THE UTILIZATION OF SQUALENE IN THE BIOSYNTHESIS OF CHOLESTEROL*

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It has been adequately demonstrated that acetic acid is the principal ultimate carbon source in the biosynthesis of cholesterol (1, 2) and ergosterol (3). Evidence also exists that acetoacetic acid may be utilized directly for the synthesis of cholesterol by animal tissues (4). However, the nature of intermediates of larger molecular weight has remained obscure. After elucidating the structure of the terpenoid hydrocarbon squalene, Heilbron et al. in 1926 suggested that this compound might be an intermediate in the biosynthesis of steroids (5). Balance studies which were carried out to test this hypothesis gave conflicting results (6–8).

In the preceding paper (9) the biological synthesis of squalene from acetate has been described. Labeled squalene can therefore be prepared biosynthetically, and with the aid of such material a very efficient conversion of the hydrocarbon to cholesterol by mammalian tissue has been demonstrated. Attempts to obtain C14-squalene by organic synthetic methods have been unsuccessful because the available methods lead to isomeric hydrocarbons which are biologically inactive. Preliminary reports of this work have appeared (10, 11).

RESULTS AND DISCUSSION

The material which has been employed to study the biochemical relationship between squalene and cholesterol is the labeled hydrocarbon obtained from the livers of rats which had been fed 1-C14-acetate together with natural squalene. Evidence for the identity of this hydrocarbon with natural squalene has been presented in the preceding paper (9). The hydrocarbon is further characterized by the infra-red absorption spectrum (Fig. 1) which closely resembles that of natural squalene from shark liver oil (Fig. 3, I).

The results of two experiments in which biosynthesized C14-squalene was fed to mice are shown in Table I. It is seen that all the sterol samples

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isolated contained significant quantities of isotopic carbon. The specific activities were not affected by successive purification steps and therefore the isotopic label must reside in cholesterol. About 50 per cent of the administered C\textsuperscript{14} was found in the fecal lipides, indicating that the absorption of squalene from the gut was incomplete. It may be estimated that approximately 8 per cent of the absorbed squalene had been converted to liver cholesterol. An additional 8 to 12 per cent of the absorbed squalene could be recovered in the form of carcass cholesterol\textsuperscript{1}. This represents a conversion efficiency approximately 20 to 40 times as great as that observed for the utilization of acetic acid in mice. Data for isovalerate, heretofore the most efficient carbon source in steroid biosynthesis (12) in the intact animal, are available only for the rat. If it is assumed that the relative efficiencies of acetate and isovalerate are the same in the mouse as in the rat, then squalene exceeds the efficiency of isovalerate by a factor of 6. Assuming from the data of Rittenberg and Schoenheimer (13) and Pihl \textit{et al.} (2) a half time value of 3 to 5 days for the liver cholesterol of mice, it may further be calculated that approximately one-fifth to one-third of the liver cholesterol which was synthesized during the test period had its origin in the squalene fed. Specificity of the conversion process is indicated not only by the high efficiency but also by the virtual absence of radioactivity in the fatty acids. All compounds which have previously been found to serve as sources of cholesterol carbon in the intact animal have also been shown to contribute appreciable quantities of carbon for fatty acid synthesis, indicating that these compounds are metabolized at least partially by way of 2-carbon intermediates. The virtual absence of C\textsuperscript{14} in the fatty acids precludes such a pathway in the conversion of squalene to cholesterol.

\textsuperscript{1} These recoveries must be regarded as minimum values because no attempts were made to isolate cholesterol quantitatively or to isolate the hydrocarbon fractions from the tissues of these animals.
Brief mention may be made of an experiment which further implicates squalene in steroid biosynthesis. The purpose of this experiment was to determine the effect of exogenous squalene upon cholesterol synthesis in vitro. If squalene were an intermediate in this process, it would be expected to depress the incorporation of acetate into the steroid. Livers from rats which had received natural unlabeled squalene in the diet and

