On the Mechanism of Squalene Biogenesis from Mevalonic Acid*  

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The essentially quantitative conversion of mevalonic acid to cholesterol by isolated liver (1) was the first finding to demonstrate the important role played by this branched-chain acid in the biogenesis of the steroids and their terpenoid precursors. Much evidence has since accumulated to show that mevalonic acid is indeed universally employed as the building stone for isoprene-derived natural products (2). During the transformation to materials of higher molecular weight mevalonic acid loses its carboxyl group (3), and C4 from 2-C4-mevalonate enters the various reaction products (squalene (4-6), cholesterol (7), soyasapogenol (8), rubber (9)) in the six positions anticipated from C-5 to C-2 (head to tail) condensations of mevalonic acid or its derivatives. The two bond-forming groups of mevalonic acid, the hydroxymethyl group at C-5 and the methylene group at C-2, are in a reduced state and as such insufficiently activated for forming carbon-carbon bonds. Modification of the molecule before condensation was therefore predictable. Exploring various possibilities for activation of the bond-forming groups we have employed tritium and deuterium as tracers to examine the loss and gain of carbon-bound hydrogen during the enzymatic conversion of mevalonic acid to squalene (10, 11). The results of these experiments were unexpected and led to the conclusion that the isoprenoid units, when undergoing head to tail condensations, must be in a state of oxidation which is unchanged from that of mevalonic acid itself. It was therefore proposed that the new carbon-carbon linkages are produced in an unprecedented manner by the coupling of two active methylene groups (11). Additional experiments have now permitted a more quantitative measurement of the losses of hydrogen from mevalonic acid in the course of squalene synthesis. The positions from which hydrogen is removed and those where it enters from external sources have been determined by analysis of the chemical degradation products of squalene. The results obtained bear out the conclusion that the head to tail condensations of the isoprenoid units occur without loss and reintroduction of hydrogen at the bond-forming centers.

EXPERIMENTAL

Materials—D2O, 99.9 atom per cent excess D was obtained from the Stuart Oxygen Company, and lithium aluminum deuteride, (LiAlD4 98 per cent D) from Metal Hydrides, Inc. T2O and lithium aluminum triteride were purchased from the New England Nuclear Corporation. 2-C4-bromoacetic acid was a product of Nuclear Chicago Corporation. ATP, DPN, and DPNH were obtained from the Sigma Chemical Company, β-hydroxy-β-methylglutaric acid from the Califormia Foundation for Biochemical Research, and farnesol from Givaudan-Delanowa, Inc. Samples of unlabeled mevalonic acid and 2-C4 mevalonic acid were kindly provided by Dr. James M. Sprague of the Merck, Sharp and Dohme Laboratories.

Enzyme Preparations—A crude yeast extract was used as the enzyme system for the present experiments. Fleischman's dried bakers' yeast was ground to a fine powder with a Bantam micro pulverizer and, after screening, autolysed in 3 times its weight of a 0.066 M (NH4)2HPO4 solution for 5 hours at 37°. The autolyte was kept overnight at 4°. centrifuged at 25,000 × g for 30 minutes and filtered through cotton gauze. This extract was stable for about a month when kept at -15°.

For the experiments with D2O it was necessary to employ an enzyme system containing a minimum of ordinary water. Direct lyophilization of the crude extract caused a 60 per cent loss of enzymatic activity and was therefore unsatisfactory. An active, nearly dry preparation was obtained in the following manner. The particulate fraction of the extract was separated from the yeast autolysate by centrifugation at 104,000 × g for 2 hours and washed once with 0.066 M (NH4)2HPO4 buffer. Three gm. of a firm jelly-like pellet were obtained from 100 ml. of crude extract. The particles retained enzymatic activity for 3 to 6 days when kept at -15°. Particles of fluffy appearance which were obtained occasionally were discarded since they were less active. The supernatant fluid from the first high-speed centrifugation was separated from the yeast autolysate by centrifugation at 104,000 × g for 2 hours and washed once with 0.066 M (NH4)2HPO4 buffer. Three gm. of a firm jelly-like pellet were obtained from 100 ml. of crude extract.

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Preparation of Labeled Substrates

2-C\textsuperscript{14}H\textsubscript{5}-T-DL-mevalonic Acid—Tritium-labeled mevalonic acid was obtained in low yield on direct reduction of \(\beta\)-hydroxy-\(\beta\)-methylglutaric acid with LiAl\textsubscript{4}H\textsubscript{4}. Ten ml. of dioxane, previously distilled from LiAIH\textsubscript{4}, were mixed with 0.56 ml. of an ethereal solution of 15 mg. of LiAl\textsubscript{4}H\textsubscript{4} (specific activity 85 mc. per gm.), brought to reflux temperature, and 50 mg. of \(\beta\)-hydroxy-\(\beta\)-methylglutaric acid (n.p. 106\textdegree) dissolved in 1.5 ml. of dioxane were added. After 15 minutes of heating, the excess LiAl\textsubscript{4}H\textsubscript{4} was destroyed by addition of a few drops of ethanol and the dioxane removed in a vacuum. The residue was dissolved in a few ml. of water, the pH adjusted to 1 with H\textsubscript{2}SO\textsubscript{4}, and mevalonic acid extracted continuously with chloroform. After addition of 10 ml. of water to the extract, the chloroform was distilled off. The solution was made alkaline with NaOH, warmed for 10 minutes at 60\textdegree to convert any mevalonolactone to the free acid, and then placed on a Dowex 1 (formate-form) column. Neutral materials were eluted from the column by washing with water, and mevalonic acid with 80 ml. of 0.1 \(\times\) formic acid. This eluate, containing 2.74 \(\times\) 10\textsuperscript{6} d.p.m. of \(T\) was lyophilized and added to an ethanolic solution of 25 mg. of the dibenzethylenediamine salt of unlabeled mevalonic acid (12). The solution was allowed to stand overnight, the salt precipitated by the addition of ether and recrystallized 4 times from alcohol-ether. The specific activity of the salt was 70 d.p.m. per \(\mu\)g. (6.5 per cent of the value calculated from the tritium content of the Dowex eluate). The crystalline material containing a total of 2.9 \(\times\) 10\textsuperscript{6} d.p.m. of \(T\) was dissolved in dilute NaOH and combined with about 10\textsuperscript{6} d.p.m. of 2-C\textsuperscript{14}H\textsubscript{5}-mevalonic acid. After removal of the dibenzethylenediamine by extraction with ether, the mixture of C\textsuperscript{14} and tritium-labeled mevalonic acids was subjected to paper chromatography on Whatman No. 1 paper with ethanol-NH\textsubscript{3}-water, 80:5:15, as the solvent system. A single but relatively broad radioactive band (\(R\textsubscript{p} 0.46\) to 0.60) was obtained. Three adjoining portions: A, \(R\textsubscript{p} 0.46\) to 0.50; B, \(R\textsubscript{p} 0.50\) to 0.53, and C, \(R\textsubscript{p} 0.53\) to 0.60, were cut from this band, eluted separately, and aliquots of the eluates analyzed for C\textsuperscript{14} and \(T\). As shown in the results in Table I, the tritium-containing product and the authentic 2-C\textsuperscript{14}H\textsubscript{5}-mevalonic acid were chromatographically indistinguishable. Fractions A, B, and C accounted for 78 per cent of the radioactivity placed on paper.

