ON THE MECHANISM OF ENZYMATIC CYCLIZATION
OF SQUALENE*

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The demonstration that lanosterol is formed from acetate (1) and is
converted to cholesterol (2, 3) has provided strong support for the hypothe-
sis that lanosterol is an early intermediate in the conversion of squalene
to cholesterol (4, 5). The cyclization of squalene not only to lanosterol,
but also to the various cyclic triterpenes, has, in fact, been postulated to
proceed in a concerted manner without the formation of any stabilized
intermediate (6, 7). Experiments designed to test this hypothesis are
now reported.

In the preceding paper liver preparations are described which convert
squalene in good yield to lanosterol, but neither synthesize squalene from
acetate nor metabolize lanosterol further to cholesterol (8). It has thereby
been possible to isolate and study the cyclization reaction without inter-
ference by other reactions of steroid biogenesis. To test whether inter-
mediates occur, the squalene-lanosterol conversion has been investigated
in a medium of D₂O. Moreover, in order to ascertain the origin of the
hydroxyl group of lanosterol, analogous experiments were carried out with
H₂O¹⁸ and O₂¹⁸. The results support the hypothesis of a concerted cycliza-
tion and establish molecular oxygen as the source of the hydroxyl group
of lanosterol. A preliminary report of this work has appeared previ-
ously (9).

EXPERIMENTAL

For the present experiments, the rat liver homogenates were prepared
under the conditions for optimal formation of lanosterol described in the
preceding paper (8).

D₂O (99.5 per cent) and H₂O¹⁸ (1.4 per cent) were obtained from the
Stuart Oxygen Company. 11.1 atom per cent O¹⁸ oxygen gas was gen-
erously supplied by Dr. D. Rittenberg of Columbia University. D anal-
yses were kindly performed for us by Dr. H. S. Anker of the University
of Chicago. O¹⁸ analyses were carried out according to Doering and Dorf-

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man (10). The authors also wish to express their gratitude to Dr. F. H. Westheimer for the use of analytical equipment for oxygen analysis. All other materials used were the same as those in the previous paper.

In the experiments with D2O, livers from 100 gm. male rats were homogenized for 15 seconds in a Waring blender with approximately one-sixth the volume of ice-cold potassium phosphate buffer (0.1 M, pH 7.4). After aging of the homogenates for 10 minutes at room temperature, 2 volumes of ice-cold phosphate buffer (0.1 M, pH 7.4) in D2O were added. This was homogenized again for 15 seconds with a Waring blender and centrifuged for 10 minutes at 700 X g. The homogenate was decanted and nicotinamide added (10 mg. per ml. of homogenate). The aging before the addition of D2O was performed as a precaution against the possible formation of D-labeled squalene from endogenous precursors. C14-labeled squalene in the amounts indicated in Table I (2690 c.p.m. per mg.) was dissolved in a small volume of acetone (0.2 ml.) and added to 5 ml. of homogenate. The acetone was removed by placing the homogenate under a rapid stream of N2 for 5 to 10 minutes until the odor of acetone was no longer detectable. The remainder of the homogenate was added and incubation was carried out for 3 hours at 38° in a Dubnoff shaker. The same procedure was used in the experiment with H2O18. For the determination of the O18 concentration in the incubation medium, an aliquot of the homogenate was frozen and the water was sublimed in vacuo to another vessel. It was found to contain 0.9 atom per cent excess O18.

In the experiment with O18-enriched oxygen gas, the incubation was carried out in flasks designed after Thunberg tubes with 125 ml. suction flasks replacing the tubes. The homogenate was placed in the main compartment of the flasks. Squalene was suspended in the supernatant fluid of liver homogenate (centrifugation for 40 minutes at 144,000 X g) and placed in the top compartment. The supernatant fluid was found on assay to be inactive by itself for the cyclization of squalene. The flasks were evacuated and flushed with helium three times. A mixture of O18-enriched oxygen and helium (roughly 1:4 by volume) was introduced. The flasks were closed off, the squalene suspension was tipped in, and the mixture was incubated for 3 hours at 38° in a Dubnoff shaker. Analyses on a sample of the oxygen-helium mixture showed it to contain 10.9 atom per cent excess O18.

