Studies on the Biosynthesis of Cholesterol

XVI. CHEMICAL SYNTHESIS OF 1-H3-2-C14- AND 1-D2-2-C14-TRANS-TRANS-FARNESYL PYROPHOSPHATE AND THEIR UTILIZATION IN SQUALENE BIOSYNTHESIS

G. Popják,* J. W. Cornforth, Rita H. Cornforth, R. Ryhage, and DeWitt S. Goodman

From the Medical Research Council, Experimental Radiopathology Research Unit, Hammersmith Hospital, London, England; National Institute for Medical Research, Mill Hill, London, England; Department of Chemistry I, Karolinska Institutet, Stockholm, Sweden; and Section on Metabolism, National Heart Institute, Bethesda 14, Maryland

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We have recently presented evidence indicating that during biosynthesis of squalene from farnesyl pyrophosphate by rat liver microsomes, 1 hydrogen atom attached to carbon 1 of 1 of the 2 precursor molecules condensing to squalene was exchanged for a hydride ion derived from reduced triphosphopyridine nucleotide (1). This conclusion was drawn from the observations, (a) that during synthesis of squalene by microsomes biosynthetically prepared C14-farnesyl pyrophosphate in the presence of H3-labeled reduced triphosphopyridine nucleotide, each micromole of squalene formed contained nearly 1 microgram atom of labeled hydrogen; and (b) that squalene biosynthesized from mevalonate-2-Cl4-5-Dz contained 11 atoms of deuterium, 3 of these being arranged asymmetrically around the 2 central carbon atoms thus: -CHD . CD2-. Uptake of a proton from the water of the incubation medium into squalene during its synthesis from farnesyl pyrophosphate with washed liver microsomes did not occur.

Since it is known that the conversion of mevalonate to squalene proceeds through the intermediate formation of farnesyl pyrophosphate (2, 3), and that mevalonate-2-C14-5-Dz yields, in all probability, 4, 8, 12-C14-1-D2-5-Dz-9-D2-farnesyl pyrophosphate,1 our conclusions were well founded. Nevertheless, further formal proof for the exchange of the 1 hydrogen atom, referred to, was required. We have therefore synthesized chemically 1-H3-2-C14- and 1-D2-2-C14-TRANS-TRANS-FARNESYL PYROPHOSPHATE and examined these as substrates for squalene synthesis.

We found, in confirmation of our previous data, that only 3 of the hydrogen isotope were contained in squalene biosynthesized from mevalonate-2-Cl4-5-Dz, and found it to contain 6 atoms of deuterium, 3 of these being attached to carbon 1, in confirmation of our former assumptions (1). Some of these observations have already been reported briefly (5).

EXPERIMENTAL PROCEDURE

Synthesis of Methyl Farnesoate-2-C14—This substance, methyl 3, 7, 11-trimethylododeca-2, 6, 10-trienoate-2-C14, was synthesized by the Reformatsky reaction, which gave methyl 3-hydroxy-3, 7, 11-trimethylododeca-6, 10-dienoate-2-C14 (1.86 g). The ester of this hydroxy acid was dehydrated with POC13 (1.6 ml) in pyridine (10 ml) during 3 days at room temperature. After the normal extraction procedures, 1.68 g of methyl farnesoate-2-C14 were obtained which, according to gas-liquid radiochromatographic analysis (6), consisted of methyl cis-trans-farnesoate (45%), trans-trans-farnesoate (43%), and some other unidentified radioactive impurities (12%).

The separations of the two farnesols (7).

