demethylase activity that was lost upon snake venom treat-
ment.

The endogenous reducing system of microsomes also helped to ex-
plain an important paradox, which developed from the first report of
DPN as the only required cofactor for C-4 demethylation. Since that
time, it has been shown that 4α-sterol carboxylic acid was formed
through attack of the 4α-methyl group by mixed function oxidase (Miller
et al., 1970a and 1070b). Oxidation presumably was stepwise and
sequential: \( \text{RCH}_3 \rightarrow \text{RCH}_2\text{OH} \rightarrow \text{RCHO} \rightarrow \text{RCOOH} \) (Miller et al.,
1971). Thus, for each mole of carboxylic acid formed, three moles
of reduced pyridine nucleotides should be consumed. However, in
previous incubation studies only DPN was added, and reducing equiva-
lents had to arise from some other sources. The presence of the en-
dogenous reducing system and the known dependence of the mixed func-
tion oxidase upon a source of reducing equivalents readily explained
the paradox observed earlier that fully active C-4 demethylase of rat
liver microsomes was observed in the absence of reduced pyridine
nucleotides when DPN was the only added cofactor.

Their observation leads me to suspect that a similar reducing
system is involved in the case of C-14 demethylation. The existence
of such an endogenous reducing system would then account for my own
paradox: the fact that aerobic incubation of \([3α^{-3}H]-14α\)-hydroxy-
methyl-5α-cholest-7-en-3β-ol with Triton-treated rat liver microsomes
in the presence of either added DPN or added TPNH generating system,
both yielded labeled cholest-8(14)-en-3β-ol as one of the major prod-
ucts.

Using $[3\alpha^3H]$-14α-hydroxymethyl-5α-cholest-7-en-3β-ol as the substrate, the following experiments were carried out:

A. In the presence of added DPN and TPNH generating system
   Aerobic incubation of the substrate with snake venom-treated rat liver microsomes for 2 hours in the presence of added DPN and TPNH generating system yielded labeled cholest-8(14)-en-3β-ol, cholest-7-en-3β-ol, and cholesterol as the major products.

B. In the presence of added TPNH generating system
   Aerobic incubation of the substrate with snake venom-treated rat liver microsomes for 2 hours in the presence of added TPNH generating system yielded labeled cholest-8(14)-en-3β-ol, cholest-7-en-3β-ol, and cholesterol.

C. In the presence of added DPN
   Aerobic incubation of the substrate with snake venom-treated rat liver microsomes for 2 hours in the presence of added DPN yielded labeled cholest-8(14)-en-3β-ol as the major product.

D. In the absence of added cofactors
   Aerobic incubation of the substrate with snake venom-treated rat liver microsomes for 2 hours in the absence of added cofactors yielded cholest-8(14)-en-3β-ol as the major labeled product.

Although a positive result (no reaction of substrate in the presence of added DPN due to inactivation of DPN-dependent cytochrome $b_5$-reducing system by snake venom) would tell us little because of possible effects of snake venom on other enzymic processes which might
produce similar results, a negative result at least tells us one thing — either some other enzyme system is generating the needed reducing equivalents (DPNH from DPN) or more likely, C-14 demethylation does not involve a mixed function oxidase type reaction as in C-4 demethylation. To differentiate between these two alternatives, further enzymatic and/or anaerobic incubation studies will have to be carried out.
EXPERIMENTAL

A crude preparation of \([3\alpha^{3}\text{H}]-14\alpha\text{-hydroxymethyl}-5\alpha\text{-cholesten}-3\beta\text{-ol}\), synthesized by Dr. Shaw in 1973, was applied to a column (1 x 100 cm) of silicic acid-Super Cel. Using a mixture of benzene and ether (8:2, v/v) as the eluting solvent, fractions 2.0 ml in volume (30 minutes per fraction; 0.067 ml/min) were collected. The resulting chromatogram is shown in Figure 151.

The contents of fractions 80-88 were pooled and the solvent removed under reduced pressure. The resulting residue was dissolved in 1 ml of propylene glycol and was sonicated for about an hour to assure complete dissolution. This preparation contained 4.96 x 10^{7} cpm/ml.