### Table I

*Feeding of Biosynthetic C\(^{14}\)-Squalene; 2080 C.p.m.*

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1*</th>
<th>Experiment 2†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity</td>
<td>Isotope recovery per cent</td>
</tr>
<tr>
<td>Liver + gut lipides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol digi-tonide</td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>21.7</td>
<td>132</td>
<td></td>
</tr>
<tr>
<td>Regenerated cholesterol</td>
<td>4.2</td>
<td>121</td>
</tr>
<tr>
<td>Cholesterol dibromide</td>
<td>121</td>
<td>158</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>41.0</td>
<td>0</td>
</tr>
<tr>
<td>Carcass lipides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude steroid</td>
<td>25.0</td>
<td>41</td>
</tr>
<tr>
<td>Cholesterol digi-tonide</td>
<td>63.7</td>
<td>43</td>
</tr>
<tr>
<td>Cholesterol dibromide</td>
<td>43</td>
<td>37</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>211</td>
<td>0</td>
</tr>
<tr>
<td>Fecal lipides</td>
<td>127</td>
<td>54</td>
</tr>
</tbody>
</table>

* 4 mg. of squalene fed per day for 2 days to a 12 gm. mouse.
† 5 mg. of squalene fed per day for 2 days to a 28 gm. mouse.
‡ Counts per minute of an infinitely thick BaC\(_{13}\) sample.
§ mm carbon isolated \(\times\) S.A. of compound isolated \(\times\) 100.
‖ mm squalene carbon fed \(\times\) S.A. squalene \(\times\) 100.
¶ Calculated for free cholesterol.
|| The fecal lipides were assumed to have a carbon content of 80 per cent.

livers from control rats were sliced and the incorporation of \(1-C^{14}\)-acetate and \(1-C^{14}\)-ethanol into the lipides of the sliced tissue was determined. The data in Table II reveal that the feeding of squalene depresses by a factor of 100 the isotope incorporation into liver cholesterol. In contrast, the isotope concentration in the fatty acids amounts to one-half of the control values; this is within the range of variation normally encountered among different animals and cannot be regarded as being an effect of squalene. Since labeled squalene is rapidly converted to cholesterol in the intact animal, it appears likely that the suppression of acetate incorporation into
cholesterol observed here is the result of preferential utilization of squalene as a cholesterol precursor.

The present findings demonstrate an efficient and specific utilization of squalene carbon for cholesterol biosynthesis. The possibility that squalene is an intermediate in the transformation of small units to the steroid structure may be considered in the light of existing knowledge concerning cholesterol biosynthesis. Recently the distribution pattern of the methyl and carboxyl carbons of acetate in the isooctyl side chain and in some positions of the ring structure of cholesterol has been determined (14–16). The results of these studies are shown in Fig. 2.

On the basis of this distribution it was suggested that cholesterol might arise by the condensation of isoprenoid units (16). Squalene is composed of 6 isoprene units and has in the past been suggested as a cholesterol precursor (5–7). If the isoprene units of squalene were synthesized from acetate in the same manner as the terminal 5-carbons of the cholesterol side chain, then squalene would contain 18 methyl and 12 carboxyl carbons of acetate. In accord with this hypothesis, both carbon atoms of acetate have now been found to serve as carbon sources in the biosynthesis of squalene (9). Robinson (17) has proposed a direct cyclization of the hydrocarbon to the steroid molecule, as indicated in Fig. 2. If this mechanism were correct, not only would the carbon skeletons of squalene and cholesterol coincide, but the postulated distribution of acetate carbons in squalene would be identical in every detail with that found experimentally in cholesterol. In the course of this transformation the branched methyl groups that are attached to carbon atoms corresponding to positions 4, 13, and 14 of cholesterol would have to be eliminated. The resulting product would contain the methyl and carboxyl carbons of acetate in a ratio of 15:12. This ratio is identical with that found experimentally for the whole

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Squalene fed*</th>
<th>Cholesterol isolated Specific activity†</th>
<th>Fatty acid Specific activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1‡</td>
<td>0</td>
<td>3990</td>
<td>8450</td>
</tr>
<tr>
<td>2†</td>
<td>+</td>
<td>39</td>
<td>4160</td>
</tr>
<tr>
<td>3§</td>
<td>0</td>
<td>3285</td>
<td></td>
</tr>
<tr>
<td>4§</td>
<td>+</td>
<td>61</td>
<td></td>
</tr>
</tbody>
</table>

* The animals used in Experiments 1 and 3 received a stock diet. Those used in Experiments 2 and 4 received in addition 0.4 gm. of carrier squalene per rat per day for 2 days.
† Counts per minute of infinitely thick BaCO₃ samples.
‡ Substrate 0.006 M 1-C¹⁴-acetate; specific activity, 5 × 10⁵ c.p.m.
§ Substrate 0.004 M 1-C¹⁴-ethanol; specific activity, 1 × 10⁴ c.p.m.
cholesterol molecule with doubly labeled acetate (15). Thus the hypothesis that squalene is an intermediate in the conversion of acetate to cholesterol is in accord with all experimental data. However, the evidence for the scheme depicted in Fig. 2 is so far indirect.