In the preparation of the tritium-labeled compound, 40.8 mg of methyl trans-trans-farnesolate-2-C\textsuperscript{14} were diluted with 367.6 mg of unlabeled ester (synthesized and purified as the C\textsuperscript{14} compound) and added in 5 ml of ether to a solution of tritium-labeled LiAlD\textsubscript{4} (10 mg of LiAlD\textsubscript{4} + 85 mg of LiAlD\textsubscript{4}) in 10 ml of ether at \(-30^\circ\) during 30 minutes; the reaction mixture was stirred at \(-30^\circ\) for a further 90 minutes (7). After the usual methods of working up and exchange of labile H with ethanol and water, 396 mg of farnesol-1-D\textsubscript{2}-2-C\textsuperscript{14} were obtained. According to gas-liquid chromatographic analysis, 95\% of the specimen consisted of the trans-trans isomer. The specific activity of this farnesol was 89,100 d.p.m. per pmole. of C\textsubscript{14} per pmole as measured with a Packard Tri-Carb scintillation spectrometer.

trans-trans-Farnesol-1-D\textsubscript{2}-2-C\textsuperscript{14} was made similarly from 506 mg of methyl trans-trans-farnesolate-2-C\textsuperscript{14} (specific activity, 0.02 \(\mu\) c per pmole) and 422 mg of LiAlD\textsubscript{4} (99.2\% D). The yield of farnesol-1-D\textsubscript{2}-2-C\textsuperscript{14} was 425 mg (93\% trans-trans isomer). Mass spectrometric analysis of this specimen showed it to have a molecular weight of 224 with a barely detectable amount (<2\%) of nonisotopic component with a molecular weight of 222. Assay for C\textsuperscript{14} with a Tri-Carb scintillation spectrometer gave a specific activity of 43,400 d.p.m. per pmole.

The infrared spectra of the synthetic farnesols were the same as those of a specimen of nonisotopic trans-trans-farnesol (separated by gas-liquid chromatography) and of geraniol except for absorption peaks in the spectrum of the 1-D\textsubscript{2}-2-C\textsuperscript{14}farnesol in the region of 2100 to 2220 cm\textsuperscript{-1} which were due to the --CD\textsubscript{3} groups.

The yield, 301 mg.

\textbf{Synthesis of Farnesyl Pyrophosphate—}The labeled farnesols were phosphorylated according to the procedure of Cramer and Böhm (8). As these authors gave no experimental details of their method, we report briefly the technical details as worked out by ourselves. To 333 mg (1.5 millimoles) of farnesol-1-D\textsubscript{2}-2-C\textsuperscript{14} there were added 1.3 g (9 millimoles) of trichloroacetoneitrile in a flask fitted with a stirrer and dropping funnel. Di-triethylamine phosphate\textsuperscript{4} (1.04 g, 3.6 millimoles) dissolved in 30 ml of acetonitrile was then introduced through the dropping funnel over a period of 4 hours, the reaction mixture being kept at room temperature and stirred continuously. After standing for 2 hours more at room temperature, 50 ml of 0.1 M \(\text{HCl}\) were added, and the solution was concentrated to approximately 5 ml when a precipitate of the lithium salt of farnesyl pyrophosphate appeared. After standing overnight at \(4^\circ\), 100 mg of the crystalline monophosphate were collected. The mother liquor was then concentrated on a rotary evaporator at \(40^\circ\) to about 0.5 ml. To this solution 2 equivalents of triethylamine (e.g., 20 g of \(\text{H}_{3}\text{PO}_{4}\) dissolved in 100 ml of acetonitrile plus 41.3 g of triethylamine) were added to it, and the mixture was then stirred for 2 hours more at room temperature, 50 ml of 0.1 N aqueous ammonium and 150 ml of ether were added to the reaction mixture in a separatory funnel, and the phosphates were extracted into the aqueous phase.\textsuperscript{5} The extraction with the dilute ammonia was repeated twice more. The combined aqueous phases were extracted three times with 100 ml of ether and then concentrated on a rotary evaporator at \(40^\circ\) to approximately 20 ml. 0.5 ml of cyclohexylamine was then added and the con-

\textsuperscript{4} Specific activity unknown because the suppliers of H\textsuperscript{3}-labeled LiAIH\textsubscript{4} informed us after the preparation was made that they had reasons to believe that the LiAIH\textsubscript{4} was unsatisfactory. The radioactive yield of H\textsuperscript{3}-labeled farnesol was one-hundredth of that expected, thereby confirming the suppliers' suspicion of the unsatisfactory nature of their preparation.

