Artificial Substrates in Squalene and Sterol Biosynthesis*

(Received for publication, December 13, 1971)

ALAN POLITO, G. POPJÁK, AND THOMAS PARKER

From the Departments of Biological Chemistry and Psychiatry, Mental Retardation Center, School of Medicine, University of California at Los Angeles, Los Angeles, California 90024

SUMMARY

Trans-[1-14C]isopentenyl pyrophosphate and [1-14C]isopentenyl pyrophosphate were incubated with 10,000 X g supernatants of rat liver homogenates. Analyses of the unsaponifiable material extracted from the incubations by gas-liquid radiochromatography and mass spectrometry revealed the synthesis not only of [14C]squalene but also of [1H, 14C]1-methylsqualene and of [1H, 14C]1,24-dimethylsqualene. The sterol fraction of the unsaponifiable extracts contained two components labelled with 1H and 14C, the 1H:14C ratios of which were identical with the isotope ratios of the 1-methylsqualenes and which had retention volumes in gas-liquid chromatography expected of 27-methylcholesterol and of 27-methylstanol. The sterol fractions did not contain components that might have been derived from the cyclization of 1,24-dimethylsqualene.

It is suggested that 1-methylsqualene is epoxidized enzymically only at its isopropylidene end, but not at its isobutylidene end.

It has been shown that an analog of geranyl pyrophosphate (6,7-dihydrogeranyl pyrophosphate) and several homologs of 3,3-dimethylallyl (3-methylbut-2-enyl) pyrophosphate (cis- and trans-3-methylpent-2-enyl, 3-ethylpent-2-enyl, and cis- and trans-3-methylhex-2-enyl pyrophosphate) acted as condensing partners with isopentenyl (3-methylbut-3-enyl) pyrophosphate in the reaction catalyzed by prenyltransferase of liver (EC 2.5.1.1), the products of synthesis being 10,11-dihydrofarnesyl pyrophosphate (1) and the appropriate homo- and bishomo-farnesyl pyrophosphates (2). Ogura et al. (3), using the prenyltransferase of pumpkin, confirmed these observations and showed that even trans-3-methyldec-2-enyl pyrophosphate reacted with isopentenyl pyrophosphate to produce a homolog of geranyl pyrophosphate.

It became of interest to examine whether squalene synthetase could accept as substrate a homofarnesyl pyrophosphate and, if so, whether the resulting homo- and bishomosqualenes might be cyclized to sterol.

In order to explore these possibilities we have incubated trans-

* This investigation was supported by United States Public Health Service Research Grant HE-12745 and by the Department of Mental Hygiene, State of California.

MATERIALS AND METHODS

[1-14C]isopentenyl Pyrophosphate—[1-14C]3-Methylbut-3-enoic acid was synthesized by the carbonation with 14CO₂ of the Grignard reagent made from 2-methylallylchloride in tetrahydrofuran (5). The resulting [1-14C]3-methylbut-3-enoic acid, purified by vacuum distillation and identified by nuclear magnetic resonance and mass spectrometry, was reduced to isopentenol with LiAlH₄.

The pyrophosphate ester was synthesized by a previously described method (6). The product obtained had a specific activity of 0.47 Ci per mole.

Trans-[1-3H₃]3-methylpent-2-enyl Pyrophosphate—This was pre-
pared as described previously (2), except that the intermediary product of synthesis, methyl trans-3-methylpent-2-enolate, separated from the cis isomer by preparative gas-liquid chromatography, was reduced to the alcohol with LiAlH₄. The specific activity of the pyrophosphate ester was 6 Ci per mole.

Enzyme Preparations—The 10,000 × g supernatants (S₁₀) of rat liver homogenates were made in 0.1 m Tris-HCl buffer containing 5 mM MgCl₂, 30 mM nicotinamide, and 10 mM KF as described (4) for the study of total sterol biosynthesis from mevalonate. The S₉ preparations were kept frozen at -20° for 4 to 5 days before use. The conditions of incubation are described in the footnote to Table I.

