Further Observations on the Biosynthesis of Squalene*

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We have previously reported that in the synthesis of squalene from 6 molecules of 5-Dt-mevalonic acid in crude yeast extracts, 10 out of a possible 12 atoms of deuterium are retained, and that the deficit of 2 atoms of deuterium is due to losses occurring when the central carbon-carbon bond of squalene is formed (1, 2). These conclusions were based on the following experimental findings: (a) squalene synthesized from 5-Dt-mevalonate contains an average of 9.5 atoms of deuterium per molecule; (b) the 2 central carbon atoms of squalene, which represent the tail-to-tail junction of isoprene units, contained 1.8 atoms of deuterium instead of the expected 4; and (c) in a medium of 100% D2O, squalene synthesis from mevalonic acid is associated with an average uptake of 3.75 atoms of deuterium of which 1 to 2 are located in the center of the molecule. From these studies the general mechanism for the head-to-tail condensations of isoprene units was correctly predicted and verified by the isolation of isoprene units joined by a single atom of hydrogen. This issue has also been clarified by studying the biosynthesis of squalene from 1-Dt-farnesyl pyrophosphate.

The present investigation was originally undertaken to obtain further insight into the mechanism of the final steps of squalene biosynthesis. While these studies were under way, we learned of results obtained by Popjak et al. (4) showing that, in the formation of the central carbon-carbon bond of squalene, only 1 of the 2 hydrogens linked to the bond-forming carbon atoms is replaced and not 2 as we had earlier concluded. We have therefore repeated our earlier experiments with 5-Dt-mevalonic acid and have modified the procedure for degrading squalene in order to minimize nonenzymatic losses of labeled hydrogen. This issue has also been clarified by studying the biosynthesis of squalene from 1-Dt-farnesyl pyrophosphate. The present results differ in some respects from those obtained earlier, and we now conclude, in harmony with the recent findings of Popjak et al. (5-7), that the formation of the central bond of squalene entails the replacement of only a single atom of hydrogen.

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† Holder of Predoctoral Fellowships from the National Science Foundation (1 year) and the United States Public Health Service (3 years).


EXPERIMENTAL PROCEDURE

Enzyme Preparations—A crude extract from Fleischmann’s dried baker’s yeast was the source of enzymes for converting mevalonic acid to squalene and was prepared as described previously (1). For the enzymatic experiment with farnesyl pyrophosphate, a yeast particulate fraction was prepared as follows: the crude yeast extract was centrifuged at 105,000 x g for 3 hours, and the sediments were suspended in 0.066 M ammonium phosphate buffer, pH 7.4, and reconstituted for 3 hours at 105,000 x g. The particles were resuspended in the same buffer with the aid of a Potter-Elvehjem homogenizer.

For the preparation of C14-farnesyl pyrophosphate, 10 ml of crude yeast extract were centrifuged at 105,000 x g, and the supernatant fluid was passed through a column containing 10 g of Sephadex G-50 in ammonium phosphate buffer, pH 7.4. The column was eluted with 0.066 M ammonium phosphate buffer, and 6-ml fractions were collected. Fractions 5, 6, and 7 were combined and used as the source of enzyme. Incubation was carried out for 4 hours at 30°C with the following additions: ATP, 30 μmoles; MnSO4, to a final concentration of 0.001 M; and 5.0 μmoles of the N,N'-dibenzylethylenediamine salt of 2-C14 mevalonic acid (1.12 × 109 d.p.m. per mg). The farnesyl pyrophosphate was isolated as described by Goodman and Popjak (8).

Incubation and Isolation Procedures—The experimental conditions for the incubations and the concentrations of cofactors and substrates added are given in the tables. Incubations were carried out for 4 hours at 30°C in a Dubnoff metabolic shaker and stopped by the addition of an equal volume of methanol. Carrier was added at this point and squalene isolated by alumina chromatography by way of the thiourea adduct as described (1).

Chemicals, isotopic materials, and coenzymes were obtained from the sources described previously (1).

Isotope Analyses—Deuterium analyses were performed by following the technique described by San Pietro (9) as modified by Rilling (10). Samples were counted for tritium, C14, and tritium and C14 in a Packard Tri-Carb scintillation counter.