\textsuperscript{5} Crystalline di-triethylamine phosphate may be prepared by dissolving 85 to 100\% orthophosphoric acid in acetonitrile and adding to it 2 equivalents of triethylamine (e.g., 20 g of \(\text{H}_{3}\text{PO}_{4}\) dissolved in 100 ml of acetonitrile plus 13.5 g of triethylamine); di-triethylamine phosphate crystallizes overnight at room temperature.

Because farnesyl phosphate and pyrophosphate decompose below pH 5.2 at room temperature (3) it is essential to keep the pH of the aqueous extract at neutrality or on the alkaline side.
labels. For this purpose the anaerobic microsomal squalene synthetase system described previously has been used (3). For preliminary tests and experiments with the 1-2,4,6 &-farnesyl pyrophosphate, the standard 1-m1 incubations were employed.

For the large-scale synthesis of squalene from the 1-2,4,6 &-farnesyl pyrophosphate, microsomes were prepared from the liver of 75 young rats as described previously. The volume of the suspension of the microsomes, washed twice with buffer, was 55 ml, and it was added to a 500-ml incubation of the following composition: 0.1 m Tris- HCl buffer, pH 7.6; 5 mM MgCl₂; 10 mM NaF; 90 mM nicotinamide; 1 mM TPN; 3 mM glucose-6-phosphate; 0.2 mM lithium 1-2,4,6 &-farnesyl pyrophosphate (total amount of substrate in 500 ml, 100 µmoles); 2 mg of glucose-6-phosphate dehydrogenase; and 1 g of bovine serum albumin. Before addition of enzymes and protein, all ingredients in the appropriate volume of water were mixed and saturated with N₂ at 0° by bubbling the gas through the solution for 10 minutes. After addition of the glucose-6-phosphate dehydrogenase, contained in 50 ml of 2% bovine serum albumin, and of the microsomal suspension, the flask was flushed with N₂ and incubated at 37° for 3 hours with occasional shaking. At the end, 4 mg of squalene, 100 ml of 40% KOH, and 200 ml of ethanol were added, and the mixture was heated at 70° for 1 hour. The extraction and purification of the squalene were described previously (1, 3). A total of 10.65 mg of the hydrocarbon (biosynthetic + carrier) was obtained, one-fiftieth of which was used for radioactive counting, one-tenth for mass spectrometric analysis, and the remainder for degradation by ozonolysis.

Ozonolysis of Squalene The purpose of this degradation was to obtain the 4 central carbon atoms of squalene in the form of succinic anhydride for mass spectrometric analysis. Details of the technique have been described (1). In addition to 520 µg of succinic anhydride, 290 µg of succinic acid were also obtained from the ozonolysis of approximately 9 mg of squalene; the anhydride was used for mass spectrometry and the acid for radioactive counting.

Mass spectrometric analysis of organic compounds was done with the instrument of the Chemistry Department of the Karolinska Institutet which was described by Ryhage and Stenhagen (9). The following substances were examined: (a) nonisotopic trans-trans-farnesol; (b) trans-trans-farnesol-1-2,4,6 &-C⁴, the synthesis of which is described in this paper; (c) trans-trans-farnesol obtained by hydrolysis with intestinal alkaline phosphatase (3) of a preparation of farnesyl pyrophosphate biosynthesized from mevalonate-2,4,6 &-D₂; (d) nonisotopic squalene; (e) squalene biosynthesized from 1-2,4,6 &-farnesyl pyrophosphate; (f) nonisotopic secoisoeic acid, and (g) secoisoeic acid obtained from squalene biosynthesized from it. All mass spectra were taken with an electron beam of 70 e.v. energy.

Sources of Reagents The suppliers of reagents not specifically mentioned here were those reported previously (1, 3). Methylbromoacetate-2-C⁴ was purchased from the Radioclinical Centre, Amersham, Bucks, England. The Aldrich Chemical Company Inc., supplied acetonitrile, trichloroacetonitrile, triethylamine, and cyclohexylamine. Orthophosphoric acid (100%) was the product of Hopkin and Williams, Ltd., Chadwell Heath, Essex, England. Tritium-labeled LiAlD₄ was bought from the New England Nuclear Corporation. Intestinal alkaline phosphatase was the product of the Worthington Biochemical Corporation.