Extraction, Fractionation, and Analysis of Products Synthesized—The incubation mixture was hydrolyzed with 2 N KOH in the presence of 50% ethanol at 70° for 1 hour. The neutral material was extracted with light petroleum (b.p. 40-60°) and divided into a hydrocarbon and crude sterol fraction by chromatography on neutral alumina (4) with light petroleum followed by acetone-diethyl ether (1:1, v/v). The acetone-ether eluate could contain not only sterols, but also prenols.

The hydrocarbon fraction was first analyzed by the gas-liquid radiochromatographic technique of Popják et al. (7) devised for the simultaneous assay of ¹³C and ³H. The scintillation counter (7) was connected to a Packard two-channel rate meter (model 3022, Packard Instrument Co., Ambar Industries Inc., Las Grange, Ill.), the output of the rate meter and of the gas chromatography-mass spectrometry analyses. The background spectra, of the specimen at each step of fractionation was determined in a Packard Tri-Carb scintillation spectrometer.

Table I: Isotope content of products synthesized from [1-³H₃]3-methylpent-2-enyl pyrophosphate and [1-¹⁴C]isopentenyl pyrophosphate

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Total Isoprenes</th>
<th>Hydrocarbons</th>
<th>Crude sterols</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>³H</td>
<td>¹³C</td>
<td>³H</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>197,120</td>
<td>48,260</td>
<td>114,475</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>476,847</td>
<td>106,311</td>
<td>311,277</td>
</tr>
</tbody>
</table>

In Experiment 2 the sterols were precipitated with digitonin; the digitonides contained 40,172 dpm of ³H and 47,874 dpm of ¹³C. Chromosorb W. The gas-liquid chromatographic analyses were made at 350-200°C. Background mass spectra were taken both before and after the completion of the gas-liquid chromatography-mass spectrometry analyses. The background spectra, of remarkably low intensity for a “bleed” from a column with a 10% stationary phase, were subtracted from those of the specimens.

The crude sterol fraction from Experiment 1 was analyzed by the gas-liquid radiochromatography method after addition of unlabelled desmosterol, lanosterol, and dihydroergosterol to the specimen and conversion of the alcohols to trimethylsilyl ethers (9). In Experiment 2 the 3β-hydroxysterols were first precipitated as the digitonides which were then decomposed with pyridine. The sterols were extracted by partitioning between diethyl ether and dilute HCl and were examined as in Experiment 1 by the gas-liquid radiochromatography method.

Radioactivity Measurements—The ³H and ¹³C content of every specimen at each step of fractionation was determined in a Packard Tri Carb scintillation spectrometer.

RESULTS

Both the [1-³H₃]3-methylpent-2-enyl and the [1-¹⁴C]isopentenyl pyrophosphates were used for the synthesis of unsaponifiable products. In the two experiments recorded in Table I, 24.3% and 14.7% of the ³H and 75.8% and 72.7% of the ¹³C of the two substrates, respectively, were found in the unsaponifiable extract, most of the ³H appearing in the hydrocarbon fraction and about one-half of the ¹³C in the crude sterol fraction. In Experiment 2 the sterol digitonides accounted for 40,172 dpm of ³H and 47,874 dpm of ¹³C, i.e. for 34.3% and 63.2% of the ³H and ¹³C, respectively, in the crude sterol fraction.

Analysis of Hydrocarbon Fraction—Fig. 1 illustrates that the hydrocarbon fraction obtained from an incubation made with [2-¹³C]mevalonate, or with [1-¹³C]isopentenyl pyrophosphate, contains squalene as the sole radioactive component and also that in the gas-liquid radiochromatography instrument the ³H channel of the recording shows no change from the mean background counting level when a ¹³C-labelled fraction enters the counter. In contrast, examination by gas-liquid radiochromatography of the hydrocarbon fraction from the experiments recorded in Table I revealed three radioactive components (Fig. 2). a small component of [¹⁴C]squalene and two others containing both ³H and ¹³C. One of these doubly labelled components had a retention volume (Rv) of 1.26 and the other of 1.65 relative to that of squalene suggesting, in analogy with previous observations on homofarnesols (2), that they were homosqualene (1-methylsqualene) and bishomosqualene (1,24-dimethylsqualene) respectively.