Degradation of Squalene—(a) Experiment 2A, Table II: squalene was isolated after addition of 191 mg of carrier (obtained by decomposing the thiourea adduct of squalene), and a 100-μl aliquot, dissolved in 10 ml of purified pentane, was ozonized at −40°C. The ozonolysis products were oxidized by H2O2, and the resulting levulinic and succinic acids chromatographed on silicic acid as described (11). (b) Experiment 1B, Table I, and Experiment 2B, Table II: approximately 200 mg of squalene were dissolved in 10 ml of purified pentane and ozonized at −40°C. The excess ozone was expelled with purified nitrogen.
and the residue dissolved in ether precooled to -40°. To this solution 1 g of LiAIH₄ in 10 ml of ether was added dropwise; the reaction mixture was allowed to reach room temperature gradually and then heated to reflux for 12 hours. The ether was removed in a vacuum, 5 ml of benzene were added, and the chilled solution was acidified with sulfuric acid to destroy the excess LiAIH₄ and to decompose the aluminum complexes. After thorough mixing, the benzene layer was separated, and the solution was re-extracted with 5 ml of benzene; 2 g of anhydrous sodium sulfate were added, and the aqueous solution was extracted twice more with 5-ml portions of benzene; 2 g more of anhydrous sodium sulfate were added, and the aqueous solution was extracted twice more with 5-ml portions of benzene. These extractions must be performed quickly, as a gel will form if the solution containing the added sodium sulfate is allowed to stand for any length of time. The purpose of these extractions was to separate isoamopanol from, 1,4-butanediol and 1,4-pentanediol. (This separation was omitted in Experiment 1B, Table 1, since the isoamopanol fraction was not expected to contain any deuterium.) The combined benzene extracts were dried over magnesium sulfate, the solvent removed in a vacuum, and the residue taken up in a small amount of 20% sulfuric acid, 2% sodium carbonate solution, and several times with distilled water. The remaining benzene solution was dried over magnesium sulfate, the solvent removed in a vacuum, and the residue taken up in a small amount of 20% benzene in Skellysolve B. This solution was placed on a Woelm alumina II (23 g) column for chromatography. On elution with 15% benzene in Skellysolve B, the isopropyl-3,5-dinitrobenzoyl chloride were added, and the mixture was allowed to stand for 12 hours. After filtration, the solution was extracted successively with 1% sulfuric acid, 2% sodium carbonate solution, and several times with distilled water. The aqueous solution remaining after the extraction of isopropanol, containing the two dials, was extracted continuously with ether for 48 hours. The ether extract was reduced in volume to approximately 5 ml; it was mixed with 50 ml of acetone and with 1 g each of sodium carbonate and magnesium sulfate to remove sulfuric acid and water. After filtration and evaporation of the solvent in a vacuum, the residue, 195 mg, was distilled in a microstil at 2 mm of Hg and at an oil bath temperature of 110°. The two diols in the distillate were separated by gas chromatography on a column (0.210 inch, inside diameter; 6 feet long) packed with diethylene glycol-succinate on Chromosorb W. The column temperature was 110°, the carrier gas was argon, and the flow rate was approximately 40 ml per minute. The 1,4-pentanediol was eluted first. Because of the large quantity of the mixture injected (5 μl), quite large, broad peaks, which were off-scale, were obtained. This made it impossible to determine the retention times accurately. On rechromatography, the 1,4-pentanediol fraction gave only a single peak, identical with that shown by the authentic substance. The purity of the 1,4-pentanediol was further confirmed by its C¹⁴ content. This can be calculated from the known distribution of C¹⁴ in the C₃ fragmentes of squalene formed biosynthetically from 2-C¹⁴ mevalonic acid (12, 13). The chromatographic separations were run on 4- to 5-μl aliquots a sufficient number of times to yield a total of 4 to 5 mg of 1,4-pentanediol per U-tube.

