Stereochemistry at the Center of Squalene during
Its Biosynthesis from Farnesyl Pyrophosphate
and Subsequent Conversion to Cholesterol*

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Recent studies on cholesterol biosynthesis have demonstrated that the biosynthesis of squalene from its immediate precursor, farnesyl pyrophosphate, is accompanied by the exchange of 1 hydrogen atom at C-1, in 1 of the 2 precursor molecules, for a hydride ion derived from reduced triphosphopyridine nucleotide (1-3). Thus, Popjak et al. observed that the farnesol of farnesyl pyrophosphate biosynthesized from mevalonate-2-14C-5-D2 contained 6 atoms of deuterium (2), whereas squalene biosynthesized from the same precursor contained only 11 atoms of deuterium (1). Three of these deuterium atoms were attached to the central 2 carbon atoms of squalene (1). Furthermore, squalene formed from 1-D2-2-14C-trans,trans-farnesyl pyrophosphate contained only 3 atoms of deuterium, attached to the 2 central carbon atoms of the molecule (2). The fourth hydrogen present at the center of the squalene was derived entirely from TPNH, and not at all from the water of the incubation medium (1).

Further studies from the same laboratories have indicated that this exchange of 1 hydrogen atom during biosynthesis of squalene is a stereospecific process. This was suggested by the absence of a visible isotope discrimination during squalene formation from 1-H2-2-14C-trans,trans-farnesyl pyrophosphate (2). Subsequently it was shown that the hydrogen transferred to the center of squalene is entirely transferred from the "β" position of TPNH (4).

This report is concerned with the stereochemistry of this hydrogen transfer with respect to squalene. Squalene was biosynthesized from 14C-farnesyl pyrophosphate with rat liver microsomes and TPNH, and then directly converted to cholesterol. The 2 central carbon atoms of squalene, which become labeled during squalene biosynthesis from farnesyl pyrophosphate with TPNH, appear in cholesterol as carbon atoms 11 and 12. Determination of the stereochemistry and distribution of the tritium label in cholesterol confirmed the fact that the hydride ion transfer is a stereospecific reaction, and allowed assignment of the absolute configuration of the asymmetrical carbon atom formed by introduction of the tritium label.

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EXPERIMENTAL PROCEDURE

Enzyme Preparations—Washed microsomes and the soluble supernatant fraction (104,000 × g supernatant) were prepared from rat liver homogenates as described in detail previously (5,6).

Before collection of the soluble supernatant fraction, the topmost layer of floating fat was removed, to produce a "defatted" preparation.

14C-Farnesyl Pyrophosphate—This compound was one of the preparations used by Popják, Goodman, Cornforth, Cornforth, and Ryhage in their studies of the mechanism of squalene biosynthesis (1). This preparation had been obtained biosynthetically from mevalonate-5-D2-2-14C, and initially had a specific radioactivity of 0.015 μc per μmole. After 21 months of storage in frozen aqueous solution at −20° under nitrogen, it was found to be 92% intact. Repeated extraction with light petroleum ether resulted in recovery of only 8% of the total 14C content of the solution, indicating that only 8% of the farnesyl pyrophosphate had hydrolyzed to liberate free allylic alcohol (mostly nerolidol) during the period of storage. The presence of deuterium in this preparation of 14C-farnesyl pyrophosphate was irrelevant for the purpose of this study and will be disregarded.

Tritium-labeled TPNH (TPN3H) preparation was carried out as described by Popják, et al. (1) in a reaction medium containing approximately 1 curie of 3H2O per ml. The specific radioactivity of the TPN3H was approximately 1.5 μc per μmole.

Biosynthesis of 14C-3H-Squalene and Conversion to 14C-3H-Cholesterol—A single, continuous, two-stage incubation was used. In the first stage, the anaerobic squalene-synthesizing system described by Goodman and Popják (6) was employed, together with TPNH as cofactor. The single 30-ml incubation mixture contained 7.5 ml of washed microsomes (concentrated 10 times; see (5, 6)); 2.2 μmoles of 14C-farnesyl pyrophosphate; 25 μmoles of TPN3H; 0.1 m potassium phosphate buffer, pH 7.4; 0.03 m nicotinamide; 0.005 m MgCl2; 0.01 m NaF; and 40 mg of bovine serum albumin. After 60 minutes of incubation under nitrogen at 37°, 15 ml of soluble supernatant fraction of the liver homogenate were added, and the incubation was continued under nitrogen for 5 minutes more. During this 5-minute period it was expected that much of the tritium of the TPN3H would be lost from the pyridine nucleotide by equilibration with the hydrogens of water, since such an equilibration has been shown to occur in

the presence of soluble supernatant plus microsomes (1). Immediately thereafter, a small aliquot was removed for analysis, 25 mg of unlabeled TPNH and 50 μmoles of glucose 6-phosphate were added, and the gas phase was changed from nitrogen to oxygen. Incubation under oxygen was continued for 3 hours, in order to convert the squalene formed during the anaerobic first stage to cholesterol.