Squalene synthesized biologically from radioactive acetate may be assumed to be randomly labeled and is for this reason not suitable for mechanism studies. Attempts were therefore made to prepare squalene labeled in selected positions by organic synthesis. Labeled geranylacetone was condensed with 1,4-dibromobutane, as described in the experimental section. The product of this reaction was the 1,6-glycol (18), which could be converted to squalene hexahydrochloride. Dehydrohalogenation of this derivative by pyridine yielded a hydrocarbon having the same boiling point and refractive index as natural squalene. However, this labeled hydrocarbon, in contrast to the biologically synthesized squalene, was inert in cholesterol synthesis in vivo. After the feeding of this compound to mice at a level of 20 mg. per 100 gm. per day for 2 days, the cholesterol isolated

![Chemical structures](image-url)
from the livers of these animals was found to be devoid of radioactivity. The 1,6-glycol obtained in the course of the synthesis was fed for 4 days in doses of 40 mg. per 100 gm. per day. The compound was absorbed but conversion to cholesterol did not occur. "Squalene" containing 9.35 atoms per cent excess deuterium was prepared by conversion of natural squalene to squalene hexadeuteriochloride and subsequent dehydrohalogenation of the derivative with pyridine. This regenerated hydrocarbon was likewise fed to rats at a level of 40 mg. per 100 gm. per day for 4 days. The liver cholesterol from these animals, as in the experiment with synthetic C\textsuperscript{14}-"squalene," did not contain a significant excess of isotope. Since in the case of the deuterio compound natural squalene was the starting material, it was suspected that treatment of the hexahydrochloride with pyridine led to a hydrocarbon which was not identical with natural squalene. This conclusion was confirmed by the following experiment. A portion of the biologically prepared C\textsuperscript{14}-squalene which had been found to be efficiently utilized for cholesterol biosynthesis was converted to squalene hexahydrochloride. The hydrochloride was dehydrohalogenated with pyridine, and the resulting hydrocarbon was isolated by alumina chromatography. It was fed at a level of 16 mg. per 100 gm. per day for 2 days. No radioactivity was present in the cholesterol isolated from the pooled liver and gut. The extent of conversion of this hydrocarbon to cholesterol must have been less than 5 per cent that of natural squalene. Passage of biologically active squalene through the hydrochloride had rendered it inert for cholesterol biosynthesis. These results suggest that a specific double bond sequence and possibly also a specific geometrical configuration are necessary for biological utilization of squalene. Chemical manipulation of the molecule appears to cause the formation of either structural isomers or steric isomers, or both. That structural isomerization does indeed occur during regeneration of squalene from the hexahydrochloride is demonstrated by the infrared absorption spectra of various "squalene" preparations. The spectrum of squalene from shark liver oil is shown in Fig. 3, I (see also Thompson and Torkington (19)). The hydrocarbon obtained after the passage of natural squalene through the hexahydrochloride gave the spectrum shown in Fig. 3, II. The spectrum of synthetic "squalene," not reproduced here, is identical with that shown in Fig. 3, II. The differences in the regions of 6.05 and 11.18 m\(\mu\) can be interpreted as resulting from a double bond shift, as shown below.

\[
R-\text{C}=\text{CH}-\text{CH}_2-\text{R'} \rightarrow R-\text{C}-\text{CH}_2-\text{CH}_2-\text{R'}
\]

\[
\text{CH}_4 \quad \text{CH}_2
\]

It is conceivable that, in addition to a specific double bond sequence, a unique cis-trans configuration, as in the carotenoids (20), is also necessary.
for biological utilization. However, the importance of this steric factor cannot be assessed at the present time.

The major effort in the study of steroid biosynthesis during recent years has been directed toward the identification of intermediates in the conversion of acetate to cholesterol. The distribution pattern of acetate carbon in cholesterol led to the hypothesis that such intermediates might be isoprenoid in nature. The present findings (9) clearly establish that the isoprenoid hydrocarbon squalene, like cholesterol, is synthesized in rat tissue from acetate as a carbon source. The normally low concentration and the rapid rate of synthesis of the hydrocarbon are properties exhibited by active intermediates in metabolism. Squalene itself is converted to cholesterol at a rapid rate and with a high degree of specificity. Thus, all available evidence is compatible with the earlier hypothesis that this hydrocarbon lies on the direct path of steroid synthesis from acetate.