The 1,4-butanediol collected from the same runs was contaminated by 10 to 20% of the 1,4-pentanediol, as revealed by rechromatography of the fractions. A complete separation of the two diols is possible with the size of column and packing described on considerably smaller quantities. Since it was desirable to have a minium of 1 mg of 1,4-butanediol for a measurement of specific activity, it was considered impractical to run the column under conditions allowing complete separation. One can, however, correct for these contaminations, because the 1,4-pentanediol contains C¹⁴, whereas the 1,4-butanediol is unlabeled. The C¹⁴ content of the 1,4-butanediol is therefore a measure of the contamination by 1,4-pentanediol.

Synthesis of 1-D₂-Farnesyl Pyrophosphate—Farnesol was oxidized to farnesal by the method employed by Bowden et al. (14) for the oxidation of acetylenic carbinols and glycols to acetylenic ketones. Farnesol was oxidized to farnesenic acid by the method of Caliez and Schinz (15) as modified by Campagne and LeSter (16). Methyl farnesate prepared with diazomethane in the usual manner was distilled at 1 mm of Hg through a 5 cm column packed with stainless steel metal sponge; b.p., 90-95°. For reduction, 9.2 mmoles of methyl farnesate in 10 ml of ether were added dropwise and with magnetic stirring to a flask containing 8.7 mmoles of LiAlH₄ (96% pure) and 15 ml of ether cooled to -30°. The reaction mixture was allowed to come to room temperature and then heated under reflux for 8 hours. The reaction products were worked up in the usual manner, and the 1-D₂-farnesol was distilled in a vacuum. Deuterium analysis (Josef Nemeth, Urbana, Illinois): found, 7.17 atom % excess; calculated, 7.69 atom % excess.

For tracing purposes and for calculation of the yield of squalene in the enzymatic synthesis, the 1-D₂-farnesol was mixed with trace amounts of C¹⁴-farnesol. This was obtained by hydroylizing biosynthetic C¹⁴-farnesyl pyrophosphate with snake venom phosphatase (Naja naja). Part of the C¹⁴-farnesyl pyrophosphate was kindly supplied by Dr. G. Popižak, and another batch was synthesized enzymatically as described above.

Specific Activity of trans-trans-1-D₂-C¹⁴-Farnesol—The 1-D₂-farnesol to be used for the synthesis of 1-D₂-farnesyl pyrophosphate was a mixture of the cis trans and the trans-trans isomers. For the purpose of the present experiment, it was necessary to know the specific activity of the biosynthetically active cis-trans isomer. To approximately 1 g of the 1-D₂-farnesol, biosynthetic C¹⁴-farnesol, and 1-μl aliquots of the mixture were subjected to gas chromatography. The column (diethylene glycol-succinate on Chromosorb W) was kept at 125°, and the argon flow rate was 40 ml per minute. The two stereoisomers gave well separated peaks, and these were collected individually in tarod U-tubes. The cis-trans isomer was eluted before the trans-trans isomer. The retention time of the two isomers was not measured because the peaks obtained were broad because of the large quantities injected. The vapor phase chromatography of these two isomers has also been described by Popižak and Cornforth (17).

A sufficient number of runs were made to yield three 1- to 3-mg samples of each isomer. The specific activities, determined by scintillation counting, were 38.2 i. d.p.m. per mg for the trans-trans-1-D₂-C¹⁴-farnesol and approximately 265 i. d.p.m. per mg for the cis-trans isomer. Since the specific activity of the cis-trans isomer was unimportant to the experiment, it was not measured very accurately. These results show, however, that the C¹⁴-farnesol derived from the biosynthetic farnesyl pyrophos-
ether. The aqueous phase was lyophilized to approximately 1 
dielectric heating tape, and was topped by a reflux condenser.  
A. Results from (1)  
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<th>Substance</th>
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<tr>
<td>1,4-Butanediol</td>
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B. Present results  
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<th>Substance</th>
<th>Rp value</th>
<th>D atoms per molecule</th>
</tr>
</thead>
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<tr>
<td>Squalene</td>
<td>0.1139 ± 0.0003</td>
<td>2.40 ± 0.13</td>
</tr>
<tr>
<td>1,4-Pentanediol</td>
<td>0.0593 ± 0.0027</td>
<td>1.51 ± 0.07</td>
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a Average of three analyses.  
b Errors are given as average deviation.  
c If these values are corrected for a contamination of the 1,4-
butanediol with 1,4-pentanediol of 10 to 20%, they become 2.64
to 2.77 and 2.35 to 2.46 atoms of D per molecule.