2-C\textsuperscript{14}H\textsubscript{5}-\(\beta\)-d-mevalonic Acid— Doubly labeled mevalonic acid containing deuterium and C\textsuperscript{14} in the same molecule was prepared by condensing 1-D\textsubscript{3}-3-ketobutanol acetate with 2-C\textsuperscript{14}H\textsubscript{5}-bromoacetic acid methylester (4) (procedure kindly furnished by Dr. F. W. Cornforth before the procedure was published). For the preparation of labeled ketobutanol, 11.1 gm. of 2-methyl-2-carboxethylideneoxaloxine were reduced in ethereal solution with 1.27 gm. of LiAl\textsubscript{4}H\textsubscript{4} ("98 per cent \(T\)) and the resulting 2-methyl-2-carboxethylideneoxaloxine converted to 1-D\textsubscript{3}-3-ketobutanol acetic acid. The product, 3.33 gm. (40 per cent of theory) had a b.p. of 97\textdegree at 25 mm. (reported, 92-95\textdegree at 25 mm. (12)). 2-C\textsuperscript{14}H\textsubscript{5}-bromomethylacetate was obtained by esterifying 21 mg. of 2-C\textsuperscript{14}H\textsubscript{5}-bromomethylglutaric acid (0.033 mc. per mmole), with an excess of diazomethane and this was diluted with 3.67 gm. of redistilled, unlabeled bromomethylacetate. The C\textsuperscript{14} ester and 1-D\textsubscript{3}-3-ketobutanol acetate were condensed in the presence of Zn (5), yielding methyl-2-C\textsuperscript{14}H\textsubscript{5}-D\textsubscript{3}-hydroxy-3-methyl-5-acetoxyvalerate in 50 per cent yield. The ester was saponified by standing in 54 ml. of 1 \(\times\) methanolic KOH at room temperature for 40 hours whereupon the resulting mevalonic acid was distilled. The final product, 1.1 gm. (61 per cent, b.p. 125\textdegree at 0.05 mm, reported, 110\textdegree at 0.1 mm. (12)) was converted into the \(N\),\(N\)'-dibenzylethlenediaminobis-mevalonate, m.p. 125\textdegree (reported, 123-126\textdegree (12)). The deuterium analysis of a sample, diluted with 115 parts of unlabeled material, gave 0.076 atom per cent excess \(D\) or 8.7 per cent for the undiluted salt (calculated for C\textsubscript{58}H\textsubscript{56}D\textsubscript{14}N\textsubscript{2}O\textsubscript{4} with "98 per cent LiAI\textsubscript{4}D\textsubscript{8} 8.9 per cent). The specific activity of the doubly-labeled acid was 1170 c.p.m. per amole. In biological experiments 50 per cent of the \(D\)-compound were converted to nonsaponifiable materials, 85 per cent of which was squalene.

1-T-farnesol—Farnesol, purified by distillation (b.p. 117 119\textdegree at 10 mm.) was oxidized to farnesol by chromic acid (13). The aldehyde was isolated by way of the bisulfite addition product and distilled (b.p. 97\textdegree at 0.2 mm., reported 110\textdegree at 0.4 mm. (14)). For storage, the farnesol was sealed under nitrogen in 1 gm. lots. For the reduction to 1-T-farnesol, 0.6 gm. of farnesol was dissolved in 50 ml. of dry ether (distilled from LiAIH\textsubscript{4}) and an ethereal solution of 23 mg. of LiAI\textsubscript{4} (specific activity 85 mc. per gm.) was added. After heating under reflux for 15 minutes, the reaction mixture was poured into 51 ml. of cold 10 per cent H\textsubscript{2}SO\textsubscript{4}, the ether decanted, and the aqueous layer extracted with petroleum ether. The combined ether and petroleum ether extracts were washed with sodium bicarbonate solution and dried over anhydrous Na\textsubscript{2}SO\textsubscript{4}. The reduction product was stored in solution without further purification. Before use the farnesol was purified by chromatography on Woelm alumina, activity No. 5. The specific activity of an aliquot was 1.35 \(\times\) 10\textsuperscript{6} d.p.m. per mg. 1-T-farnesol—1-T-farnesol, 30 mg., in 5 ml. of petroleum ether was oxidized with 100 mg. of acid-washed MnO\textsubscript{2}. The suspension was agitated on a rotary shaker for 5 days, the manganese salts filtered off, and the filtrate diluted to 30 ml. with petroleum ether. For enzymatic experiments, portions of this solution were used without further purification. An aliquot of the 1-T-farnesol solution containing an estimated 5 mg. was added to the mixture obtained by oxidizing 1 gm. of unlabeled farnesol with MnO\textsubscript{2}, and the diluted aldehyde converted to the 2,4-dinitrophenylhydrazone, m.p. 99-102\textdegree. The specific activity, determined in the gas flow counter was 3,000 c.p.m. per mg. or 1.08 \(\times\) 10\textsuperscript{4} c.p.m. per mg., calculated for undiluted farnesol. This value is in the range expected but cannot be directly compared to the specific activity of the 1-T-farnesol from which the aldehyde was prepared because the two materials were counted by different procedures. Scintillation counting was necessary for determining the radioactivity of the volatile farnesol, whereas the dinitrophenylhydrazone of farnesol had to be counted in a gas flow counter because of its quenching properties.