Thin-layer radiochromatographic analyses of the above preparation on plates of Silica Gel G, with solvent systems of chloroform-acetone (95:5, v/v; Figure 152) and ethyl acetate-benzene (5:2, v/v; Figure 153), showed a single labeled component corresponding in chromatographic mobility to that of authentic 14\alpha\text{-hydroxymethyl}-5\alpha\text{-cholesten}-3\beta\text{-ol}. The radiopurity of \([3\alpha^{3}\text{H}]-14\alpha\text{-hydroxymethyl}-5\alpha\text{-cholesten}-3\beta\text{-ol}\) was judged to be in excess of 97% on the basis of above chromatographic analyses.

A. IN THE PRESENCE OF ADDED DPN AND TPNH GENERATING SYSTEM

The livers (36.0 g) of four male rats (Sprague-Dawley strain; 150-200 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (360 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by differential centrifugation. This was then centrifuged at 105,000 x g for 60 minutes in a Beckman L2-65B Ultracentrifuge, using a Type 35 (35,000 rpm) rotor and polycarbonate screw-capped tubes. The pellets were removed and resuspended in 8 ml of
Figure 151. Silicic acid-Super Cel column chromatogram of the crude preparation of $[3\alpha^-3\text{H}]-14\alpha$-hydroxymethyl-5α-cholestan-7-en-3β-ol. The column was eluted with benzene-ether (8:2, v/v) and fractions 2.0 ml in volume were collected. o-o, Tritium radioactivity.
Figure 152. Thin-layer chromatogram of [3-H]-14-hydroxy-5-methyl-cholesterol-7-en-3-ol on a plate of Silica Gel G, developed twice in chloroform-acetone (95:5, v/v). Authentic 14-hydroxy-5-methyl-cholesterol-7-en-3-ol and cholesterol are shown at 4 cm and 7.5 cm, respectively.
Figure 153. Thin-layer radiochromatogram of [3α-3H]-14α-hydroxymethyl-5α-cholesterol-7-ene-3β-ol on a plate of Silica Gel G, developed in ethyl acetate-benzene (5:2, v/v). Authentic 14α-hydroxymethyl-5α-cholesterol-7-ene-3β-ol and cholesterol are shown at 8.5 cm and 11.5 cm, respectively.
potassium phosphate buffer (0.1 M, pH 7.4) to give a protein concentration of 22 mg/ml.

For snake venom treatment, the microsomal protein (176 mg) was incubated with 100 μg of anhydrous snake venom (Crotalus adamanteus) for 10 minutes at 37⁰ in 8 ml of potassium phosphate buffer (0.1 M, pH 7.4), to which 40 μmoles of calcium chloride were added. Digestion of phospholipid was arrested by the addition of 200 μmoles of EDTA. Potassium phosphate buffer (50 ml; 0.1 M, pH 7.4) was then added, and the treated microsomes were collected by centrifugation at 105,000 x g for 60 minutes. The isolated snake venom-treated pellets were resuspended in 8 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride to give a final protein concentration of 16.6 mg/ml.

To a test tube containing 2 ml of the above microsomal preparation, DPN (3 mg) and TPNH generating system (0.9 ml) were added to give final concentrations of 1.5 mM of DPN and 1.2 mM of TPN. [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol (2.48 x 10⁶ cpm. 7.9 μg) in propylene glycol (0.05 ml) was added last. Aerobic incubation was carried out for 2 hours at 37⁰ with constant shaking.

At the end of incubation, acetone (40 ml) was added and the mixture was homogenized. The resulting coagulum was removed by filtration; the filter paper and the coagulum were washed several times with fresh acetone. The combined filtrates were evaporated to dryness and the residue (2.41 x 10⁶ cpm; 97.2% recovery of incubated radioactivity) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 6 mg of unlabeled cholesterol. Using benzene–ether (9:1, v/v) as the eluting solvent (changed to benzene–ether
(8:2, v/v) at fraction 110), fractions 1.5 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 110; 0.075 ml/min) were collected. The resulting chromatogram (Figure 154) indicated that a major portion (46.7%) of the recovered radioactivity was eluted right after cholesterol (fractions 48-58), while another 35.8% of the eluted radioactivity corresponded chromatographically to the mobility of the unreacted substrate (fractions 144-159).