results and discussion  
Squalene Synthesis from &Dz-Mevalonic Acid—In Table I are
recorded the new and the previously reported deuterium analyses
and also the D concentrations of the products obtained on
degradation of squalene. The present figure for the total num-
ber of D atoms incorporated into squalene (10.4 atoms) is
slightly, and possibly significantly, higher than those reported
previously. An identical experiment with the corresponding
enzyme system from liver has recently been reported by Popjak
et al. (6, 7), and, according to their data, the number of D atoms
retained in squalene synthesized from 5-Dz-mevalonic acid is 11
out of a possible 12 and not 10. That the value of 11 is correct
and possible reasons why our earlier values were too low will
become apparent from the discussion later in this paper.

phosphate is essentially free of cis-trans isomer. From the above
value for the specific activity of the trans-trans isomer and from
the specific activity of the enzymatically formed squalene, the
dilution of the squalene was calculated, and from the latter value
and the D concentration in the squalene, the number of D atoms
incorporated could be determined (Table III).

1-Dz-C64-Farnesyl Pyrophosphate—The procedure used was
that of Cramer and Doham (18), as amplified in a private com-
unication from Professor Cramer. The di-triethylammonium
salt of phosphoric acid was prepared by mixing 4.5 moles of
anhydrous crystalline phosphoric acid, 4.0 ml of acetonitrile, and
9.0 moles of triethylamine. The mixture was gently
heated until the solids had dissolved and then transferred to a
dropping funnel which was kept at approximately 80°, by means
of electrical heating tape, and was topped by a reflux condenser.
This solution was then added dropwise in the course of 25 minutes
to a solution of 2.25 moles of 1-Dz-C64-farnesol in 8.3 moles
of trichloroacetonitrile. The reaction mixture was stirred vig-
ously with a magnetic stirrer and gradually warmed to 45-
50°. Stirring was continued at this temperature for 6 hours.
After standing in an ice bath overnight, the reaction mixture
was diluted with 8 ml of water and then repeatedly extracted with
ether. The aqueous phase was lyophilized to approximately 1

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squalene was isolated and analyzed as succinic acid. On the basis of these data alone, it would be difficult to decide whether, in the central condensation to squalene, the number of hydrogen atoms removed from the C-5 positions of mevalonic acid (or the corresponding carbon atoms of farnesyl pyrophosphate) is 1 or 2. However, these data, in conjunction with the convincing evidence presented by Popják et al. (5-7) and with the results reported below, leave little doubt that 1 atom is the correct value, i.e., that squalene synthesized from 5-D₂-mevalonic acid contains 3 atoms of D at the 2 central carbon atoms. Moreover, this value follows logically from the conclusion that in the synthesis of squalene from 6 molecules of 5-D₂-mevalonic acid, 11 of the possible 12 atoms of D are retained.

**Synthesis of Squalene from Farnesyl Pyrophosphate**—Since farnesyl pyrophosphate is known to be the essequiterpenoid precursor of squalene (4), and since suitable methods for the synthesis of this compound are available (18), the oxidation-reduction occurring during the condensation of C₁₅ units is susceptible to direct investigation. For this purpose the 2 hydrogen atoms of C-1 of farnesol were replaced with deuterium by reducing methyl farnesate with LiAlD₄ to 1-D₂-farnesol. C₃₄-Farnesyl obtained by phosphatase hydrolysis of biosynthetic farnesyl pyrophosphate was added, and the mixture, consisting of the cis-trans and the trans-trans isomers of 1-D₂-farnesol and of the predominantly trans-trans isomer of C₁₅-farnesol, was converted to farnesyl pyrophosphate by published procedures (18). This product was incubated with washed yeast particles and TPNH, and the squalene was isolated with the aid of carrier. The specific activity (C₃⁴) of the biologically active trans-trans component of farnesyl pyrophosphate was determined as described in "Experimental Procedure," and from the value obtained, the dilution of squalene and the number of D atoms incorporated were calculated. As shown in Table III, the resulting squalene contained 2.75 atoms of D; this demonstrates very clearly that, during the dimerization of farnesyl pyrophosphate, only 1 of the 4 hydrogen atoms at the bond-forming carbon atoms is removed. Popják et al. (6, 7) have already investigated the conversion of 1-T₂-C₄-farnesyl pyrophosphate to squalene and have reported results showing a loss of tritium corresponding to the removal of only 1 of the 4 labeled hydrogen atoms. Whereas these experiments already gave the correct answer, the possibility of fortuitous results due to isotope effects could not be excluded, and this made it desirable to obtain confirmatory evidence by using deuterium-labeled farnesyl pyrophosphate.