Degradation of Squalene—For the ozonolysis of squalene to yield acetone, levulinic acid, and succinic acid, the method used was essentially that of Cornforth and Poppjek (16). In following the published procedure we were unable to recover levulinic acid in satisfactory yield. It was then noted that under the conditions specified levulinic acid is further degraded to succinic acid.\textsuperscript{1} For the interpretation of the present experiments it was crucial that the succinic acid isolated was derived only from the central four carbon atoms of squalene and not contaminated by succinic acid from other sources. The degradation procedure was there-

\textsuperscript{1} Dr. Cornforth has informed us that under the conditions employed in this procedure (16) up to 20 per cent of the succinic acid isolated on degradation of squalene arises by oxidation of levulinic acid.
fore modified as follows, to avoid secondary oxidation of levulinic acid: 180 mg. of squalene, dissolved in 5 ml. of Skellysolve B, were ozonized for 3 hours at 0° with a 3 per cent stream of ozone in oxygen. After the addition of 1 ml. of acetic acid, ozonization was continued for another 3 hours. Thereafter, 0.2 ml. of 30 per cent H₂O₂ was added and the solution shaken at room temperature for 1 hour. After a second addition of 0.2 ml. of 30 per cent H₂O₂ and shaking for another hour, 1 ml. of 30 per cent H₂O₂ and 1.5 ml. of water were added and the solution, protected by a reflux condenser, warmed to 70° and kept at this temperature for 3 hours. After cooling and addition of 3 ml. of water the pH of the solution was brought to 5 with NaOH. Acetone was distilled in a nitrogen stream into ice water and isolated in the form of the HgO - HgSO₄ complex (17). The remaining solution was acidified with 5 ml. of 2 N H₂SO₄ and the excess H₂O₂ destroyed by adding, in the cold, an excess of solid ferrous sulfate. Succinic and levulinic acids were obtained by continuous extraction with ether for 16 hours, followed by removal of ether and acetic acid in a vacuum. Levulinic acid was extracted from the dry residue by three portions of chloroform and the remaining succinic acid crystalized successively from water and chloroform-ethanol (m.p. 185-187°). For the isolation of levulinic acid, the chloroform solution was placed on a column packed with a suspension of 12 gm. of silicic acid and 7.5 gm. of water in water-saturated chloroform. The eluting solvents were 100 ml. of chloroform, followed by 100 ml. of 5 per cent n-butanol in chloroform. Levulinic acid emerged with the last 40 ml. of chloroform and the first 10 ml. of the 5 per cent n-butanol-chloroform mixture. It was converted to the 2,4-dinitrophenylhydrazone by adding 200 mg. of 2,4-dinitrophenylhydrazine in a mixture of 1 ml. of concentrated H₂SO₄, 1.5 ml. of water and 10 ml. of 50 per cent H₃PO₄. For each milligram of levulinic acid, a volume of reagent containing 3 mg. of the hydrazine was added. After standing at room temperature for 15 minutes, the reaction mixture was diluted with an equal volume of water, the resulting precipitate collected by centrifugation and washed with water. It was redissolved in saturated sodium bicarbonate solution, cleared by centrifugation, and then reprecipitated by addition of acid. The dried material, when crystallized from chloroform or ethyl alcohol, melted at 205-209°, undepressed by authentic levulinic acid 2,4-dinitrophenylhydrazone.

Incubation and Isolation Procedures—The conditions for converting mevalonic acid to squalene varied from experiment to experiment and specific details are therefore given in the tables, along with the results. In general, the enzyme system was supplemented with ATP, 1 mg. per ml., DPN, 1 mg. per ml., and 0.001 N MnSO₄. Mevalonic acid was added either as the potassium salt or as the dibenzylethlenediamine salt, with no difference in results. Farnesol and farnesal were added to the reaction mixture dissolved in small volumes of acetone. Incubations were carried out at 30° in air for periods varying from 4 to 10 hours and terminated by addition of an equal volume of methanol. The reaction mixtures were digested in 20 per cent aqueous KOH and heated on the steam bath for 15 minutes. Squalene was isolated from the saponified mixtures by three extractions with ether or petroleum ether. The hydrocarbon was purified by alumina chromatography (18), formation of the thiourea adduct (19), and in some cases by preparation of the hexahydrochloride derivative (20, 21). In one experiment the identity of the radioactive hydrocarbon was established as follows. The nonsaponifiable material from an experiment identical with Experiment 1, Table II, and weighing 40 mg. was combined with 706 mg. of carrier squalene. After conversion to the crystalline thiourea adduct, the hydrocarbon was regenerated, chromatographed on silicic acid (22), and finally converted to the hexahydrochloride. As shown by the specific activities of the hydrocarbon at successive stages of purification, the amounts of contaminating hydrocarbons are small and are removed during the first purification step:

<table>
<thead>
<tr>
<th>Isotope Analyses</th>
<th>Description</th>
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<tbody>
<tr>
<td>1. Nonsaponifiable fraction plus carrier squalene...</td>
<td>36</td>
</tr>
<tr>
<td>2. From thiourea adduct</td>
<td>40</td>
</tr>
<tr>
<td>3. After silicic acid chromatography</td>
<td>41</td>
</tr>
<tr>
<td>4. Squalene hexahydrochloride</td>
<td>40</td>
</tr>
</tbody>
</table>

Isotope Analyses—For deuterium analysis samples were burned, the water collected and reduced to hydrogen which was analyzed in a Consolidated-Nier model 20-201 isotope mass spectrometer. The techniques used for combustion and converting water to hydrogen were essentially those described by San Pietro (23). The error of the D analyses varied from day to day between 0.002 to 0.005 atom per cent excess but never exceeded the larger of these values. All the D values given in the tables are averages of 2 or more analyses.

Samples were counted for C¹⁴ in a windowless gas flow counter (Packard Instrument Company). Specific activities were determined in triplicate and samples counted for a sufficient length of time to give less than 5 per cent standard error. Organic materials were counted either as infinitely thin layers or, after combustion, as BaCO₃ corrected for infinite thickness. Analyses of all tritium samples and samples containing both tritium and C¹⁴ were performed by the New England Nuclear Corporation with a Packard scintillation counter.

RESULTS AND DISCUSSION

Squalene is a symmetrical dihydroterpene and, in a formal sense, the condensation product of two farnesyl residues which in turn are composed of 3 isoprene units each. During the synthesis of squalene from 6 subunits, 5 carbon to carbon bonds are newly formed. Four of these result from head to tail interactions between C-2 and C-5 groups of mevalonic acid (or derivatives) while the fifth or central carbon to carbon bond is established by a C-5 to C-5 (tail to tail) linkage of isoprenoid or terpenoid intermediates. The condensations involved in squalene synthesis are therefore of two types, but the over-all result is the same for both, namely the formation of R-CH₂CH₂-R type structures by the coupling of two R-CH₂ groups. For simplicity it will be assumed that all the C-2 to C-5 condensations occur by the same mechanism.

Our previous experiments (10, 11) and those reported here were designed to characterize the functional groups of whatever intermediates are formed during squalene synthesis from mevalonic acid. For this purpose, isotopic hydrogen is uniquely suitable as a labeling agent because it allows detection of any changes in the state of oxidation at the reacting centers. In the first series of such experiments with a yeast extract as enzyme source, 2-C¹⁴-5-T-mevalonic acid was the substrate used and it was found that the squalene synthesized contained tritium as well as C¹⁴ (Table I and 10). The fact that hydrogen bound to C-5 was retained at all rules out reactions such as R-CH₂OH → R-COOH, i.e. the conversion of mevalonic acid to β-hydroxy-
The deuterium-bearing carbon atoms being adjacent and in the center of the molecule (Fig. 1). Had all the deuterium been lost by deuterium and therefore analogous experiments have been carried out with 2-C\(^14\)-5-D\(\text{\textit{d}}\)-mevalonic acid containing nearly 100 atom per cent excess D at C-5. The results were similar to those obtained with tritium but there were slight quantitative differences (Table II). In the experiments with deuterium the ratio of D to C\(^14\) again declined somewhat and in this case was found to be 17 to 25 per cent lower in squalene than in the added substrate. This loss corresponds to \(\frac{1}{2}\) or less of an atom of deuterium per molecule of mevalonic acid which is equivalent to 2 or 3 of the 12 deuterium atoms originally present in the six substrate molecules. The losses measured by tritium and deuterium labeling thus differ by 1 to 2 (out of 12) atoms, presumably because of analytical errors, isotope discrimination, or both. At any rate it is now conclusively shown that 9 to 10 of the deuterium (or hydrogen) atoms bound to C-5 of mevalonic are retained during squalene formation and hence that mevaldic acid (3-hydroxy-3-methylglutaraldehye acid (25-27)) or any other 5-aldehydes cannot be intermediates in the formation of squalene from mevalonic acid. Nevertheless, it is obvious that some changes occur at one or several of the C-5 positions, since the observed loss of 2 to 3 atoms is significantly outside the error of the analytical method. To explain this loss, the following possibilities have been considered. There is good evidence that mevalonic acid is synthesized in yeast from \(\beta\)-hydroxy-\(\delta\)-methylglutaryl-CoA by way of mevaldic acid (28). If in the enzyme system the reduction of mevaldic acid to mevalonic acid was partially reversible, deuterium bound to C-5 would gradually "leak out" and be replaced by normal hydrogen. Such a loss of deuterium would be unrelated to the obligatory events in the squalene synthesis. Alternatively the partial loss of D can be explained by the selective oxidation of one or two of the six participating hydroxymethyl groups to R-CHO or R-CO\(\text{OH}\). To distinguish between some of these possibilities, squalene derived from 5-D\(\text{\textit{d}}\)-mevalonic acid was degraded by ozonation according to the method of Cornforth and Popjak (16) with the results shown in Fig. 1 and Table III. It can be predicted on the basis of earlier information (4-8) that 5-D\(\text{\textit{d}}\)-mevalonic acid will label a total of six positions of squalene, namely every fifth carbon atom, counting from the ends of the hydrocarbon chain, two of the deuterium-bearing carbon atoms being adjacent and in the center of the molecule (Fig. 1). Had all the deuterium been retained, then the distribution of D in the degradation products should be as follows: a total of eight of the labeled atoms should be recovered at the \(\alpha\) positions of the four leucinie acid molecules and the remaining four atoms at the methylene carbons of seucic acid which is derived from the central portion of squalene. Experimentally it was found that leucinie acid contained, at least in one case (Table III), 1.80 atoms of D per molecl or nearly the theoretical 2, while the seucic acid had 1.8 atoms of deuterium instead of the possible 4. Therefore all four of the C-5 to C-9 interactions must have occurred virtually without loss of hydrogen bound to C-5. On the other hand, the low D content of seucic acid shows that hydrogen is being removed from C-5 when the central carbon to carbon bond of squalene is formed.
Deuterium content of degradation products of squalene synthesized from 2-C14-D5-DL-mevalonic acid