The contents of fractions 48-58 from previous column were pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (6.37 x 10^5 cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8,14-cholestadiene (5 mg), 3β-acetoxy-Δ^5,7-cholestadiene (6 mg), and cholesteryl acetate (4 mg). The column was eluted with hexane-benzene (7:3, v/v) and fractions 4.1 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 70; 0.205 ml/min) were collected. The resulting profile is shown in Figure 155. Most (93%) of the recovered radioactivity corresponded in chromatographic mobility to that of cholesteryl acetate (fractions 12-20).

Fractions 12-20 were pooled and applied (3.23 x 10^5 cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8(14)-cholestene (5 mg), 3β-acetoxy-Δ^7-cholestene (6 mg), and cholesteryl acetate (6 mg). The column was eluted with hexane-benzene (9:1, v/v) and fractions 4.2 ml in volume (20 minutes per fraction; 0.21 ml/min) were collected. The results of this column chromatography are shown in Figure 156. Approximately 10% of the recovered radioactivity cochromatographed with authentic
Figure 15A: Silicic acid–Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesten-3β-ol with stimulated rat liver microsomes in the presence of added DPNH and TPPI generating system. ○-○, Tritium radioactivity. △-△, Cholesterol measured colorimetrically.
Figure 155. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholestan-7-en-3β-ol with snake venom-treated rat liver microsomes in the presence of added DPN and TPNH generating system. △-△, Tritium radioactivity. •-•, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ5,14α-cholestadiene, and the third is due to 3β-acetoxy-Δ5,7-cholestadiene.
Figure 156. Alumina-Super Cel-silver nitrate column chromatogram of the acetate derivatives of monounsaturated sterols recovered from aerobic incubation of [3α-H]-14α-hydroxymethyl-5α-cholester-7-en-3β-ol with snake venom-treated rat liver microsomes in the presence of added DPN and TPNH generating system. O-O, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to 3β-acetoxy-Δ7(14)-cholestene, the second is due to 3β-acetoxy-Δ⁷'-cholestene, and the third is due to cholesteryl acetate.
3β-acetoxy-Δ^8(14)-cholestone (fractions 21-26), 71% was associated with 3β-acetoxy-Δ^7-cholestone (fractions 36-47), and 19% corresponded to the chromatographic mobility of cholesteryl acetate (peak at fraction 69).

The percent recovery of labeled sterols isolated from this experiment is tabulated in Table 59.

Table 59

Percent Recovery of Labeled Sterols Isolated from Aerobic Incubation of [3α-^3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol with Snake Venom-treated Rat Liver Microsomes in the Presence of DPN and TPNH

<table>
<thead>
<tr>
<th>Sterols Isolated</th>
<th>% Recovery of Total Incubated Substrate</th>
<th>% Recovery of Total Reacted Substrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-8(14)-en-3β-ol</td>
<td>4.3%</td>
<td>6.8%</td>
</tr>
<tr>
<td>Cholest-7-en-3β-ol</td>
<td>30.8%</td>
<td>48.0%</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>8.4%</td>
<td>12.9%</td>
</tr>
<tr>
<td>Total</td>
<td>43.5%</td>
<td>67.7%</td>
</tr>
</tbody>
</table>

*Total reacted substrate = total incubated substrate - unreacted substrate recovered from silicic acid-Super Cel column.

B. IN THE PRESENCE OF ADDED TPNH GENERATING SYSTEM

The livers (36.0 g) of four male rats (Sprague-Dawley strain; 150-200 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (360 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by differential centrifugation. This was then centrifuged at 105,000 x g for 60 minutes and the resulting pellets were resuspended in 8 ml of potassium phosphate buffer (0.1 M, pH 7.4) to yield a protein concentration of 22 mg/ml.
For snake venom treatment, the microsomal protein (176 mg) was incubated with 100 μg of anhydrous snake venom (Crotalus adamanteus) for 10 minutes at 37° in 8 ml of potassium phosphate buffer (0.1 M, pH 7.4), to which 40 μmoles of calcium chloride were added. Digestion of phospholipid was arrested by the addition of 200 μmoles of EDTA. Potassium phosphate buffer (50 ml; 0.1 M, pH 7.4) was then added, and the treated microsomes were collected by centrifugation at 105,000 x g for 60 minutes. The isolated snake venom-treated pellets were resuspended in 8 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM of magnesium chloride to yield a final protein concentration of 16.6 mg/ml.