Assay of H Authentication of C⁴—These two isotopes were usually measured with the Packard Tri-Carb scintillation spectrometer with diphenylxazole dissolved in toluene as scintillator. For one set of C⁴ analyses (1-2,4,6 &-farnesyl pyrophosphate and squalene biosynthesized from it) and succinic acid obtained by ozonolysis of this squalene), counting of solid samples of negligible thickness was carried out with a thin window Geiger-Müller tube; the efficiency of this type of counting was approximately 6%.

RESULTS

Labeling of Farnesyl Pyrophosphate Biosynthesized from Mevalonate-5-D₂—One important assumption in the interpretation of our previous results on squalene biosynthesized from mevalonate-2,4,6 &-D₂ was that the farnesyl pyrophosphate formed from the mevalonate, as intermediate in squalene biosynthesis, contained 6 atoms of deuterium, of which were attached to C-1 of farnesol. In order to obtain concrete evidence on this question, 10 µmoles of a specimen of "allyl pyrophosphates" (the "C⁴-D₂-farnesyl pyrophosphate" used in previous work (1), biosynthesized from mevalonate-2,4,6 &-D₂ were hydrolyzed with intestinal alkaline phosphate in 0.1 m Tris - HCl buffer, pH 8.6, containing 5 µM MgCl₂. As the preparation contained some dimethylallyl and geranyl pyrophosphate (3), the alcohol liberated by the phosphatase and extracted with ether were separated by gas-liquid chromatography on an ethylene glycol adipate polyester column at 197° (7). The farnesol collected (1.95 mg) was analyzed in the mass spectrometer; the mass spectrum obtained from this specimen was compared with that of nonisotopic trans-trans-farnesol and with the spectrum of the synthetic trans-truns-farnesol-2,4,6 &-C⁴, (Table I). The nonisotopic farnesol gave a molecular peak at m/e (mass/electronic charge ratio, 31).
tronic charge) 222 and, in addition to a complex “cracking spectrum,” a peak at m/e 191. The synthetic farnesol-2-C¹⁴-1-D₂ gave, as expected, a molecular peak at m/e 224 and, like the normal farnesol, also a peak at m/e 191; the ratios of the intensities of the molecular ions to those at m/e 191 were approximately the same for the two specimens. These observations indicated that the ions at m/e 191 were formed from the molecular ions by the elimination of the group represented by C-1, i.e. CH₂OH from the normal and CD₂OH from the farnesol-2-C¹⁴-1-D₂. The farnesol obtained from the C⁴-D-farnesyl pyrophosphate gave molecular ions at m/e 228, indicating the presence of 6 atoms of deuterium in the molecule, and, instead of a large ion peak at m/e 191, a peak appeared at m/e 195, as might be expected if 2 of the 6 atoms of deuterium were attached to C-1 of this farnesol. This information, then, gives full support to our previous assumption and shows that the hydrogen atoms attached to C-5 of mevalonate were transferred without any change to farnesyl pyrophosphate.

Conversion of Synthetic Farnesyl Pyrophosphates into Squalene—

The data of Table II show that the chemically synthesized farnesyl pyrophosphates (1-H²-2-C¹⁴-, and 1-D₂-2-C¹⁴-) were effective substrates for squalene biosynthesis by microsomes. The yields of squalene in these experiments were of the same order of magnitude as previously observed (1, 3) in our standard 1-ml incubations from biosynthetically prepared farnesyl pyrophosphate. In the experiments with the 1-H²-2-C¹⁴-farnesyl pyrophosphate, the ratio of H²: C¹⁴ in the squalene relative to that in the precursor was reduced to 0.75 to 0.77. Since 2 farnesyl pyrophosphate molecules must have supplied 2 labeled carbon atoms and could have supplied 4 labeled hydrogen atoms to squalene, it appears that 1 labeled hydrogen atom out of these possible 4 was lost during the synthesis, in confirmation of our previous data. That the elimination of this 1 hydrogen atom should have occurred without an apparent “isotope effect,” i.e. without discrimination against tritium, is significant in relation to the biosynthetic mechanism and will be discussed further on.