At the end of the incubation, 50 ml of ethanol and 4 ml of 90% KOH were added, and the mixture was saponified under nitrogen at 60° for 1 hour. The nonsaponifiable fraction was obtained by three extractions with 1.5 volumes of light petroleum ether, and was separated into squalene and sterol fractions by chromatography on alumina (7). Portions of each fraction were then dissolved in 0.5% diphenylxazole in toluene and assayed simultaneously for 14C and 3H in a Packard liquid scintillation spectrometer.

The small sample removed from the incubation at the end of the anaerobic period was similarly treated and analyzed.

**Conversion to Bile Acids in Vivo**—The newly biosynthesized 14C-3H-cholesterol was suspended in a 0.5% bovine serum albumin solution and injected intraperitoneally into a 300-g rat with a bile fistula. Cannulation of the bile duct was performed as described by Bergström, Sjövall, and Voltz (8). Four serial 2-day collections of bile were then made. At the end of each collection the proteins were precipitated with ethanol and the ethanolic extracts were evaporated to dryness with nitrogen.

Before proceeding further with the isolation of bile acids, two of the samples were dissolved in 65% ethanol and extracted twice with equal volumes of light petroleum ether. The lipid extracts so obtained were largely decolorized by passage over small columns of alumina, with elution of cholesterol, and all less polar lipids, with acetone-ethyl ether (1:1). The extracts were then simultaneously assayed for 14C and 3H.

**Isolation of Bile Acids**—The conjugated bile acids were hydrolyzed in 2 N NaOH solution at 115° for 6 hours, and the free bile acids were extracted with ethyl ether after acidification with HCl.

Cholic and chenodeoxycholic acids1 were isolated by reversed phase partition chromatography in Solvent Systems C and F (9, 10): Solvent C, methanol-water, 150:150 v/v (moving phase), chloroform-isooctanol, 15:15 v/v (stationary phase); Solvent F, methanol-water, 165:155 v/v (moving phase), chloroform-heptane, 43:5 v/v (stationary phase).

Four milliliters of the stationary phase were supported on 4.5 g of hydrophobic Super-Cel (Johns Manville). The temperature was 23° (constant temperature room). The yield of chenodeoxycholic acid (m.p. 143-145°) was 28 mg from bile Samples 1 and 2 and 23 mg from Samples 3 and 4. Corresponding figures for cholic acid (m.p. 198-199°) were 103 mg and 98 mg. These crystalline acids were converted into the corresponding methyl esters by treatment with diazomethane.

**Methyl 3a,7a-Diacetoxy-12a-hydroxy-5β-cholanoate**—A solution of 88 mg of labeled methyl cholate in 0.42 ml of benzene and 0.1 ml of pyridine was treated with 0.11 ml of acetic anhydride and kept at 25° for 24 hours (12). Benzene (5 ml) was added, and the solution was washed five times with 2 ml of water. The solvent was evaporated under reduced pressure, and the residue was crystallized twice from aqueous methanol. The yield was 58 mg, m.p. 187.5-188°.

**Methyl 3a,7a-Diacetoxy-12 keto-5β-cholanoate**—Oxidation with chromium trioxide-pyridine complex (13) was chosen in order to avoid exchange of the hydrogens at C-11 with the medium through enolization (cf. (14)). Earlier and partly unpublished experiments have demonstrated that the 3H:14C ratio of methyl 3a-cathaloyloxy-7a,12α-dihydroxy-5β cholanoate 6α, 6β, 8α, 8β-H 24-14C is unchanged after oxidation to methyl 3a-cathaloyloxy-7,12-diketo-5β-cholanoate with the chromium trioxide-pyridine complex or with potassium chromate in sodium acetate-acetic acid buffer solution (15), thus excluding enolization in an analogous case under similar conditions. To a suspension of chromium trioxide-pyridine complex, prepared fresh from 60 mg of chromium trioxide and 0.6 ml of pyridine, was added a solution of 52 mg of methyl 3a,7a-diacetoxy-12a-hydroxy-5β-cholanoate in 0.6 ml of pyridine. The reaction mixture was kept at 25° for 24 hours, and the product was isolated by ether extraction after addition of water. Crystallization from aqueous methanol gave 41 mg of methyl 3a,7a-diacetoxy-12-keto-5β-cholanoate, m.p. 178-179° (Fieser and Rajagopalan have reported 177-178° (12)).