The distribution of ³H and ¹³C in the components of the hydrocarbon fractions, based on gas-liquid radiochromatography analysis, is shown in Table II. Although the relative amounts of radioactivity in the three components differed in the two experiments (different enzyme preparation was used in each), the

1 Recovery of cholesterol as the digitonide from standard solutions, in the range of 0.2 to 2.0 mg, is about 90% in our laboratory (data of Dr. J. Edmond). Thus, probably 70% of the ¹³C and 38% of the ³H in the crude sterol fraction were associated with sterols.

2 The retention volumes (Rv) of radioactive components seen in gas-liquid radiochromatography, which had no corresponding mass detector responses on account of the low sensitivity of the mass detector, were taken from the midpoints of the integral curves of radioactivity.
Homosqualenes and Homosterols

Fig. 1. Gas-liquid radiochromatographic analysis of the hydrocarbon fraction, with added unlabelled squalene, obtained from an incubation of a 10,000 × g supernatant of liver homogenate with [14C]isopentenyl pyrophosphate. The radioactive counts form an integral curve because the radioactive substance accumulates in the scintillator solution circulating in the counter. The increased “noise” in the 3H channel after the appearance of [14C]-squalene is due to the fact that the standard error of the difference increased “noise” in the 3H channel after the appearance of %I-

Fig. 2. Gas-liquid radiochromatographic analysis of the hydrocarbon fraction from Experiment 2 recorded in Table I. The column used was the same as the one employed in the analysis of [14C]squalene shown in Fig. 1 except that it was run at 255°. The records of the 3H and 14C channels overlapped during the emergence of the first radioactive fraction after squalene. The mean counting rates have been drawn over the original record and these have been accentuated at some places by white paint around them.

### Table II

Percentage of distribution of 3H and 14C among components of hydrocarbon fraction calculated from data of gas-liquid radiochromatography

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Squalene</th>
<th>Homosqualene</th>
<th>3H:14C</th>
<th>Bishomosqualene</th>
<th>3H:14C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>pm/pm</td>
<td>%</td>
<td>pm/pm</td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3H</td>
<td>0.0</td>
<td>25.40</td>
<td>3.10</td>
<td>69.80</td>
<td>6.93</td>
</tr>
<tr>
<td>14C</td>
<td>4.80</td>
<td>41.60</td>
<td></td>
<td>51.00</td>
<td>6.93</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3H</td>
<td>0.0</td>
<td>49.35</td>
<td>3.66</td>
<td>50.65</td>
<td>6.11</td>
</tr>
<tr>
<td>14C</td>
<td>14.82</td>
<td>55.70</td>
<td></td>
<td>31.48</td>
<td>6.11</td>
</tr>
</tbody>
</table>

* The 3H:14C ratios were derived by converting the radioactive counts to disintegrations per min by the use of counting efficiencies determined with 3H- and 14C-labelled toluenes.
* The sum of the percentages for each isotope in Experiment 1 does not add to 100 because the specimen contained a small unidentified doubly labelled component, which emerged at the solvent front.

(endogenous) which was followed by two fractions with R<sub>g</sub> values of 1.26 and 1.65 relative to squalene. The mass spectra of the latter are compared with that of squalene in Fig. 3.

The molecular weight of the substance with a R<sub>g</sub> of 1.26 was 424, C<sub>50</sub>H<sub>80</sub>, and of the one with a R<sub>g</sub> of 1.65 was 438, C<sub>52</sub>H<sub>84</sub>, i.e. 14 and 28 mass units, respectively, higher than the molecular weight of squalene.

The mass spectrum of squalene is characterized by two lines of active. The substances found here were not of a random series of odd- and even-numbered n-paraffins, but only of the four monoenes; they were not radio active.

### Notes

3. The maximum slopes of the radioactivity tracings, at the half-value of the ascending limb of the gas chromatographic peak. The maximum slopes of the radioactivity tracings, at the half-value of the ascending limb of the gas chromatographic peak.