After incubation, the homogenates were saponified with methanolic KOH and the non-saponifiable material was extracted with petroleum ether (b.p. 30–60°). A few mg. of carrier lanosterol were added and the mixture was chromatographed (8). The quantity of lanosterol formed from squalene was estimated from the total radioactivity in the lanosterol fraction recovered after chromatography. After further dilution with
suitable amounts of carrier lanosterol, the solvent was removed in vacuo and lanosterol was recrystallized twice from dichloromethane-methanol. After drying at 100° in vacuo overnight, the samples were analyzed for D and O^{18}. The specific activities of the recrystallized lanosterol samples were determined and the dilution of biosynthesized lanosterol by carrier lanosterol was calculated. The lanosterol samples were characterized as described in the previous paper (8).

Results

The results on the squalene-lanosterol conversion when conducted in a medium containing D_2O are summarized in Table I. As seen from the

**Table I**
*Cyclization of Squalene in D_2O*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Homogenate used (ml.)</th>
<th>Atom per cent D in medium</th>
<th>Squalene added (mg.)</th>
<th>Lanosterol formed (mg.)</th>
<th>Dilution with carrier lanosterol</th>
<th>Atom per cent excess D in lanosterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>350</td>
<td>62.7</td>
<td>60</td>
<td>4.47</td>
<td>4.12</td>
<td>0.114 0.005</td>
</tr>
<tr>
<td>2</td>
<td>56</td>
<td>39.4</td>
<td>15</td>
<td>1.27</td>
<td>6.48</td>
<td>0.040 -0.003</td>
</tr>
<tr>
<td>3</td>
<td>62</td>
<td>41.7</td>
<td>15</td>
<td>0.81</td>
<td>7.2</td>
<td>0.038 0.001</td>
</tr>
</tbody>
</table>

Each ml. of the reaction mixtures contained 10 mg. of nicotinamide and 0.5 mg. of diphosphopyridine nucleotide. The incubations were carried out for 3 hours at 38° in a Dubnoff shaker.

* Calculated for incorporation of 1 proton from the medium per molecule of lanosterol, assuming that the reaction rate for H^+ is 5.5 times that for D^+.

The data in Table II show that, in the course of the conversion of squalene to lanosterol in a medium of H_2O^{18}, no O^{18} is incorporated. On the other hand, if formed in the presence of O^{18} gas, lanosterol contains O^{18} in considerable excess. Thus, the oxygen atom in lanosterol is clearly de-
rived from molecular oxygen. The fact that the O\textsuperscript{18} concentration in the lanosterol obtained from the experiment with O\textsubscript{2},\textsuperscript{18} was lower than required by theory is probably due to inadequate control of the temperature of the furnace in the oxygen analysis train.\textsuperscript{2}

For the discussion of the implications of these results, the scheme of concerted cyclization proposed by Ruzicka (6) is reproduced in Fig. 1.\textsuperscript{3} In this formulation, the initiation of the cyclization process is represented as an electrophilic attack by a hypothetical oxidizing agent, OH\textsuperscript{+}. The

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|}
\hline
Experiment & Homogenate used & Atom per cent excess O\textsubscript{18} in medium & Squalene added & Lanosterol formed & Dilution with carrier lanosterol & Atom per cent excess O\textsubscript{18} in lanosterol \\
\hline
H\textsubscript{2}O\textsubscript{18} & 200 & 0.9 & 60 & 2.20 & 10.5 & 0.085 \\
O\textsubscript{2},\textsuperscript{18} & 210 & 10.9 & 90 & 0.673 & 81.3 & 0.134 \\
\hline
\end{tabular}
\caption{Cyclization of Squalene in Presence of H\textsubscript{2}O\textsubscript{18} or O\textsubscript{2},\textsuperscript{18}}
\end{table}

The conditions are the same as those for Table I.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Mechanism of cyclization according to Ruzicka (6).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Reaction 1, initiation of cyclization by removal of hydride ion. The hypothetical removal of a hydride ion is assumed to be effected by transfer to oxidized diphosphopyridine nucleotide. Only ring A of the steroid molecule is shown in this and Figs. 3 and 4.}
\end{figure}

cyclization, once initiated, goes to completion and yields lanosterol as the result of various hydride and methyl shifts.