Identical results were obtained from the analysis of the squalene biosynthesized from 1-D₂-2-C¹⁴-farnesyl pyrophosphate in the large-scale incubation, details of which were described in “Experimental Procedure.”

The 10.65 mg of squalene isolated contained 78,690 c.p.m. of C¹⁴, as counted in a negligibly thin layer with an end window Geiger-Müller counter. Since the specific activity of the 1-D₂-2-C¹⁴-farnesyl pyrophosphate, counted similarly, was 2083 c.p.m. per μmole, the yield of newly synthesized squalene was 78,690/2083 x 2 = 14.7 μmoles, equivalent to 6.07 μg of the hydrocarbon (calculated with a molecular weight of 413; see below); the remainder of the substance isolated, 4.65 μg, can be accounted for mostly by the 4.0 mg of carrier added. One-tenth, 1.065 mg, of this squalene specimen was analyzed by mass spectrometry, and approximately 9 mg were degraded by ozonolysis.

Squalene, like other organic molecules, gave a complex “cracking spectrum” in the mass spectrometer, but this need not be detailed here. Broadly, there were two groups of ionic fragments formed: the first one contained ions smaller than one-half of squalene, which are of no interest for the present purpose; the second contained larger ones up to the molecular (M) ion.

In this latter group we present data on three sets of ions related to the (M - 69), (M - 43), and the molecular (M) ions, all of which were very measurable. The (M - 69) ion, which was the most abundant one in this group, is derived in all probability by the cleavage of one terminal isoprenoid unit, [(CH₃)₃C = CH - CH₂], and the (M - 43) ion arose probably by the elimination of one terminal isopropyl group plus 1 proton, [(CH₃)₂C = CH], from squalene. The abundances of these various ions are shown in Table III as a percentage of that of (M - 69). Simple inspection of the data shows that the squalene from the biosynthetic experiment contained at least two molecular species, one with a molecular weight of M₁ = 410 and another of M₂ = 413; correspondingly, there were large peaks both at (M₁ - 43) and (M₂ - 43), and at (M₁ - 69) and (M₂ - 69) in the spectrum of the biosynthetic specimen, indicating the presence of 3 atoms of deuterium in more than one-half of the squalene molecules. In order to decide whether the specimen contained in addition a significant number of molecules with 1, 2 or 4 atoms of deuterium, a more detailed analysis is needed. Taking the group of fragments related to (M - 69) as an example for such analysis, we have, in the spectrum of the normal squalene, ions at m/e 330, 340, 312 and 324 in definite proportions of the principal ion at m/e 341. These ions can be attributed (a) to a loss of 1 or 2 protons from the main fragment (m/e 339 and 340); (b) to the natural abundance of C¹⁴ and of D (most of the ion at m/e 342); and (c) to the capture of 1 or 2 protons by the ions m/e 341 and 342. Assuming that the proportions of these “secondary” ions in the spectrum of the biosynthetic squalene were the same as in that of the normal substance, then the abundance of the ions, e.g. at m/e 343 in the spectrum of the biosynthetic specimen, can be accounted for almost entirely by the expected relative abundances of the [(M₂ - 69) - 1] and [(M₁ - 69) + 2] ions, 12.2 and 3.3, respectively. There is a small excess of [17.3 - (12.2 + 3.3)] -
TABLE III

**Mass spectrometric analysis of normal squalene and of squalene biosynthesized from 1-D$_2$-C$^{14}$-farnesyl pyrophosphate**

The relative intensities of only three groups of ions derived from squalene are shown. These ions are related to the molecular ion (M), to the ion (M - 43), and (M - 69). In these groups (M - 69) was the most abundant; the intensity of all other ions is expressed as a percentage of (M - 69). The molecular weight of the normal, nonisotopic squalene is denoted by $M_1 (= 410)$ and that of the biosynthetic hydrocarbons by $M_2 (= 412)$.