To a test tube containing 2 ml of this microsomal suspension, a TPNH generating system (0.9 ml) was added to give a final concentration of 1.2 mM of TPN. \( [3\alpha^{-3}H]-14\alpha\)-Hydroxymethyl-5α-cholest-7-en-3β-ol (2.48 x 10^6 cpm, 7.9 μg) in propylene glycol (0.05 ml) was added last. Aerobic incubation was carried out for 2 hours at 37° with constant shaking.

At the end of incubation, acetone (40 ml) was added and the mixture was homogenized. The resulting coagulum was removed by filtration; the filter paper and the coagulum were washed several times with fresh acetone. The combined filtrates were evaporated to dryness and the residue (2.45 x 10^6 cpm; 98.8% recovery of incubated radioactivity) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 6 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 110), fractions 1.4 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 110; 0.07
ml/min) were collected. The results (Figure 157) indicated that approximately half (50%) of the recovered radioactivity was eluted after the cholesterol sample (peak at fraction 60), and another 28% of the eluted radioactivity corresponded in chromatographic mobility to that of the unreacted substrate (peak at fraction 152).

Fractions 52-65 were pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (5.11 x 10^5 cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled carriers of 3β-acetoxy-Δ^8,14-cholestadiene (6 mg), 3β-acetoxy-Δ^5,7-cholestadiene (5 mg), and cholesteryl acetate (3 mg). The column was eluted with hexane-benzene (7:3, v/v) and fractions 4.1 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 70; 0.205 ml/min) were collected. The resulting chromatogram (Figure 158) showed that approximately 90% of the recovered radioactivity cochromatographed with authentic cholesteryl acetate (fractions 13-18).

The contents of fractions 13-18 were pooled and applied (2.14 x 10^5 cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8(14)-cholestene (5 mg), 3β-acetoxy-Δ^7-cholestene (5 mg), and cholesteryl acetate (6 mg). The column was eluted with hexane-benzene (9:1, v/v) and fractions 4.4 ml in volume (20 minutes per fraction; 0.22 ml/min) were collected. The resulting chromatogram is shown in Figure 159. Approximately 10% of the recovered radioactivity corresponded chromatographically to the mobility of authentic 3β-acetoxy-Δ^8(14)-cholestene (peak centered at fraction 24), 77% was associated with 3β-acetoxy-Δ^7-cholestene (peak
Figure 157. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of \([3\alpha-^3\text{H}]\)-14α-hydroxymethyl-5α-cholesten-7-en-3β-ol with snake venom-treated rat liver microsomes in the presence of added TPNH generating system. ○-○, Tritium radioactivity. △-△, Cholesterol measured colorimetrically.
Figure 158. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesta-7-en-3β-ol with snake venom-treated rat liver microsomes in the presence of added TPNH generating system. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ5,14-cholestadiene, and the third is due to 3β-acetoxy-Δ5,7-cholestadiene.
Figure 159. Alumina-Super Cel-silver nitrate column chromatogram of the acetate derivatives of monounsaturated sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesta-7-en-3β-ol with snake venom-treated rat liver microsomes in the presence of added TPNH generating system. O-O, Tritium radioactivity. △-△, Steryl acetate measured colorimetrically. The first peak is due to 3β-acetoxy-Δ7(14)-cholestene, the second is due to 3β-acetoxy-Δ7-cholestenol, and the third is due to cholesteryl acetate.
at fraction 42), and 12% cochromatographed with cholesteryl acetate (peak at fraction 70).

Percent recovery of labeled sterols isolated from this experiment is given in Table 60.