The outcome of the above experiment and the results reported simultaneously by Popják et al. (22), in this Journal, lead to the firm conclusion that in the central condensation leading to squalene, only 1 atom of hydrogen is replaced and not 2, as we had previously reported. Popják et al. (5, 7) have pointed out that this result is not compatible with any of the mechanisms that had earlier been proposed for the final step of squalene synthesis.

**Role of TPNH in Squalene Synthesis**—The over-all transformation of mevalonic acid to squalene includes a reductive step involving reduced pyridine nucleotides as coenzymes (23). As Lynen et al. (4) have shown, this reduction occurs during the coupling of 2 molecules of farnesyl pyrophosphate. According to our earlier work, the synthesis of squalene from mevalonic acid in a medium of 98% D₂O is associated with an average uptake of 3.75 atoms of D, and our data obtained by degrading squalene synthesized in T₂O tended to show that approximately half of the labeled hydrogen incorporated is located in the 2 central carbon atoms of squalene. The more recent results with 5-D₂-mevalonic acid and with 1-D₂-farnesyl pyrophosphate provide, however, for the replacement of only 1 atom of hydrogen at the squalene center, and therefore our published data were either in error or our interpretation was incorrect. Repeating these experiments with tritium-labeled water, we have again found an uptake of labeled hydrogen into squalene, over half of which was located at the 2 central carbon atoms. From a medium containing T₂O, approximately 0.7 atom of T is incorporated per molecule of squalene (analyzed to 3.75 atoms of D in 98% D₂O); this shows that the reactions involved are subject to a very marked isotope effect. Of the total tritium incorporated from T₂O, 50 to 60% is located at the two central positions of squalene (analyzed by degrading squalene to 1,4-butenediol), approximately 10% in the C₅ fragment (1,4-pentanediol), and the remainder in the terminal isopropyl groups (Table II). From the known details of the mechanism of squalene synthesis, one would expect two-thirds of the total tritium to enter the two terminal isopropylidene groups as the result of the isopentenyl pyrophosphate to dimethylallyl pyrophosphate isomerization (24, 25) and only one-third of the total at the squalene center. However, since the relative proportions are reversed, i.e., approximately twice as much of the tritium appears in the squalene center as at the ends of the squalene chain, the isotope effects operating in the two processes must differ greatly in magnitude. Calculation shows that the uptake of T at the two ends of the squalene molecule, presumably by a protonation reaction (24, 25), has an isotope effect of 6 to 8, whereas the over-all isotope effect for the introduction of T at the squalene center is only 2 to 4. The existence of this large and differential isotope effect in the incorporation of 1 into squalene is one of the reasons we were led to the erroneous conclusion that 2 protons enter squalene during the formation of the central carbon carbon bond (1).

The source and the quantity of hydrogen taken up during the central condensation have recently been clarified by Popják et al. (5-7). They have found that in a crude liver system, as in our yeast system, tritium is incorporated into squalene when mevalonic acid is the precursor, but that no tritium is incorporated from T₂O when squalene is synthesized from farnesyl pyrophosphate by washed liver microsomes in the presence of TPNH. When TPNH was used instead in a H₂O medium, 0.6 to 0.8 atom of T appeared at the two central positions of squalene. It is clear from these results that the source of the hydrogen entering during the formation of the central carbon-carbon bond of squalene is hydride ion, and that in crude enzyme systems the uptake of T from T₂O at the squalene center is most likely due to the exchange reaction TPNH + T⁺ → TPN + H⁺. These findings also explain why, in our earlier experiments with DPN (generated from DPN and 1-D₂-ethanol), only 0.3 atom of D was incorporated into squalene (2).