**RESULTS**

Analysis of the small sample removed from the incubation at the end of the anaerobic period revealed the expected finding that almost all of the nonsaponifiable radioactivity in this sample was in the squalene fraction. Extrapolation of the results for this sample to the entire incubation indicated that during the 65 minutes of anaerobic incubation 0.99 μmole of squalene had been newly synthesized from 14C-farnesyl pyrophosphate (yield, 90%). There was 0.5 μg atoms of hydrogen incorporated per μmole of squalene, as calculated from the 14C content. This latter value is similar to that observed previously (1) and differs from unity because of isotope discrimination.

In contrast to the anaerobic first stage, at the end of the aerobic period of incubation less than 4% of the total nonsaponifiable radioactivity was recovered in squalene. The cholesterol fraction was not purified or analyzed other than by the single chromatography on alumina described above. It contained 95.2% of the 3H and 77.2% of the 14C present in squalene at the end of the anaerobic period of incubation. Since 1 of the 6 14C-labeled atoms in squalene derived from 14C-2-mevalonate is lost during the anaerobic period of incubation. Since 1 of the 6 14C-labeled atoms in squalene derived from 14C-2-mevalonate is lost during the conversion of squalene to cholesterol, it was expected that the 3H:14C ratio in cholesterol would be 1.20 times the corresponding ratio observed in the initially formed squalene. The observed value was 1.23, indicating that no significant loss or gain of 3H occurred during the conversion of the 14C-3H-squalene to cholesterol.

In order to ascertain whether the 14C-3H-cholesterol sample might have contained significant amounts of 4-methylated sterol precursors of cholesterol, a small scale incubation was later conducted, containing the same components employed in the large incubation described above, but with only one-tenth as much of each constituent. Unlabeled TPNH was used. After incubation for 1 hour under nitrogen and 3 hours under oxygen, carrier squalene, lanosterol, and cholesterol (1 mg each) were added and the mixture was saponified as usual. The nonsaponifiable frac-
Stereochemistry at Center of Squalene

The experiments described in this report were designed to study the stereochemistry of the hydride ion transfer to squalene, by examining the steric distribution of tritium in cholesterol derived from newly synthesized, labeled squalene. During the cyclization of squalene to sterol, the central 2 carbons of squalene become C-11 and C-12 of the sterol ring system. The method of biological hydroxylation was employed, since all known reactions involving the enzymatic hydroxylation of a steroid have been shown to involve replacement of the hydrogen in the position which is hydroxylated, leaving the other hydrogen intact (14, 18-20). The hydrogen isotope present in 12α or 12β positions in cholesterol was hence determined by converting cholesterol to cholic acid (3α, 7α, 12α-trihydroxycholic acid) in a bile fistula rat, followed by oxidation to the 12-keto derivative. The hydrogen isotope attached to C-11 was found by determining the 3H content after subjecting the 12-keto compound to enolizing conditions.

A continuous two-stage incubation was used for biosynthesis of 3H-3H-squalene and further conversion to 3H-3H-cholesterol. The first step involved conversion of 3H-farnesyl pyrophosphate to 3H-3H-squalene by washed rat liver microsomes and TPNH under anaerobic conditions. The synthesis of cholesterol was then achieved by adding the soluble supernatant fraction of the liver homogenate and by incubation under oxygen. A short incubation under nitrogen after addition of the soluble fraction was used to exchange the 3H in TPNH for ordinary hydrogen in the medium (1). That this exchange was effective and that no tritium label was introduced in the subsequent reactions leading from squalene to cholesterol follows from the fact that the 3H:3H ratios in squalene isolated at the end of the anaerobic incubation and in cholesterol were practically identical when corrected for the loss of 1 3H-labeled carbon atom during this conversion. The results from the 3H:3H ratio determinations of cholesterol and bile acid derivatives clearly showed that cholic acid and chenodeoxycholic acid had both practically the same 3H content (relative to 14C) as cholesterol. This means that only a negligible amount of tritium label might have been present in the 12α position of the biosynthesized cholesterol. Oxidation to the 12-keto derivative under nonenolizing conditions resulted in loss of approximately half of the 3H content present in cholesterol. Exchange of the hydrogens at C-11 by enolization during treatment of the 12-keto compound with alkali resulted in almost complete loss of the tritium label (29% retention). These data