Table 60

Percent Recovery of Labeled Sterols Isolated from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol with Snake Venom-treated Rat Liver Microsomes in the Presence of Added TPNH

<table>
<thead>
<tr>
<th>Sterols Isolated</th>
<th>% Recovery of Total Incubated Substrate</th>
<th>% Recovery of Total Reacted Substrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-8(14)-en-3β-ol</td>
<td>4.5%</td>
<td>6.3%</td>
</tr>
<tr>
<td>Cholest-7-en-3β-ol</td>
<td>34.7%</td>
<td>48.1%</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>5.4%</td>
<td>7.5%</td>
</tr>
<tr>
<td>Total</td>
<td>44.6%</td>
<td>61.9%</td>
</tr>
</tbody>
</table>

*Total reacted substrate = total incubated substrate - unreacted substrate recovered from silicic acid-Super Cel column.

C. IN THE PRESENCE OF ADDED DPN

The livers (36.0 g) of four male rats (Sprague-Dawley strain; 150-200 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (360 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by differential centrifugation. This was then centrifuged at 105,000 x g for 60 minutes and the resulting pellets were resuspended in 8 ml of potassium phosphate buffer (0.1 M, pH 7.4) to yield a protein concentration of 22 mg/ml.

For snake venom treatment, the microsomal protein (176 mg) was incubated with 100 μg of anhydrous snake venom (Crotalus adamanteus)
for 10 minutes at $37^\circ$ in 8 ml of potassium phosphate buffer (0.1 M, pH 7.4), to which 40 $\mu$ moles of calcium chloride were added. Digestion of phospholipid was arrested by the addition of 200 $\mu$ moles of EDTA. Potassium phosphate buffer (50 ml; 0.1 M, pH 7.4) was then added, and the treated microsomes were collected by centrifugation at 105,000 x g for 60 minutes. The isolated snake venom-treated pellets were resuspended in 8 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM of magnesium chloride to yield a final protein concentration of 16.6 mg/ml.

To a test tube containing 2 ml of the above microsomal preparation, DPN (3 mg) was added to give a final concentration of 1.5 mM of DPN in the incubation sample. $[3^\alpha-^3H]-14\alpha$-Hydroxymethyl-5$\alpha$-cholest-7-en-3$\beta$-ol (2.48 x $10^6$ cpm, 7.9 $\mu$g) in propylene glycol (0.05 ml) was added last. Aerobic incubation was carried out for 2 hours at $37^\circ$ with constant shaking.

At the end of incubation, acetone (40 ml) was added and the mixture was homogenized. The resulting coagulum was removed by filtration; the filter paper and the coagulum were washed several times with fresh acetone. The combined filtrates were evaporated to dryness and the residue (2.50 x $10^6$ cpm; 100.8% recovery of incubated radioactivity) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 6 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 80), fractions 1.6 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 80; 0.08 ml/min) were collected. The results (Figure 160) showed a major portion (49%) of the radioactivity was recovered as unreacted substrate
Figure 160. Silicic acid-Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with snake venom-treated rat liver microsomes in the presence of added DPN. o-o, Tritium radioactivity. Δ-Δ, Cholesterol measured colorimetrically.
(peak centered at fraction 134) and another 39% of the radioactivity was eluted right after the cholesterol standard (peak at fraction 50).

Fractions 44-56 from previous column were pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (2.94 x 10^5 cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8,14-cholestadiene (6 mg), 3β-acetoxy-Δ^5,7-cholestadiene (6 mg), and cholesteryl acetate (4 mg). The column was eluted with hexane-benzene (7:3, v/v) and fractions 4.4 ml in volume (20 minutes per fraction; 0.22 ml/min) were collected. The resulting chromatogram, shown in Figure 161, indicated that approximately 70% of the recovered radioactivity corresponded chromatographically to the mobility of cholesteryl acetate (fractions 31-43). A significant portion (22%) of the recovered radioactivity was eluted prior to 3β-acetoxy-Δ^8,14-cholestadiene, corresponding to the supposed mobility of 3β-acetoxy-Δ^7,14-cholestadiene (peak at fraction 83).

The contents of fractions 31-43 were pooled and applied (1.35 x 10^5 cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ^8(14)-cholestene (5 mg), 3β-acetoxy-Δ^7-cholestene (5 mg), and cholesteryl acetate (5 mg). The column was eluted with hexane-benzene (9:1, v/v) and fractions 3.5 ml in volume (20 minutes per fraction; 0.175 ml/min) were collected. The results of this column chromatography are shown in Figure 162. Most (95%) of the recovered radioactivity corresponded chromatographically to the mobility of authentic 3β-acetoxy-Δ^8(14)-cholestene (fractions 24-32).