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<th></th>
<th>Degradation 1</th>
<th>Degradation 2</th>
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<tr>
<td></td>
<td>Atoms D per cent excess D</td>
<td>Atoms D per molecule</td>
</tr>
<tr>
<td>Squalene*</td>
<td>0.071</td>
<td>9.9</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>0.105</td>
<td>1.3</td>
</tr>
<tr>
<td>Levulinic acid†</td>
<td>0.066</td>
<td>1.5</td>
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* Sample diluted 279 fold (see Table II).
† Calculated as the 2,4 dinitrophenylhydrazone and calculated for free levulinic acid.
§ Since squalene yields on degradation 1 molecule of succinic acid and 4 of levulinic acid, the total recovery of D in these products is: 1.8 + 4 × 1.8 = 9.0 of the 9.9 atoms retained in squalene.

Dilution X atom per cent excess D X No. of H atoms in compound

100

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Dilution X atom per cent excess D X No. of H atoms in compound

100

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<tr>
<th></th>
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<td>2 CH3COClH3</td>
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<tr>
<td>4 CH3COCH2H2CO2H</td>
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<tr>
<td>1 CH2CO2H</td>
<td>4</td>
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</table>

Fig. 1. Expected and observed distribution of deuterium in squalene formed from 5-Dz-mevalonic acid. The encircled numbers refer to the carbon atoms of mevalonic acid which form the new carbon-carbon bonds.

Synthesis of squalene from 2-C14-DL-mevalonic acid in 99 per cent D2O

All flasks contained 1 mg. of ATP, 1 mg. of DPN per ml., and 0.001 M Mn++ except for Experiment B, in which DPN was replaced by the same quantity of DPNH. The gas phase in Experiments A, B, and D was air, and helium in Experiment B. The volumes of D2O in the 4 experiments were 80, 20, 18, and 18 ml., respectively.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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<tr>
<td>Mevalonic acid added, mg...</td>
<td>11</td>
<td>2</td>
<td>1.8</td>
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<tr>
<td>Mevalonic acid, c.p.m./µmole</td>
<td>490</td>
<td>490</td>
<td>670</td>
<td>670</td>
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<tr>
<td>Squalene, c.p.m./µmole</td>
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<td>25</td>
<td>42</td>
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<tr>
<td>Dilution</td>
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<td>118</td>
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<td>82</td>
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<tr>
<td>Squalene formed, mg...</td>
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<td>Squalene, atom per cent excess D§</td>
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<td>4.00</td>
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* Average of 2 or more analyses.

and these reactions, whatever they are, fully account for the losses of deuterium observed in the over-all reaction. Since the two methylene groups of succinic acid are indistinguishable, no decision can be made whether one atom each is removed from two R-CH2OH groups or both atoms from one and the same molecule.

Taking the data in Table II alone it is difficult to decide whether a total of 9 or 10 of the 12 deuterium atoms are retained in squalene. However, the good recoveries of deuterium in levulinic and succinic acids (Experiment 3, Table III) make it reasonably certain that 10 is the correct number. The data moreover suggest that in the yeast system used very little if any hydrogen at C-5 is enzymatically labilized due to reversal of the mevalonic-acid conversion.

Next, by studying squalene synthesis in D2O, we have attempted to ascertain whether any changes in oxidation state occur at C-2, the second of the bond-forming groups of mevalonic acid. Labeled hydrogen is taken up in the over-all process and from the extent of the incorporation it is possible to deduce certain aspects of the synthetic mechanism. As previously reported (11), approximately four atoms of deuterium are incorporated when squalene is synthesized from mevalonic acid in a medium containing nearly 100 per cent D2O (Table IV). Structural considerations had suggested that 2 of these D atoms are located at the two ends of the squalene chain and that the other 2 enter during a reductive step (11). Degradation of the hydrocarbon has now shown that 2 of the 4 atoms incorporated are present in the succinic acid from the squalene center, 0.8 atom (2 × 0.4 atom) in the acetoxy group derived from the two terminal isopropyl groups, and essentially none in levulinic acid (Table V). Thus approximately 2.5 to 3 of the 4 atoms of deuterium in squalene are accounted for in the degradation products. The low recovery of D is probably due to some loss of carbon-bound deuterium during the degradation of squalene and particularly during isolation of acetone in the form of the mercury derivative.2 3 If this is the correct explanation for the incomplete recovery of deuterium, then hydrogen uptake from the D2O environment must be limited to the two terminal positions, and to the center of the squalene chain where two hydrogens originally attached to C-5 groups are replaced by hydrogen from the medium. It is of particular significance that no hydrogen enters the four positions which represent the C-5 to C-2 junctions of mevalonic acid units (Fig. 2.C). These results with D2O thus fully confirm and amplify the findings made with deutero-mevalonic acid. They show independently that 10 of the 12 hydrogen atoms bound to C-5 of the six participating mevalonic acid molecules are retained in the end product and that 2 atoms are removed from the C-5 groups which form the squalene center.