<table>
<thead>
<tr>
<th>Ions related to (M - 69)</th>
<th>Normal squalene</th>
<th>Biosynthetic squalene</th>
</tr>
</thead>
<tbody>
<tr>
<td>339</td>
<td>5.5</td>
<td>4.0</td>
</tr>
<tr>
<td>340</td>
<td>12.2</td>
<td>3.9</td>
</tr>
<tr>
<td>341 (M - 69)</td>
<td>100.0</td>
<td>53.4</td>
</tr>
<tr>
<td>342</td>
<td>33.6</td>
<td>20.0</td>
</tr>
<tr>
<td>343</td>
<td>6.1</td>
<td>17.3</td>
</tr>
<tr>
<td>344 (M - 69)</td>
<td></td>
<td>100.0</td>
</tr>
<tr>
<td>345</td>
<td></td>
<td>34.6</td>
</tr>
<tr>
<td>Ions related to (M - 43)</td>
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<td></td>
</tr>
<tr>
<td>365</td>
<td>2.5</td>
<td>4.0</td>
</tr>
<tr>
<td>366</td>
<td>2.5</td>
<td>4.0</td>
</tr>
<tr>
<td>367 (M - 43)</td>
<td>33.0</td>
<td>17.3</td>
</tr>
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<td>368</td>
<td>11.6</td>
<td>9.3</td>
</tr>
<tr>
<td>369</td>
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<td>6.7</td>
</tr>
<tr>
<td>370 (M - 43)</td>
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<td>36.0</td>
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<tr>
<td>371</td>
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<td>14.6</td>
</tr>
<tr>
<td>Ions related to M</td>
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<td></td>
</tr>
<tr>
<td>408</td>
<td>6.1</td>
<td>4.0</td>
</tr>
<tr>
<td>409</td>
<td>4.3</td>
<td>3.0</td>
</tr>
<tr>
<td>410 (M$_1$)</td>
<td>68.3</td>
<td>39.5</td>
</tr>
<tr>
<td>411</td>
<td>25.0</td>
<td>17.8</td>
</tr>
<tr>
<td>412</td>
<td>6.7</td>
<td>12.5</td>
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<tr>
<td>413 (M$_2$)</td>
<td></td>
<td>71.0</td>
</tr>
<tr>
<td>414</td>
<td></td>
<td>29.6</td>
</tr>
</tbody>
</table>

TABLE IV

**Mass spectrometric analysis of succinic anhydride derived by ozonolysis from squalene biosynthesized from 1-D$_2$-C$^{14}$-farnesyl pyrophosphate**

The fragments of succinic anhydride related to the (M - 44), i.e. [CH$_2$-CH$_2$-CO]$^+$ ions are only shown. The complete "cracking spectrum" of this substance has been described previously (1). The abundance of the fragments is expressed as a percentage of the most abundant ion, found at m/e 56, in the group. The fragments are denoted by their mass/electronic charge (m/e) ratios. The values shown in parentheses for normal succinic anhydride are those reported previously (1).

<table>
<thead>
<tr>
<th>Fragment (m/e)</th>
<th>34</th>
<th>35</th>
<th>36</th>
<th>37</th>
<th>38</th>
<th>39</th>
<th>40</th>
<th>41</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal succinic anhydride</td>
<td>1.0</td>
<td>7.4</td>
<td>100</td>
<td>3.4</td>
<td>0.3</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1.0)</td>
<td>(6.2)</td>
<td>(100)</td>
<td>(4.4)</td>
<td>(0.4)</td>
<td>(0.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinic anhydride from biosynthetic squalene</td>
<td>2.8</td>
<td>7.6</td>
<td>100</td>
<td>6.8</td>
<td>10.0</td>
<td>78.4</td>
<td>3.0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