Examination of the scheme proposed for this oxidative cyclization raises the following questions. Are any intermediates formed, or does the reaction go in one step? What agent initiates the cyclization? Does the

\textsuperscript{2} Private communication from Dr. W. von E. Doering.

\textsuperscript{3} As pointed out by Eschenmoser et al. (7), throughout the process of concerted ring closures and hydride and methyl shifts, the positive charge of the intermediary carbonium ion is at no time centered on 1 single atom. In the present paper, such carbonium ions are used for the purpose of simplifying the illustration. It should be borne in mind that they do not imply the existence of such "free" carbonium ions during the process.
oxidation precede, follow, or take place simultaneously with the cyclization process? Is the oxidation effected by dehydrogenation (or removal of hydride ion) or by direct oxygenation? These questions are really overlapping, as will become evident from the following considerations.

Three distinct mechanisms for initiation of the cyclization and the subsequent ring closure are represented in Reactions 1 to 3. For purposes of illustration, the reactions are represented as if carried out in a medium of D₂O in the presence of O₂: (1) In Reaction 1 initiation occurs by removal of hydride ion, followed by hydration (Fig. 2). This mechanism is ruled out because neither D nor O¹⁸ from water is incorporated into lanosterol. (2) Reaction 2 is proton-initiated, followed by stereospecific oxygenation (Fig. 3). This mechanism cannot be eliminated solely on the basis of D and O¹⁸ data. However, in this case one would expect squalene to be transformed to cyclic products such as Δ⁵,⁷-lanostadiene in the absence of oxygen.

In order to test this possibility, carrier-free C¹⁴-labeled squalene was incubated with homogenate under anaerobic conditions. The hydrocarbon fraction, obtained by chromatography of the non-saponifiable material on alumina grade II, was chromatographed on alumina grade I (Woelm) together with 1 mg. of Δ⁴-cholestene. This hydrocarbon was chosen as a marker on the assumption that the hypothetical intermediate, Δ⁵,⁷-lanostadiene, travels on the columns closely together with Δ⁴-cholestene, but not with squalene. By using a 10 gm. Al₂O₃ column and petroleum ether (30–60°) as eluent, Δ⁴-cholestene was separated completely from squalene. It contained no radioactivity, indicating that a cyclic hydrocarbon is not formed from squalene under anaerobic conditions.

(3) In Reaction 3 direct oxygenation of squalene occurs (Fig. 4). This mechanism, the same as that shown in Fig. 1 with the exception that activated molecular oxygen takes the place of OH⁺, is consistent with all experimental data and will be discussed in detail later.
The conclusion that the cyclization is initiated by oxygen already answers the question of intermediates to the extent that non-oxygenated cyclic compounds are eliminated. Another type of intermediate may be visualized to arise from squalene if an intermediary carbonium ion is stabilized by uptake of OH⁻ or ejection of proton before hydride and methyl shifts take place (Reactions 4 to 6, Fig. 5). This would yield structures of the isoeuphol type (12, 13) which possess a completed tetracyclic ring system but differ from the steroids by having a methyl substituent at C-8 instead of C-13. Isoeuphol itself has been obtained by acid-catalyzed isomerization of euphol (14) and tetracyclic triterpenes containing the isoeuphol carbon skeleton have recently been isolated from dammar resin (15). Isoeuphol, with a β-methyl group at C-8 and an α-methyl group at C-14, can give rise in theory to either lanosterol, by one 1,3-methyl shift, or to euphol, by two 1,2-methyl shifts (Fig. 6) (16), and therefore is of special interest in regard to the question whether the routes of the biogenesis of the steroids, on the one hand, and of euphol, on the other, part company.
at the stage of squalene or at the stage of a tetracyclic intermediate. Compounds having methyl substituents at carbons 8 and 14 are, however, unlikely intermediates in the synthesis, at least of lanosterol, for the following reasons. In the case of isoeuphol itself or of a double bond isomer, conversion to lanosterol would have to be started by an electrophilic attack on the double bond by a proton as illustrated in Fig. 6 and this would result in an incorporation of D into lanosterol from a D₂O-containing reaction medium. Reactions 5 and 6 (Fig. 5) are, therefore, ruled out by the D₂O experiments.