In the light of the results discussed above, an attempt can be made to reconcile some of our earlier findings with the knowledge that is now available. One of the figures which is at variance with present information is the number of deuterium atoms retained during the conversion of 5-D₂-mevalonic acid to squalene. The published value was 9.5 atoms (1), the value obtained in the present investigation is 10.4 atoms, and the value reported by Popják et al. (5, 7) is 11 atoms.

Secondly, we have reported an uptake of 3.75 atoms of D (average of 4 analyses) for squalene synthesized in a medium
TABLE II
Squalene synthesis in TzO from 2-C14-mevalonic acid

Experiment 2A—The incubation mixture contained 10 ml of yeast extract, 1 ml of TzO (100 me), and the following additions: ATP, 20 amoles; DPN, 15 amoles; 0.1 ml of 0.1 M MnSO4; and 5.9 amoles of the N,N'-dibenzylethylene diamine salt of 2-C14-mevalonic acid (specific activity, 92,900 d.p.m. per mg). The C14 content of the squalene isolated after addition of 191 mg of carrier corresponds to a 873-fold dilution and a yield of 56%; the tritium content of the squalene corresponds to an incorporation of 0.736 atom of T. The squalene was ozonized, and the ozonolysis products were reduced by LiAlH4.

Experiment 2B—The conditions were the same as in Experiment 2A except that this experiment was done on twice the scale. At the end of the incubation, 318 mg of carrier was added and the squalene isolated. The C14 specific activity of the squalene corresponds to a 461-fold dilution and a yield of 88%; the tritium content of the squalene corresponds to an incorporation of 0.736 atom of T. The squalene was ozonized, and the ozonolysis products were reduced by LiAlH4.

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<th>A</th>
<th>B</th>
<th>A</th>
<th>B</th>
<th>% of total T</th>
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a Errors given as average deviation.
b Corrected for a 20% contamination by 1,4-pentanediol as indicated by the C14 content of the fraction.
c Not a measured value; calculated by difference.
d Corrected for being only 85% pure on the basis of the C14 specific activity expected versus that found.

of 98% D2O, whereas, on the basis of current information, the value should be 3.0 atoms: 1 atom each entering at the squalene ends as the result of the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate and another atom during the formation of the central carbon-carbon bond of squalene.

These differences are probably a composite of analytical errors and of nonexperimental errors arising from enzymatic side reactions in the crude yeast system. Among the likely sources of experimental error are the determinations of the dilution of squalene biosynthesized which are based on the relative specific activities (C14) of mevalonic acid and squalene, weighing errors in the determination of the specific activity of the diluted squalene samples, and possibly the failure to remove moisture or solvent completely when the squalene samples are prepared for combustion. To what extent these operations contributed to analytical errors is difficult to estimate, but the fair agreement among duplicate samples suggests that this was not the sole factor contributing to the low or high deuterium values.

It is noteworthy that in both types of experiments the results were in error by approximately 1 atom of D, the number of D atoms retained from 5-D3-mevalonic acid being too low (9.5 and 10.4 versus 11 atoms) and the uptake of D from D2O being too high (3.75 versus 3.0 atoms). We therefore believe that the deficit and excess of D are complementary effects, and that they both result from some subsidiary enzymatic reactions and not from analytical errors only. One possible cause for the excessive loss or gain of D is a limited dehydrogenation of mevalonic acid to mevalid acid and perhaps also to 3-hydroxy-3-methylglutaryl-CoA, and re-entry of hydrogen on reversal of these reactions. According to the literature, 3-hydroxy-3-methylglutaryl-CoA reductase catalyzes an essentially irreversible reaction (26, 27), but this does not preclude the possibility of a reversible dehydrogenation of mevalonic acid by other enzymes present in the crude yeast extract. An indication for a limited replacement of hydrogen at C-5 of mevalonic acid is given by the experiments on squalene synthesis in TzO (Table II). The C14 fragment obtained on degradation of the squalene (either as levulinic acid or 1,4-pentanediol) contains significant amounts of tritium, and since there is no evidence for a replacement of hydrogen at C-5 of mevalonic acid during the conversion to isopentenyl pyrophosphate or during the condensation of isopentenyl units to farnesyl pyrophosphate, this uptake of tritium is probably due to a reversible dehydrogenation of the 5-hydroxymethyl group of mevalonic acid. This presupposes the presence of enzymes catalyzing a TPNH-tritium exchange as has been demonstrated by Popjak et al. (5, 7) for the liver system.