Percent recovery of labeled sterols isolated from this experiment
Figure 161. Silica Gel G-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from aerobic incubation of \([3\alpha-^3\text{H}]-14\alpha\text{-hydroxymethyl-5\alpha-cholest-7-en-3\beta-ol}\) with snake venom-treated rat liver microsomes in the presence of added DPN. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second is due to 3β-acetoxy-Δ\(^{5\beta}\)-cholestadiene, and the third is due to 3β-acetoxy-Δ\(^{3\beta}\)-cholestadiene.
Figure 162. Alumina-Super Cel-silver nitrate column chromatogram of the acetate derivatives of monounsaturated sterols recovered from aerobic incubation of [3α-^3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with snake venom-treated rat liver microsomes in the presence of added DPN. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to 3β-acetoxy-Δ^8(14)−cholestanol, the second is due to 3β-acetoxy-Δ^7−cholestanol, and the third is due to cholesteryl acetate.
is given in Table 61.

Table 61

Percent Recovery of Labeled Sterols Isolated from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholest-7-en-3β-ol with Snake Venom-treated Rat Liver Microsomes in the Presence of Added DPN

<table>
<thead>
<tr>
<th>Sterols Isolated</th>
<th>% Recovery of Total Incubated Substrate</th>
<th>% Recovery of Total Reacted Substrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-8(14)-en-3β-ol</td>
<td>25.9%</td>
<td>50.9%</td>
</tr>
</tbody>
</table>

*Total reacted substrate = total incubated substrate - unreacted substrate recovered from silicic acid-Super Cel column.

D. IN THE ABSENCE OF ADDED COFACTORS

The livers (36.0 g) of four male rats (Sprague-Dawley strain; 150-200 g each) were homogenized in a medium of ice-cold potassium phosphate buffer (360 ml; 0.1 M, pH 7.4). The 10,000 x g supernatant fraction was isolated by differential centrifugation. This was then centrifuged at 105,000 x g for 60 minutes and the resulting pellets were resuspended in 8 ml of potassium phosphate buffer (0.1 M, pH 7.4) to give a protein concentration of 22 mg/ml.

For snake venom treatment, the microsomal protein (176 mg) was incubated with 100 μg of anhydrous snake venom (Crotalus adamanteus) for 10 minutes at 37° in 8 ml of potassium phosphate buffer (0.1 M, pH 7.4), to which 40 μmoles of calcium chloride were added. Digestion of phospholipid was arrested by the addition of 200 μmoles of EDTA. Potassium phosphate buffer (50 ml; 0.1 M, pH 7.4) was then added, and the treated microsomes were collected by centrifugation at 105,000 x g for 60 minutes. The isolated snake venom-treated pellets were resus-
pended in 8 ml of potassium phosphate buffer (0.1 M, pH 7.4) containing 4 mM magnesium chloride to yield a final protein concentration of 16.6 mg/ml.

To a test tube containing 2 ml of the microsomal preparation prepared as described above, [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol (2.48 x 10^6 cpm, 7.9 μg) in propylene glycol (0.05 ml) was added. Aerobic incubation was carried out for 2 hours at 37° with constant shaking.

At the end of incubation, acetone (40 ml) was added and the mixture was homogenized. The resulting coagulum was removed by filtration; the filter paper and the coagulum were washed several times with fresh acetone. The combined filtrates were evaporated to dryness and the residue (2.36 x 10^6 cpm; 95% recovery of incubated radioactivity) was chromatographed on a column (1 x 100 cm) of silicic acid-Super Cel, along with 6 mg of unlabeled cholesterol. Using benzene-ether (9:1, v/v) as the eluting solvent (changed to benzene-ether (8:2, v/v) at fraction 80), fractions 2.0 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 80; 0.1 ml/min) were collected. The results (Figure 163) indicated that most (72%) of the recovered radioactivity corresponded chromatographically to the mobility of the unreacted substrate (peak at fraction 123), and another 26% of the applied radioactivity was eluted right after cholesterol (fractions 42-50).