**TABLE I**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Radioactivity measured</th>
<th>Η: Η&amp; C ratio</th>
<th>Η retained</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Η</td>
<td>Η&amp; C</td>
<td>%</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>3510</td>
<td>962</td>
<td>3.65 (= 4.56*)</td>
</tr>
<tr>
<td>Methyl 3α,7α-dihydroxycholanoate (methylchenocholesterol)</td>
<td>787</td>
<td>186</td>
<td>4.22</td>
</tr>
<tr>
<td>Methyl 3α,7α-diacetoxy-12α-hydroxycholanoate</td>
<td>617</td>
<td>138</td>
<td>4.47</td>
</tr>
<tr>
<td>Methyl 3α,7α-diacetoxy-12α-ketocholanoate</td>
<td>285</td>
<td>138</td>
<td>2.06</td>
</tr>
<tr>
<td>3α,7α-Dihydroxy-12-keto-cholestanonic acid (subjected to enolizing conditions)</td>
<td>11</td>
<td>82</td>
<td>0.13</td>
</tr>
</tbody>
</table>

* This Η:Η& C ratio of 3.65 has been corrected (3.65 X 5/4 = 4.56) for the loss of 1 of the 5 14C-labeled carbon atoms during conversion of cholesterol to bile acids.

**DISCUSSION**

The experiments described in this report were designed to study the stereochemistry of the hydride ion transfer to squalene, by examining the steric distribution of tritium in cholesterol derived from newly synthesized, labeled squalene. During the cyclization of squalene to sterol, the central 2 carbons of squalene become C-11 and C-12 of the sterol ring system. The method of...
therefore indicate that the tritium label introduced into squalene from TPN\textsuperscript{3}H\textsubscript{2}, during its biosynthesis from farnesyl pyrophosphate, appeared in cholesterol in approximately equal amounts at C-11 and C-12. The tritium label present in the latter position was exclusively confined to the \( \beta \) position, whereas the sterically equivalent position of the tritium label at C-11 has not been determined. The distribution of the \( ^{3} \text{H} \) label in cholesterol between C-11 and C-12 demonstrates that cyclization of squalene proceeded to about the same extent from either end of the molecule. This randomization might have been brought about either by cyclization from either end of the molecule when still on the synthetase enzyme, or by dissociation of squalene from the synthetase before cyclization. The available data do not allow a distinction to be made between these possibilities. However, it should be mentioned that the experimental conditions designed to avoid exchange of the tritium of TPN\textsuperscript{3}H with hydrogen in the medium did allow squalene to accumulate during the first stage of the incubation, whereas it normally is subject to rapid cyclization.

The presence of the tritium label at C-12 exclusively in the \( 12\beta \) position unequivocally demonstrates that the introduction of the hydride ion to the squalene precursor is a stereospecific reaction. A nonstereospecific reaction can easily be ruled out, since this would result in the appearance of one-fourth of the introduced \( ^{3} \text{H} \) in each of the four positions \( 11\alpha, 11\beta, 12\alpha, \) and \( 12\beta \) of cholesterol, provided that cyclization is initiated from either end of the molecule. A nonstereospecific reaction followed by cyclization in one direction only would give cholesterol with \( ^{3} \text{H} \) in \( 11\alpha \) and \( 11\beta \) positions, or in \( 12\alpha \) and \( 12\beta \) positions.

The presence of the \( ^{3} \text{H} \) at C-12 exclusively in the \( 12\beta \) position further demonstrates that the asymmetrical carbon atom created in squalene by introduction of the hydrogen isotope has the \( R \) configuration (21). The sterically equivalent carbon at C-11 has not been determined. However, tritium-labeled squalene with the \( R \) configuration at the central carbon containing the tritium label will, according to the above considerations, yield cholesterol containing tritium in the \( 12\beta \) or \( 11\alpha \) position, depending upon the end of the molecule from which cyclization is initiated (Fig. 1). It can thus be assumed with reasonable confidence that the \( ^{3} \text{H} \) not in the \( 12\beta \) position was present entirely at the \( 11\alpha \) position.

The assignment of the absolute configuration as \( R \) rests, of course, on the assumption that the introduction of the \( 12\alpha \)-hydroxyl group in cholic acid involves a direct replacement of hydrogen from the position hydroxylated. This mechanism has been found to occur in all steroid hydroxylation reactions previously examined, including hydroxylations at positions \( 11\alpha \) and \( 11\beta \) (19), \( 7\alpha \) (18), \( 6\alpha \) and \( 6\beta \) (20), and \( 12\beta \) (14), and involving a variety of compounds and species. The occurrence of a similar reaction during \( 12\alpha \)-hydroxylation in cholic acid biosynthesis is therefore highly probable.

These conclusions about the absolute configuration of squalene are in complete agreement with those presented in a recent preliminary report by Cornforth et al. (22). In an elegant series of experiments, these workers unequivocally demonstrated the absolute configuration of \( R \) to be present at the center of squalene. A preliminary report of the findings now presented by us (23) was published simultaneously with the report of Cornforth et al.
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