Evidence for the loss of hydrogen from the C-5 of mevalonic acid is provided by the finding that in all our experiments with 5-D3-mevalonic acid, the C14 fragment obtained on squalene degradation (either as levulinic acid or 1,4-pentanediol) contained yeast particles from 150 ml of yeast extract.

TABLE III
Synthesis of squalene from 1-D3-C14-farnesyl pyrophosphate

The incubation mixture was patterned after that of Goodman and Popjak (8) and contained yeast particles from 150 ml of yeast extract in 150 ml of 0.066 M ammonium phosphate buffer, pH 7.4; NaF, 1.5 mmoles; nicotinamide, 4.5 mmoles; bovine serum albumen, 300 mg; glucose 6-phosphate, 900 amoles; glucose 6-phosphate dehydrogenase, 10 mg; TPN, 300 amoles; 0.75 ml of 1 M MnSO4; 0.75 ml of 1 M KCN; and the ammonium salt of farnesyl pyrophosphate, prepared from 72.5 amoles of the trilithium salt by ion-exchange with Dowex 50 trans.trans.1-D3-C14-Farnesol, from which the pyrophosphate was made, had a specific activity of 3977 d.p.m. per mg and 7.17 atom % excess D. The squalene isolated after the addition of 300 mg of carrier had 53.6 d.p.m. per mg. This corresponds to an 81-fold dilution or a yield of 37%.

<table>
<thead>
<tr>
<th>Atom % excess</th>
<th>Atoms of D per molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0620</td>
<td>2.70</td>
</tr>
<tr>
<td>0.0624</td>
<td>2.71</td>
</tr>
<tr>
<td>0.0656</td>
<td>2.85</td>
</tr>
<tr>
<td>0.0033 ± 0.0015*</td>
<td>2.75 ± 0.06</td>
</tr>
</tbody>
</table>

a Errors are given as average deviation.
liable measure of the extent of enzymatic dehydrogenation at C-5 of mevalonic acid. However, the observed figures undoubtedly exaggerate the extent of these enzymatic reactions as shown by the following considerations. When squalene synthesized from 5-D$_2$-mevalonic acid and containing 10.4 atoms of D was degraded, the products 1,4-pentanediol (1.5 atoms of D) and 1,4-butanediol (2.4 atoms of D) accounted for only 8.4 of the 10.4 atoms of D observed in squalene itself. Similarly, in our previous experiments (1) the recovery of D in the degradation products was only 7.8 to 9.0 of the 9.9 atoms of D found in the whole molecule. The degradation of squalene containing D must therefore entail labilization and exchange of hydrogen, and for this reason the D content of the fragments is not a reliable measure of the extent of enzymatic dehydrogenation at C-5 of mevalonic acid. It seems safer to estimate the magnitude of this reaction from the D content of squalene itself. The average for squalene synthesized is approximately 10 atoms, and accepting 11 as the correct value, one can calculate that 1.00/11 or 0.09 of every D atom originally bound to C-5 of mevalonic acid was replaced as a result of subsidiary enzymatic reactions. As pointed out already, the excess of 0.75 atom taken up by squalene when it is synthesized in a D$_2$O medium (3.75 versus 3.0 atoms) is explainable on the same basis. A relatively slight enzymatic replacement of hydrogen at C-5 of mevalonic acid would therefore be sufficient to account for the discrepancies between the earlier results and the present values.