1.8 at m/e 343 which might be attributed to trideuterio molecules, but this value is probably not significant. Similar considerations indicate that the size of the ions found at m/e 342 and 345 are accountable by the sum of the contribution of the "secondary" ions related to (M$_1$ - 69) and (M$_2$ - 69), respectively, and not by the presence of mono and tetradeuterio molecules. The same arguments hold true for the analysis of the fragments related to (M - 43) and M, all of which lead us to conclude that the biosynthetic specimen contained essentially normal and trideuterio molecules. Assuming that the ratios of M$_2$:M$_1$, (M$_2$ - 43):(M$_1$ - 43), and (M$_2$ - 69):(M$_1$ - 69) in the biosynthetic specimen represent the ratios of trideuterio and normal molecules, we calculated that 64.3, 67.5, or 65.2% of all the molecules contained 3 atoms of deuterium. From the C$^{14}$ content of the biosynthetic specimen, we have calculated previously that 6.07 mg out of 10.65 mg of hydrocarbon isolated from the experiment must have been synthesized de novo. The positions of the 3 atoms of deuterium in squalene could not be deduced precisely from the mass spectrometric analysis beyond the suggestion that these must have been located near the center of the molecule, for fragments smaller than one-half of the molecule contained no deuterium. The examination of the succinic anhydride, obtained by ozonolysis from the squalene, gave decisive information on this question.

We have reported previously (1) that the molecular ions in the spectrum of succinic anhydride cannot be used for evaluation of its deuterium content because of the great tendency of these ions to capture 1 or 2 protons and to give peaks at (M + 1) and (M + 2) as large, or larger than, at M. This phenomenon is a characteristic property of dibasic acids. We have shown that for succinic anhydride the fragments related to the (M - 44) ion, i.e. (CH$_3$CH$_2$CO)$^+$, are the most suitable for evaluation of the extent of deuterium labeling of this substance. As the method of analysis of the "cracking spectrum" of succinic anhydride has been described in detail before (1), this need not be repeated again. The data showed that the 3 atoms of deuterium found in the biosynthetic squalene were attached to its 2 central carbon atoms since the succinic anhydride contained only normal and trideuterio molecules (Table IV). However, the ratio of the ions found at m/e 59 (trideuterio ions) to those found at m/e 56 (normal ions) indicated that only 44% of all the molecules in the specimen contained 3 atoms of deuterium as against approximately 65% deduced from the analysis of the squalene itself. This change in the ratio of labeled to unlabeled molecules is not entirely unexpected as it is known (1) that during ozonolysis of squalene, 20%, and occasionally more, of the succinic acid obtained may come from parts of the molecule other than the 4 central carbon atoms. The degree of dilution of the labeled succinic anhydride with unlabeled molecules could be also deduced from its C$^{14}$ content since the labeled carbon atoms incorporated into squalene from 1-D$_2$-C$^{14}$-farnesyl pyrophosphate must appear as the carbonyl carbons of the succinic anhydride. The specific activity of the succinic acid from which the anhydride was made was determined on 290 µg (see "Experimental Procedure") by counting with an end window counter. It gave 2050 c.p.m. per µmole. As the specific activity of the farnesyl pyrophosphate was 2683 c.p.m. per µmole, the C$^{14}$ assay indicated that only (2050/2683 × 2) × 100 = 38.2% of all the molecules in the succinic acid specimen (and hence in the anhydride too) were labeled. In the case of the squalene also, the C$^{14}$ assay gave a lower percentage of...
labeled molecules (56.6%) than the mass spectrometric analysis (approximately 65%). This discrepancy may be due either to an error in our determinations of the specific activity of the farnesy1 pyrophosphate or to a difference in the fragmentation of normal and deuterium-labeled molecules in the mass spectrometer. If such differentiation should occur in the mass spectrometer, it is unlikely that succinic anhydride and squalene would be affected in a similar way. Since the magnitude of the discrepancy noted was the same for squalene (56.6/65 = 0.87) as for the succinic acid (38.2/44 = 0.868), we are inclined to believe that the specific activity of the farnesy1 pyrophosphate was, in fact, lower than we report. The discrepancy does not alter in any way the general conclusion that the squalene biosynthesized from 1-D2-2-C14-farnesyl pyrophosphate contained 3 atoms of deuterium attached to its 2 central carbon atoms.