The occurrence of oxido-reductions at the adjacent C-2 positions can be excluded on the basis of similar arguments. It is clear that any removal of hydrogen from C-2 would have to be followed by re-entry of hydrogen since in the end product of synthesis the corresponding groups are fully reduced. In the forma-

2 In a control experiment such an exchange of hydrogen has indeed been observed. Unlabeled squalene was degraded and the acetone isolated in the presence of 1 per cent D2O. The mercury derivative of acetone contained 0.08 atom per cent excess D.

3 Loss of deuterium from the β- and δ-positions of levulinic acid as a result of enolization is of course also a possibility. This is however less likely than the loss of deuterium from acetone, because levulinic acid contained no labeled hydrogen at all in two experiments and an insignificant excess in the third (Table V).
tion of a total of 4 new carbon-carbon bonds by head to tail linkage, a minimum of 4 hydrogen atoms should be taken up at C-2 positions if such hypothetical oxidation reductions had occurred. A re-entry of hydrogen in the form of $H^+$, or hydrogen exchangeable with $H^+$, is excluded because the total number of atoms incorporated from D$_2$O was only 4 and because 3 of these atoms are located elsewhere in the molecule. The possibility of hydrogen re-entering in the form of hydride ion, i.e. by direct hydride transfer from reduced pyridine nucleotide and therefore not exchangeable with $H^+$ (20), is likewise ruled out and on the following grounds. When a reduced pyridine nucleotide is the reductant, hydrogen is transferred as a pair of $H^+$ and $H^-$. In a heavy water medium, therefore, one D$^+$ will be introduced along with each unlabeled hydride ion. In the case under discussion, i.e. the coupling between C-5 and C-2 groups, the reaction:

$$R-CH=CH-R + H^- + D^+ + R'-CH=CH(R')$$

would be obligatory if carbon-bound hydrogen had previously been removed. In this final reductive step 4 atoms of deuterium would therefore be introduced at C-5 along with 4 $H^-$ at C-2. It is obvious that the data from the experiments with D$_2$O and 5-D$_2$-mevalonic acid are both incompatible with such reductions and hence also with any removal of hydrogen from the C-2 positions of mevalonic acid, at any intermediary stage.

The failure of hydrogen to enter any intra-chain positions of squalene except in the center of the molecule also bears on the mechanism for decarboxylating mevalonic acid. The carboxyl groups cannot be eliminated according to the equation:

$$R-CH=CH-CH=CH-R + H^- + D^+ + R'-CH=CH-CH=CH(R')$$

because D should then have entered squalene at the six C-2 positions indicated in Fig. 2,B, which is at variance with the total number of D atoms introduced and also with the observed distribution pattern. It is true that on degradation of squalene the C-2 positions become the $\beta$-methylene groups of levulinic acid and therefore susceptible to chemical exchange. However this does not weaken the argument because 3 of the 4 atoms of D incorporated into squalene have been shown to be located elsewhere in the molecule. Decarboxylation therefore occurs without protonation of the carbon chain. To account for this fact we have proposed (11) that the carboxyl group and the tertiary hydroxyl group of mevalonic acid are removed in a concerted fashion affording derivatives of $\Delta^8$-isopentenol, i.e. products with an "active" exomethylenegroup. This is in agreement both with the lack of biological activity of various unsaturated acids derived from mevalonic acid (6) and with the structure of recently isolated intermediates (30) which will be discussed below. In any event the decarboxylation and the introduction of the double bond at the tertiary carbon of mevalonic acid must be closely linked.

It will be recalled that the two isopropyl groups of squalene contain D and it could therefore be argued that the precursors of the two terminal units of the squalene chain, in contrast to the precursors of the intra-chain units, undergo decarboxylation by a nonconcerted mechanism. However, for the moment, it seems simpler to assume that only a single decarboxylation mechanism exists and that deuterium is introduced at the terminal positions, not during decarboxylation, but when the exomethylene double bonds shift to the neighboring positions (see Fig. 3).

The interpretation given to the deuterium data up to this point may be summarized as follows. The head to tail condensation of the six isoprenoid units take place with complete retention of the hydrogen bound originally to C-5 and C-2 of mevalonic acid whereas the tail to tail condensation, which affords the

### Table V

**Deuterium concentrations in degradation products of squalene synthesized in D$_2$O or T$_2$O**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Atom per cent excess D</th>
<th>Atoms of D per molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squalene</td>
<td>0.027</td>
<td>3.75</td>
</tr>
<tr>
<td>Suseinic acid</td>
<td>0.022</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Levulinic acid</td>
<td>0.003</td>
<td>0.4</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.022</td>
<td></td>
</tr>
</tbody>
</table>

Squalene from Experiment A, Table IV, diluted an additional 4.1 times.

† This sample of squalene was degraded by the unmodified procedure which gives inaccurate results for succinic acid as explained in the experimental part.

‡ The incubation mixture contained 100 mc. of T$_2$O, 10 mg. of DPN, 10 mg. of ATP, 0.001 M Mn++, and 1.0 mg. of unlabeled mevalonic acid in a total volume of 11 ml. The isolated squalene was diluted with carrier and purified by way of the thinurols adduct.

§ Assuming that the same number of atoms enter per molecule of squalene synthesized as in the experiments with D$_2$O.

¶ The actual counting rate was only 18 d.p.m. over a background of 80. This value is not considered significant.