As stated earlier, there is a relatively large discrepancy between our published data and the now accepted value for the D content of the central 2 carbon atoms of squalene synthesized from 5-D$_2$-mevalonic acid. Our original value for succinic acid obtained by degradation of squalene was 1.8 atoms. The value which we have now found is 2.4 atoms, and according to the recent studies of Popják et al. (5, 7), the value is 3.0 atoms. Assuming that mevalonic acid loses 0.09 atom of each deuterium atom at C-5 by reversible dehydrogenation, then the D content at C-1 of farnesyl pyrophosphate will be approximately 1.82 atoms instead of 2, and when the two C$_{15}$ units dimerize with the replacement of 1 of the hydrogen atoms, the squalene produced will contain 3 × 0.92 or 2.76 atoms of D. The value which we have now found by the reductive degradation of squalene, and by analysis of the central 4 carbon atoms in the form of 1,4-pentanediol and 1,4-butanediol, agrees reasonably well with this figure, but our earlier data for succinic acid are too low (1.8 atoms of D) to be explained on the same basis. It seems likely to us that losses of deuterium occurred either at some stage of the degradation of squalene, perhaps during the oxidative cleavage of the ozonolysis products or during the purification of succinic acid. Suggestive evidence for the labilization of hydrogen on degradation of squalene to succinic acid is provided by the data of Table II. When the central 4 carbon atoms were isolated as 1,4-butanediol, they contained over 60% of the total tritium incorporated into squalene, whereas succinic acid isolated from a similar experiment contained only 39% of the total tritium.

It has been reported (28) that the methylene hydrogens of succinic acid exchange with water at the rate of 1% per hour at room temperature and pH 7. It is therefore conceivable that our succinic acid samples, which were recrystallized from water before analysis, suffered considerable loss of deuterium by exchange.

The value of 1.8 atoms of D for succinic acid was reasonable at the time because it seemed to complement the independent observations that 2 out of the 12 D atoms are lost in the conversion of 6 molecules of 5-D$_2$-mevalonic acid to squalene and also that 2 atoms of hydrogen are taken up at the squalene center. From the recent investigations of Popják et al. (5, 7) and from our own studies, it is now clear that, as far as the reactions at the central 2 carbon atoms of squalene are concerned, this balancing of the losses and gains of hydrogen was fortuitous. When our studies with 5-D$_2$-mevalonic acid and D$_2$O were begun (1, 2), none of the intermediates in squalene biosynthesis were known. For obvious reasons, it was necessary at that time to employ a crude extract as an enzyme source, and it is perhaps not surprising that in this complex system our investigation was complicated by irrelevant side reactions. These studies with deuterium have, nevertheless, had the value of correctly predicting the isopentenyl nature of the condensing unit in terpene biosynthesis and also the general mechanism by which these units are formed from mevalonic acid. The only misleading conclusions derived from these experiments concerned the loss and uptake of hydrogen during the formation of the central carbon-carbon bond of squalene, and we regret that other investigators have sufficiently trusted this information to propose a mechanism of squalene synthesis from C$_{15}$ units, which the more recent results have made untenable.

The low D values for 1,4-pentanediol and 1,4-butanediol might also be due in part to contamination by "bleeding" of liquid phase from the column when the samples are collected after gas chromatography. Since the diols are liquids and difficult to handle in small quantity, the magnitude of this error cannot be readily assessed.

A second possible source of this extra amount of D is the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate which initiates the condensation of C$_{15}$ units (24). If the reaction is unidirectional, only 1 atom of D will be incorporated into each of the two ends of the squalene chain, and this will account for 2 of the 3 atoms of D expected in the whole molecule. However, in the event that the isomerization can be reversed, additional D would enter at both C-2 and C-4 of the isopentenyl units. According to Agranoff et al. (24) the equilibrium of the reaction lies far to the side of dimethylallyl pyrophosphate, and unless the rate of the reversible isomerization is very rapid, this reaction is not likely to contribute substantially to the "extra" D which enters squalene synthesized in D$_2$O.

SUMMARY

1. Further experiments on the synthesis of squalene from 5-D$_2$-mevalonic acid have given results indicating that 11, out of a total of 12, atoms of deuterium are retained in the condensation and not 10 as reported earlier from this laboratory.

2. Squalene synthesized from 1-D$_2$-farnesyl pyrophosphate was found to contain 2.75 atoms of deuterium. This result shows, in agreement with the findings of Popják et al. (5–7, 22), that the formation of the central carbon-carbon bond of squalene involves the replacement of a single hydrogen atom.

REFERENCES


Further Observations on the Biosynthesis of Squalene
C. R. Childs, Jr. and Konrad Bloch