**EXPERIMENTAL**

*Materials*—Biosynthetic C$^{14}$-squalene, having a specific activity of 2080 c.p.m. (9), was obtained by isolating the hydrocarbon fraction from the livers of rats which had been fed 1-C$^{14}$-acetate together with carrier squalene.
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Evidence for the identity of this material with squalene has been presented (9).

*Synthetic C\(^{14}\)*—Sodium acetate was prepared by carboxylation of methylmagnesium bromide with C\(^{14}\)O\(_2\) (21). Ethyl acetate was prepared by the interaction of sodium acetate and ethyl sulfate (22). The ethyl acetate was then condensed to ethyl acetoacetate under the influence of sodium triphenylmethyl (23). The resulting acetoacetic ester was coupled with geranyl chloride, with sodium ethoxide as the condensing agent (24). The geranyl acetoacetic ester was saponified with barium hydroxide, and the free acid was decarboxylated to yield geranylacetone. The 2-C\(^{14}\)-geranylacetone was allowed to react with 1,4-dibromobutane as described by Schmitt (25). The product of this reaction was the 1,6-glycol (18), b.p. 165–183° at 0.01 mm., \(n_\text{D}^\text{20}\) 1.4875. From this glycol a crystalline hydrochloride was prepared which was identical with squalene hexahydrochloride. The hydrochloride was dehydrohalogenated by heating with pyridine under a reflux, and the resulting hydrocarbon was isolated by alumina chromatography. The specific activity of this material was 7950 c.p.m.

Deuterium-Labeled *“Squalene”*—0.8 mole of DCl, generated by the method of Brown and Groot (26), was allowed to pass through a solution of 16 gm. (0.039 m) of natural squalene in 70 ml. of dry ethyl ether at -5°. The reaction mixture was allowed to stand overnight at 0°. The precipitate was collected, washed well with dry ether, and dried *in vacuo*. The hydrochloride was then refluxed for 16 hours with pyridine under a stream of nitrogen. The resulting hydrocarbon was distilled twice and the fraction boiling from 160–170° at 0.02 mm. was used in the feeding experiment. This hydrocarbon contained 9.35 atoms per cent excess deuterium.

**Analyses**—All C\(^{14}\) analyses were performed as previously described (9). Deuterium analyses were carried out after conversion of the hydrogen of the organic compounds to hydrogen gas by the method of Graff and Rittenberg (27). The abundance of mass 3 was measured in a Consolidated-Nier isotope ratio mass spectrometer.

Infra-red spectra were determined for 10 per cent solutions of the hydrocarbons in chloroform. We are indebted to Dr. Harold Boaz and Dr. Thomas V. Parke of the Lilly Research Laboratories for performing these infra-red analyses, and for interpreting the spectra.

**Isolation Procedures**—All the tissues were saponified by heating under a reflux with 10 per cent KOH in 50 per cent ethanol for 4 to 6 hours in a stream of nitrogen. The non-saponifiable fraction was extracted with petroleum ether, and the volume of the extract was reduced to approximately 30 ml. This was then poured onto a 1.5 X 15 cm. alumina column. The hydrocarbons were washed through the column with 150 ml. of petroleum
ether and the steroids were eluted with 150 ml. of 1:1 acetone-ether. Cholesterol was isolated from the sterol fraction by precipitation with digitonin. The digitonide was decomposed with pyridine and the free cholesterol was crystallized and then converted to the dibromide (28). The fatty acids were isolated by petroleum ether extraction of the acidified saponification mixture.

Liver Slice Procedures—In each 125 ml. incubation flask were placed 1.5 gm. of liver slices and 10 ml. of Krebs-Ringer phosphate buffer, pH 7.4. To this were added either 5 mg. of 1-C¹⁴-potassium acetate or 2 mg. of 1-C¹⁴-ethanol. The tissues were incubated in oxygen at 37° for 4 hours with shaking.

**SUMMARY**

1. C¹⁴-Squalene prepared biosynthetically from acetate is efficiently converted to cholesterol in the tissues of the mouse.

2. The preparation of C¹⁴-“squalene” and of deuterium-labeled “squalene” is described. These compounds, which are shown to be structural isomers of the naturally occurring hydrocarbon, are not utilized in steroid biosynthesis.

3. The rôle of squalene as an intermediate in cholesterol biosynthesis is discussed.

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