---

Fig. 2. Introduction of D into squalene synthesized from mevalonic acid in a D$_2$O medium. *A*, observed distribution of D in squalene, in atoms per molecule; *B*, distribution of D (**) in squalene expected from a nonconcerted decarboxylation of mevalonic acid derivatives; *C*, the heavy lines show the bonds formed by interaction of isoprenoid units and the expected location of D if reductions had occurred during the condensation process.
central carbon-carbon bond of squalene, is accompanied by the replacement of two of the hydrogens at the reacting carbon atoms. Secondly, the carboxyl and tertiary hydroxy functions of mevalonic acid are removed in a concerted manner yielding an unsaturated 5-carbon intermediate which is sufficiently activated for condensation at one of the two bond-forming groups. The $\Delta^3$-isopentenol derivatives so produced can be readily visualized to eliminate the $\text{OH}$ or $\text{OR}$ groups at C-5 to form dienes, and we have therefore considered the possibility that isoprene itself is the condensing unit which polymerizes in a concerted manner to a sesquiterpenoid intermediate (11). Attempts to convert labeled isoprene into squalene by or to demonstrate the formation of isoprene from mevalonic acid (2) have however been unsuccessful and hence the “isoprene” hypothesis, at least in the simplified form presented, must be abandoned. Yet it is quite clear that the deuterium data restrict the structural choice to compounds with 2 methylene groups which are sufficiently activated for condensation. Recent observations in this laboratory satisfactorily reconcile the inactivity of isoprene itself with the type of structure postulated on the basis of the deuterium data. Several phosphorylated intermediates in squalene synthesis from mevalonic acid have been isolated (31–33), one of which has been identified as the pyrophosphate ester of $\Delta^3$-isopentenol (34, 30). The close relation of this compound to isoprene is evident. The pyrophosphate ester grouping would furnish a suitable leaving group for generating an electrophilic center at C-5 and thereby facilitate reaction with a second molecule of isopentenyl ester. A condensation process involving isopentenyl phosphates was one of the possibilities which we had already considered (11). In Fig. 3 two mechanisms for the coupling process are shown, both consistent with the isotopic and structural evidence and different only with respect to the stage at which double bonds in the chain-terminal units are isomerized. Mechanism B, the more attractive one on chemical grounds, implies the existence of two distinct 5-carbon units, isopentenyl derivatives as precursors for the four internal units and dimethylallyl esters for the two terminal ones of the squalene chain. Conceivably the dimethylallyl esters are derived from the $\Delta^2$-isopentenyl derivatives by isomerization, but such a reaction has not yet been demonstrated (See, however “Addendum”).

The mechanism presented here for carbon-carbon bond formation in the synthesis of isoprene polymers has no apparent analogy in biological systems. Elongations of carbon chains, where known in some detail, e.g. in the condensation of trioses, acetoin formation, fatty acid synthesis, or carboxylation reactions, follow a general pattern$^6$ which in its simplest form may be written as shown below:

![Diagram showing mechanisms for the condensation of isopentenyl esters to geranyl derivatives.](image)

The carbonyl component is activated by enolization to an anion while the other bond-forming carbon acquires nucleophilic character by elimination of carbon-bound hydrogen. The condensation product retains the oxygen of the carbonyl component, and if the ultimate product of synthesis is fully reduced as it is in the synthesis of higher fatty acids, the oxygen function is eliminated later by dehydration. On the other hand, in the head to tail condensations of 5-carbon units during terpene synthesis, one of the bond-forming carbon atoms is made electron-deficient by elimination of an anion, either before or simultaneously with condensation. This permits the carbonium ion to attack the “active” exomethylene group of the second condensing unit and produces the saturated carbon chain in a single step. In some respects, notably the nonparticipation of hydrogen from the environment, isoprenoid condensation resembles the mechanism by which carbon-carbon bonds are formed in the cyclization of squalene to lanosterol (35). In both cases saturated carbon chains are produced directly, except that in squalene cyclization the interaction is intramolecular.

In the scheme which we have proposed earlier (11), three isoprene units were visualized as condensing to form a sesquiterpenoid carbonium ion which can stabilize alternatively by proton elimination to farnesene, by OH$^-$ addition to the allylic alcohol nerolidol or by isomerization and OH$^-$ uptake to farnesol. If however isopentenolpyrophosphate is the reacting unit, the first sesquiterpenoid condensation produced will be the stable farnesylpyrophosphate. This possibility derives strong support from the recent finding of Lynen$^6$ that farnesylpyrophosphate is formed from phosphomevalonic acid in yeast extracts. The free farnesol which is formed in plant systems could then be produced hydrolytically, and the isomeric nerolidol by anion elimination and subsequent OH$^-$ addition. Similarly, termination of isopentenyl condensations at the monoterpenic stage should afford geranylpyrophosphate and by analogous reactions free geraniol and the isomeric linalool.

As pointed out above, the central carbon-carbon bond of squalene is established by tail to tail (C-5 to C-5) interaction between the appropriate terpenoid intermediates. The formation of this

$^6$ This generalization may not apply to the pyridoxal-dependent syntheses of serine from glycine and formaldehyde, and of tryptophane from indole derivatives and serine which may well involve condensations of active methylene groups.

$^6$ F. Lynen, private communication.