4. Contamination of squalene specimens made from liver, or liver homogenates, by other hydrocarbons has been noted by one of us over many years. The origin of these hydrocarbons is unknown, but a dietary source is suspected. The substances unknown, but a dietary source is suspected.

The 3H:14C ratios in the equivalent doubly labelled fractions were very similar.

The assumption that the two doubly labelled fractions were homo- and bishomosqualene was proved by mass spectrometric analysis of the hydrocarbon fraction from Experiment 2. The specimen left over after the gas-liquid radiochromatography analysis was introduced into the combined gas chromatography-mass spectrometry instrument which was fitted with the same gas chromatography column as the one used for gas-liquid radiochromatography.

The specimen contained four uncharacterized hydrocarbon fractions of a homologous series (n-C<sub>18</sub>H<sub>36</sub>, n-C<sub>20</sub>H<sub>40</sub>, n-C<sub>22</sub>H<sub>44</sub>, n-C<sub>24</sub>H<sub>48</sub>) emerging from the column before squalene.

The mass spectrum of squalene is characterized by two lines of active. The substances unknown, but a dietary source is suspected. The substances found here were not of a random series of odd- and even-numbered n-paraffins, but only of the four monoenes; they were not radio active.
A. Polito, G. Popjak, and T. Parker

TABLE III

Percentage of distribution of \(^{1}H\) and \(^{14}C\) among components of sterol fractions as calculated from gas-liquid radiochromatographic analyses

<table>
<thead>
<tr>
<th>Chromatographic fraction</th>
<th>Experiment 1 (^{a})</th>
<th>Experiment 2 (^{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent front. . . . . .</td>
<td>(%) total (\Delta pM/\Delta pM)</td>
<td>(%) total (\Delta pM/\Delta pM)</td>
</tr>
<tr>
<td>Cholesterol, (R_{r} 1.0)</td>
<td>44.7 0.0 0.0</td>
<td>0.0 0.0</td>
</tr>
<tr>
<td>Desmosterol, (R_{r} 1.14)</td>
<td>0.0 35.6 0.0</td>
<td>0.0 47.80 0.0</td>
</tr>
<tr>
<td>Fraction, (R_{r} 1.26)</td>
<td>29.0 22.2 3.14</td>
<td>0.0 11.60 0.0</td>
</tr>
<tr>
<td>Lanosterol, (R_{r} 1.43)</td>
<td>0.0 22.2 0.0</td>
<td>0.0 13.05 0.0</td>
</tr>
<tr>
<td>Fraction, (R_{r} 1.73)</td>
<td>26.3 20.0 3.17</td>
<td>9.4 8.70 3.73</td>
</tr>
</tbody>
</table>

\(^{a}\) Data from the analysis of the crude sterol fraction (acetone-ether eluate from alumina column; cf. "Methods and Materials").

Fragmentation, one beginning with the elimination of 69 and the other by the elimination of 43 mass units, each being followed by further losses of 68 mass units, and by a base peak at \(m/e 81\) accompanied by an intense ion at \(m/e 69\) (Fig. 3A).

The substance with a \(R_{r}\) of 1.20 showed four lines of fragmentation: \([M-69]+\), \([M-43]+\), \([M-83]+\) and \([M-57]+\), each being followed by further losses of 68 mass units. Although the base peak in this spectrum was also at \(m/e 69\), there were present two ions of nearly equal intensities at \(m/e 81\) and 83, the latter being very weak in the spectrum of squalene (Fig. 3B).

The mass spectral fragmentation of the substance with a \(R_{r}\) of 1.65 was the simple two-line fragmentation seen in the spectrum of squalene except that the first fragment ions found were those of \([M-83]+\) and \([M-57]+\) each being followed by the loss of 68 mass units and that the base peak of \(m/e 69\) in the spectrum of squalene was replaced by one at \(m/e 83\) (Fig. 3C).

Thus, the mass spectra of the two new substances are those one could predict for the disymmetric 1-methylsqualene and the symmetrical 1,24-dimethylsqualene.