DISCUSSION

The experimental observations presented have confirmed in an unambiguous way our previous conclusions, which have been summarized in the introduction, regarding the exchange of 1 hydrogen atom attached to C-1 of 1 of the 2 farnesyl pyrophosphate molecules condensing to squalene. It was an essential assumption in the interpretation of our previous data on the extent of the deuterium labeling of squalene biosynthesized from mevalonate 2 C14-5 D2 (1) that the farnesyl pyrophosphate formed as an intermediate in the process should contain 6 atoms of deuterium, 2 of these being attached to the carbinol carbon (C-1) of farnesol. Experimental observation now proves this assumption to be correct.

It is of particular interest that the previously deduced exchange of the 1 hydrogen atom during squalene biosynthesis could be demonstrated equally well with either the 1-D2 or the 1-H2-farnesyl pyrophosphate. It is certain from the mode of preparation that both hydrogen atoms of C-1 of the 1-H2-2-C14-farnesyl pyrophosphate were labeled to the same extent, although, of course, only a relatively few atoms of hydrogen in the specimen were tritium. It is common in all reactions involving protium and tritium and which are not stereospecific, that a discrimination against the heavy isotope occurs. The absence of such an “isotope effect” in our experiments indicates that the removal of the 1 hydrogen atom during squalene biosynthesis is a stereospecific process. This phenomenon offers further interesting possibilities both as to the study of the final reaction of squalene biosynthesis and also of the cyclization of squalene to sterol. The stereospecificity of this hydrogen exchange could not be inferred logically from the results obtained with either mevalonate-5-D2 (1) or with the 1-D2-farnesyl pyrophosphate, as virtually all the hydrogen atoms attached to the particular carbon atom in nearly all the molecules of the two precursors were deuterium.

The results, apart from implying the stereospecificity of the removal of the 1 hydrogen atom, indicate further that, if dissociation of the farnesyl pyrophosphate-enzyme complex can occur, then the removal of the hydrogen cannot involve a molecule of farnesyl pyrophosphate but must occur in a later intermediate derived from it. Such an intermediate could be of the type suggested by us (1) as being formed by the condensation of a molecule of nerolidyl pyrophosphate with 1 of farnesyl pyrophosphate, although the removal of the hydrogen before the condensation of two farnesyl residues is also a possibility. If the removal of the hydrogen had occurred in a dissociable farnesyl pyrophosphate-enzyme complex, it would have been impossible to obtain an asymmetrically labeled squalene.

The effectiveness of the synthetic farnesyl pyrophosphates to act as squalene precursor constitutes also the first formal proof for the correctness of the structure deduced for this intermediate from the study of trace or micromole amounts (2, 3) obtained from mevalonate by enzymic synthesis.

SUMMARY

1. The chemical syntheses of methyl farnesoate-2-C14, trans-trans-farnesol-1-H2-2-C14, trans-trans-farnesol-1-D2-2-C14, 1-H2-2-C14-trans-trans-farnesyl pyrophosphate, and of 1-D2-2-C14-trans-trans-farnesyl pyrophosphate are described.

2. The synthetic farnesyl pyrophosphates were effective substrates for the biosynthesis of squalene by liver microsomes. The squalene synthesized from either of these labeled precursors contained 3 labeled hydrogen atoms. By the analysis of squalene derived from the 1-D2 precursor it was shown that the 3 atoms of deuterium were attached to the 2 central carbon atoms of the hydrocarbon. That the loss of 1 labeled hydrogen atom from C-1 of farnesyl pyrophosphate could be demonstrated, even with the H2-labeled substrate without an “isotope effect,” implies that the removal of the H atom during squalene biosynthesis is a stereospecific process and occurs probably from an intermediate formed by the condensation of two farnesyl residues.

3. The farnesol of farnesyl pyrophosphate biosynthesized from mevalonate-2-C14-5-D2 contained 6 atoms of deuterium, 2 of these being attached to carbon 1 of farnesol.

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G. Popják, J. W. Cornforth, Rita H. Cornforth, R. Ryhage and DeWitt S. Goodman

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