---

$^4$ J. Law and K. Bloch, unpublished experiments.
of the hydrocarbon farnesene which we had proposed (11) before the results of the squalene degradation were known. Also in-
densations between two acyl or two carbonyl derivatives such as which furnish the central portion of squalene, retain 2 of the 4
V). These data show that the two C-5 groups of mevalonic acid content of the degradation products of squalene (Tables III and
medium enter these positions. A number of mechanisms are thereby ruled out, among them the dimerization of two molecules of the hydrocarbon farnesene which we had proposed (11) before the results of the squalene degradation were known. Also in-
compatible with the experimental results are symmetrical con-deusions between two acyl or two carbonyl derivatives such as farnesenic acid or farnesal. For farnesenic acid this is evident since oxidation to the carboxylic acid would remove all hydrogen at the two C-5 positions which furnish the central carbon atoms of squalene. The possibility of two molecules of farnesal undergoing an acyloin condensation is excluded because in the reaction sequence:
\[
\begin{align*}
R \cdot CH_2OH & \rightarrow R \cdot CHO; \\
2 R \cdot CHO & \rightarrow R \cdot COCH_2H \cdot R; \\
R \cdot COCH_2H \cdot R & \rightarrow \rightarrow R \cdot CH_2CH_2 \cdot R
\end{align*}
\]
a minimum of 3 atoms of D should be removed from the carbon atoms involved and conversely the uptake of D from a D_2O medium should have been greater than the 2 atoms which have been found to enter at these positions. To test the possibility that farnesal or farnesol provide one or both of the C_10-condensing units, these compounds were labeled with tritium at the C-1 position which corresponds to C-5 of a mevalonic acid unit. Whereas 1-T-farnesal was inactive as a squalene precursor, 1-T-farnesol yielded squalene with a small but significant content of tritium (Table VI). Judging from the extent of the conversion farnesol is much less effective a precursor than mevalonic acid and it is therefore unlikely that the free alcohol takes part di-
rectly in the condensation process. Conceivably farnesol is con-
verted to a limited extent to farneslypyrophosphate, the inter-
mediate isolated by Lynen. A similar explanation may apply to farnesenic acid which according to Dituri et al. (22) is con-
verted to squalene but judging from the present results is more likely on a side path of squalene biogenesis.
Since squalene is a dihydroterpene, its formation from meva-
lonic acid according to the equation:
\[
6 \text{C}_4\text{H}_8\text{O}_4 + 2\text{H} \rightarrow \text{C}_{30}\text{H}_{40} + 6 \text{CO}_2 + 12 \text{H}_2\text{O}
\]
must include at least one reductive step, regardless of mechanism. The existence of this reductive step is reflected by the require-
ment for DPNH or TPNH in squalene synthesis which has been demonstrated for the enzyme systems from both yeast (10) and liver (28). Since the early transformations of mevalonic acid to various phosphorylated intermediates are not dependent on re-
duced pyridine nucleotides (30), this reductive step must occur at a relatively late stage of the over-all process. As has been pointed out, the formation of the central carbon-carbon bond of squalene is associated with the uptake of 2 atoms of hydrogen and it would seem reasonable to attribute this uptake to the re-
duction of a double bond subsequent to the coupling of ses-
quiterpenoid intermediates. The introduction of 2 hydrogens (from water) would suggest that a flavoprotein mediates this hydrogen addition since in hydrogen transfer reactions involving

### Table VI

<table>
<thead>
<tr>
<th>Additions</th>
<th>Squalene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d.p.m.</td>
</tr>
<tr>
<td>Farnesal, 1 mg., 5 \times 10^4 d.p.m.</td>
<td>0; 140</td>
</tr>
<tr>
<td>Farnesal, 1 mg., 5 \times 10^4 d.p.m. plus 0.16 mg unlabeled mevalonic acid*</td>
<td>120; 0</td>
</tr>
<tr>
<td>Farnesol, 0.5 mg., 5 \times 10^4 d.p.m.</td>
<td>860; 830</td>
</tr>
<tr>
<td>Farnesol, 0.5 mg., 5 \times 10^4 d.p.m. plus 0.16 mg unlabeled mevalonic acid*</td>
<td>980; 1230</td>
</tr>
</tbody>
</table>

* Added to provide for the possibility that the labeled substrates furnished only one of the two sesquiterpenoid precursors of squalene.

flavoproteins, hydride and hydrogen ions equilibrate (37). If, on the other hand, DPNH (or TPNH) were the immediate hydro-
gen donor, only 1 of the hydrogens incorporated at the double bond should come from the aqueous medium while the other should be directly transferred from reduced pyridine nucleotide (29). Preliminary experiments to test this point have been carried out by supplementing the enzyme system with DPN and 1-D_2-ethanol for generating DPND. Under these conditions some deuterium is introduced into squalene but the values ob-
tained, generally much less than 1 atom per molecule, have been too low and variable to decide whether DPNH is the immediate reducing agent or whether it functions by reducing another co-

### SUMMARY

1. 2-C^4-DL-mevalonic acid and 2-C^4-DL-mevalonic acid have been synthesized and converted to squalene in yeast extracts. The squalene formed from the deuterium-labeled mev-
alonic acid was chemically degraded and the ratios of D to C^4 in the various products determined.

2. It is concluded that the head to tail condensations of isop-
renoid units take place without loss of hydrogen bound to C-5 of mevalonic acid, but that two hydrogens are removed from C-5 positions when the central carbon-carbon bond of squalene is formed.

3. Experiments with heavy water show that during squalene synthesis from mevalonic acid 3.5 to 4 atoms of hydrogen are taken up from the reaction medium and that this hydrogen is incorporated at the 2 central carbon atoms and at the terminal isopropyl groups of squalene. From the same results it is con-
cluded that the loss of the carboxyl group and of the tertiary hydroxyl group of mevalonic acid is concerted and affords deriv-
atives of A^2-isopentenol.

4. Mechanisms for the condensation of isopentenyl derivatives to terpenes are presented.

### Acknowledgment

The authors gratefully acknowledge stimulating discussions with Dr. T. T. Tchen which have been invaluable for interpreting the experimental results.

Addendum—Two important papers dealing with the mechanism of squalene biogenesis have recently appeared from Lynen’s laboratory, describing the isolation of farnesylpyrophosphate and its conversion to squalene, the enzymatic and organic synthesis of isopentenylpyrophosphate and the enzymatic isomerization of isopentenylpyrophosphate to dimethylallylpyrophosphate (Lynen, F., Eggerer, H., Henning, U., and Kessel, I., Angew. Chem. 70, 739 (1955), and Agranoff, B. W., Eggerer, H., Henning, U., and Lynen, F., J. Am. Chem. Soc., 81, 1254 (1959).

REFERENCES

34. Bloch, K., Proceedings of the 4th international congress in biochemistry, Vienna, 1958, in press.
On the Mechanism of Squalene Biogenesis from Mevalonic Acid
H. C. Rilling and Konrad Bloch