**Analysis of Sterol Fraction**—The analysis of the MeSOI derivatives of the crude sterol fraction from Experiment 1 and of the digitonin-precipitable sterols from Experiment 2 by the gas-liquid radiochromatography technique as described under "Methods" gave the data recorded in Table III.

The \(^{1}H\)-labelled component, containing no \(^{14}C\), and emerging at the solvent front in the gas-liquid radiochromatography analysis of the crude sterol fraction from Experiment 1 was most probably 3-methylpent-2-en-1-ol released from the substrate by hydrolysis. It is probable that the same substance was present also in the crude sterol fraction from Experiment 2, the \(^{1}H:\(^{14}C\) ratio of which was 1.56 as compared with 0.84 in the sterol digitonides and 2.55 in the supernatant left after precipitation of sterols with digitonin.

Fig. 4 shows the analytical record from the second experiment. We found in both experiments two doubly labelled components among the sterols: one of these had a retention volume of 1.26 and the other 1.73 relative to that of cholesterol (\(- R_{r}\) of 1.21 relative to that of lanosterol). The evidence that these doubly
Homosqualenes and Homosterols

Vol. 247, No. 11

FIG. 4. Copy of gas-liquid radiochromatographic record obtained from the analysis of sterols, as the Me4Si derivatives, recovered from the digitonides of Experiment 2 (cf. Table I and text). Column: 10% Dexsil, 6 feet, 285°. Mass detector (thermal conductivity cell) response at top: Peak I, cholesterol; Peak 2, desmosterol; Peak 3, 24,25-dihydrolanosterol; Peak 4, lanosterol.1

labelled components are sterols rests at present on their precipitation with digitonin. Their retention volumes are those expected from the Me4Si ethers of a methylcholesterol ("homocholesterol") and a methylxanosterol ("homolanosterol"). However, the amounts biosynthesized up to now were insufficient for rigorous identification either by mass spectrometry or by chemical means.

In addition to the two doubly labelled components, 14C, but not 3H, was associated with the gas chromatographic peaks of cholesterol and lanosterol and also, in Experiment 2 with desmosterol.

**DISCUSSION**

The synthesis of squalene, homosqualene and bishomosqualene in incubations containing trans-[1-3H]3-methylpent-2-enyl pyrophosphate and [1-14C]isopentenyl pyrophosphate indicates that Reactions 1, 2, and 3, described in the introduction, have occurred in the enzyme preparations and also that the squalene synthetase system of liver microsomes condensed readily not only farnesyl pyrophosphate with homofarnesyl pyrophosphate, but also the latter with itself.

The theoretical ratio of 3H to 14C in homofarnesyl pyrophosphate synthesized according to Reaction 3 should be 6/2 × 0.47 = 6.35 and the same value should also apply to bishomosqualene (2 × 0.4/0.47). The found values of 6.99 and 6.41 (Table II) are reasonably close to these predicted values considering the errors of reading the levels of radioactive counts from the gas-liquid radiochromatography records and converting these into disintegrations.

Since homosqualene, synthesized by the condensation of [9-2H1,5-14C]12-methylfarnesyl pyrophosphate with [1,5,9-14C]farnesyl pyrophosphate (cf. Reactions 2 and 3), should contain only one 3H-labelled position and five positions labelled with 14C, the 3H:14C ratio in this substance should be 6/5 × 0.47 = 2.55. The values found, 3.10 and 3.96 (Table II), particularly in Experiment 2, were substantially higher than predicted. Some systematic error of counting might be the explanation for the anomalous data. An alternative explanation might be that unlabelled farnesyl pyrophosphate present in the undialyzed enzyme preparations diluted the [14C]farnesyl pyrophosphate. We have no certain information to choose between these alternatives. The mass spectrometric analysis of the specimen from Experiment 2 left no doubt that the substance with the unexpectedly high 3H:14C ratio was 1-methylsqualene. It may be significant that the 3H:14C ratios found in the substances thought to be homosterols were identical, within small experimental errors, with the 3H:14C ratios of the homosqualenes in both experiments (cf. Tables II and III), a finding that argues against an error of counting.