Fractions 42-50 were pooled and acetylated with pyridine-acetic anhydride (2 ml; 1:1, v/v) as described previously. The labeled steryl acetates (2.73 x 10^5 cpm) were then applied to a column (1 x 50 cm) of Silica Gel G-Super Cel-silver nitrate, along with unlabeled samples of
Figure 163. Silicic acid–Super Cel column chromatographic analysis of sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholesten-7-en-3β-ol with snake venom–treated rat liver microsomes in the absence of added cofactors. ○○, Tritium radioactivity. △△, Cholesterol measured colorimetrically.
Table 62

Percent Recovery of Labeled Sterols Isolated from Aerobic Incubation of [3α-3H]-14α-Hydroxymethyl-5α-cholestan-7-en-3β-ol with Snake Venom-treated Rat Liver Microsomes in the Absence of Added Co-factors

<table>
<thead>
<tr>
<th>Sterol Isolated</th>
<th>% Recovery of Total Incubated Substrate</th>
<th>% Recovery of Total Reacted Substrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-8(14)-en-3β-ol</td>
<td>21.7%</td>
<td>77.4%</td>
</tr>
</tbody>
</table>

*Total reacted substrate = total incubated substrate - unreacted substrate recovered from silicic acid-Super Cel column.

3β-acetoxy-Δ⁸,1⁴-cholestadiene (5 mg), 3β-acetoxy-Δ⁵,7-cholestadiene (6 mg), and cholesteryl acetate (5 mg). The column was eluted with hexane-benzene (7:3, v/v) and fractions 5.4 ml in volume (20 minutes per fraction, changed to 30 minutes per fraction at fraction 70; 0.27 ml/min) were collected. The resulting chromatogram (Figure 164) showed that approximately 85% of the recovered radioactivity cochromatographed with authentic cholesteryl acetate (fractions 24-36), and another 11% of the applied radioactivity was eluted prior to 3β-acetoxy-Δ⁸,1⁴-cholestadiene, corresponding to the supposed mobility of 3β-acetoxy-Δ⁷,1⁴-cholestadiene (peak at fraction 74).

The contents of fractions 24-36 were pooled and applied (1.75 x 10⁵ cpm) to a column (1 x 100 cm) of alumina-Super Cel-silver nitrate, along with unlabeled samples of 3β-acetoxy-Δ⁸(1⁴)-cholestene (4 mg), 3β-acetoxy-Δ⁷-cholestene (5 mg), and cholesteryl acetate (4 mg). The column was eluted with hexane-benzene (9:1, v/v) and fractions 3.2 ml in volume (20 minutes per fraction; 0.16 ml/min) were collected. The resulting elution profile is shown in Figure 165. Most (98%) of the
Figure 164. Silica Gel O-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monohydroxy sterols recovered from aerobic incubation of [3α-3H]-14α-hydroxymethyl-5α-cholest-7-en-3β-ol with snake venom-treated rat liver microsomes in the absence of added cofactors. o-o, Tritium radioactivity. Δ-Δ, Steryl acetate measured colorimetrically. The first peak is due to cholesteryl acetate, the second peak is due to 3β-acetoxy-Δ8,14-cholestadione, and the third peak is due to 3β-acetoxy-Δ5,7'-cholestadione.
Figure 165. Alumina-Super Cel-silver nitrate column chromatographic analysis of the acetate derivatives of monounsaturated sterols recovered from aerobic incubation of $[3\alpha^{3}\text{H}]-14\alpha$-hydroxymethyl-5$\alpha$-cholestan-7-en-3$\beta$-ol with snake venom-treated rat liver microsomes in the absence of added cofactors. ○○, Tritium radioactivity. △△, Steryl acetate measured colorimetrically. The first peak is due to 3$\beta$-acetoxyl-5$\alpha$-cholest-7-en-3$\beta$-ol, the second is due to 3$\beta$-acetoxyl-5$\alpha$-cholest-7-en-3$\beta$-ol, and the third is due to cholesteryl acetate.
eluted radioactivity corresponded chromatographically to the mobility of 3β-acetoxy-Δ^8(14)-cholestone (fractions 27-33).

Percent recovery of labeled sterols isolated from this experiment is given in Table 62.
VII. REFERENCES


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