Reaction 4 (Fig. 5) has not been eliminated definitely. For the diol to be further transformed to lanosterol or euphol, regeneration of the original carbonium ion by elimination of OH⁻ would have to take place. Although such a process would seem rather wasteful, it could be reasoned that formation of a stabilized diol is necessary to permit a transfer to separate stereospecific enzymes which determine whether lanosterol or the isomeric euphol is formed (Fig. 6). However, the unlikelihood of this reaction sequence is suggested by several lines of evidence. First of all, no evidence for any intermediate between squalene and lanosterol has been found throughout this investigation. Secondly, compounds with the isoeuphol carbon skeleton have not been encountered in the animal kingdom. Lastly, 1,3-methyl shifts, though postulated in many organic reactions, have never been experimentally established. Though none of these points is conclusive, they tend to argue against diols with the isoeuphol carbon skeleton as an intermediate.

The final possibility remains that a tetracyclic carbonium ion with the isoeuphol carbon skeleton is not stabilized by Reactions 4 to 6, but is directly transformed to lanosterol by concerted hydride and methyl shifts. As pointed out by Eschenmoser et al. (7), the squalene chain may be folded to give rise to an intermediary carbonium ion of either the "isoeuphol" or the "isolanosterol" type (Fig. 7). These would undergo two 1,2-hydride shifts and either two 1,2-methyl shifts ("isolanosterol type" carbonium ion) or one 1,3-methyl shift ("isoeuphol" type carbonium ion) to form lanosterol. Definite conclusions on this point must await the result of further work now in progress.

Though the recently isolated triterpenes with the isoeuphol carbon skeleton (15) cannot be biological precursors of lanosterol, their existence, nevertheless, furnishes additional support for a concerted mechanism of cyclization, since these compounds are the anticipated stabilization products of the cyclic carbonium ion, as indicated in Reactions 4 and 5. The formation of the various triterpenes and of the steroids from squalene thus must follow an essentially similar mechanism, the identity of the product being determined by the steric requirements of the cyclizing enzymes. In
the case of squalene cyclization by liver preparations there is no indication that products other than lanosterol are formed. This absolute steric specificity is, indeed, remarkable, since the squalene molecule has no polar groups and the squalene-enzyme interaction is limited to that between the \( \pi \) electrons in the double bonds of squalene with specific loci on the enzyme molecule. The liver enzyme being specific for lanosterol, it is not unreasonable to assume the existence of a family of similar squalene-cyclizing enzymes for the formation of the diverse cyclized products. It would, therefore, appear appropriate to designate as squalene-oxidocyclase I the liver enzyme which catalyzes the oxidative cyclization of squalene to lanosterol.

**SUMMARY**

By using \( D \) and \( O^{18} \) as tracers, it has been demonstrated that during the cyclization of squalene to lanosterol (1) no proton or \( \text{OH}^- \) from the me-
dium is incorporated into lanosterol, (2) molecular oxygen is incorporated into lanosterol, and (3) isoeuphol is not an intermediate.

The mechanism and the stereochemistry of this process have been discussed.

BIBLIOGRAPHY

ON THE MECHANISM OF ENZYMATIC CYCLIZATION OF SQUALENE
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