The evidence for the two doubly labelled components found in the sterol fractions to be truly sterols (homocholesterol and homolanosterol) rests at present only on their being precipitable by digitonin and on their having retention volumes in gas-liquid chromatography commensurate with those expected of sterols. Although we need more conclusive proof, we believe the data give strong indication of the cyclization of homosqualene, but not of bishomosqualene to a sterol. We found no evidence by the gas-liquid radiochromatography analyses of the sterol fractions from either experiment to suggest a component derived from the cyclization of bishomosqualene, i.e. a component having a 3H:14C ratio similar to that of bishomosqualene.

Oxidation of squalene to the 2,3-epoxide is known to precede cyclization to sterol (10-13). Homosqualene might be epoxidized at either end of the molecule (Scheme I), but each oxide form (II and V) would lead to a different type of homolanosterol: homolanosterol III would contain the 3H-labelled position in ring A and the 4-gemdimethyl group of lanosterol would be replaced in it by the 4-ethyl-4-methyl groups; homolanosterol VI would contain the 3H-label in the side chain and the extra methyl group at C-27. The transformation of 27-methyllanosterol (VI) to 27-methylcholesterol (and intermediates) could proceed probably without difficulty. If homolanosterol III has been formed and transformed into cholesterol the latter should have contained 3H; we found only 14C associated with cholesterol.

The fact that we did not find a component in the sterol fractions that might have been derived from the cyclization of bishomosqualene suggests that the isobutylidene structures at each end of bishomosqualene prevented enzymic epoxidation and, hence, cyclization. The apparent cyclization of homosqualene, but not of bishomosqualene, to a sterol suggests that homosqualene may be epoxidized only at its isopropylidene end.
only difference between bishomosqualene and homosqualene is that the former contains the isobutylidene group at both ends whereas the latter contains at one end an isobutylidene and at the other an isopropylidene group. We exclude, therefore, the reaction sequence I \rightarrow III in Scheme 1 and suggest that the doubly labelled substances found in the sterol fractions of the experiments were derived by the reaction sequence IV \rightarrow VI (Scheme 1) and its sequelae.

Corey et al. (14) and Crosby et al. (15), in their studies on the enzymic cyclization of homologs of squalene 2,3-oxide, observed the formation of a homolanosterol in incubations of 2,3-oxidosqualene sterol cyclase with a racemic mixture of chemically synthesized cis- and trans-epoxides of 1-methylalqualene. Crosby et al. (15) concluded that only that isomer of the squalene-epoxide homolog in which the unaltered terminal methyl group was cis to the main chain, corresponding to Structure II in Scheme 1 and expected to be synthesized from trans-3-methylpent-2-enyl-pyrophosphate, was cyclized since the homolanosterol formed was identical as the 4α-ethyl-4β, 14α-dimethylcholesta-8,24-dien-3α-ol. Thus if the bishomosqualene synthesized in our experiments had been epoxidized it should have been cyclized enzymically. The findings of Crosby et al. (15), taken in conjunction with our own, reinforce our conclusion that the isobutylidene end of homosqualene cannot be epoxidized enzymically and support our tentative assignment of the structure of the homosterols as 27-methyl compounds. This conclusion is also supported by the relative retention volumes of the homosterols in gas chromatography. If the extra methyl group had been attached to the nucleus of the sterols, the increase in the retention volumes should have been much less than observed. It is generally true that in a homologous open chain structure the addition of a methylene group in the chain increases the retention volume by a factor of 1.2 to 1.3; the effects of additions of branched methyl groups onto a ring system are less pronounced. The data of Crosby et al. (15) indicate, e.g., that the retention volume of the Me3Si ether of 4α-ethyl-4β, 14α-dimethylcholesta-8,24-dien-3α-ol relative to that of lanosterol was only 1.05.

A "unidirectional" cyclization of the asymmetrically synthesized squalene (16) to lanosterol has been explored before (17, 18) with inconclusive results. Our data suggest for the first time that a minor chemical modification of the squalene structure may induce the enzymic cyclization to be initiated from one specific end of the molecule.

We have also examined our data as to whether they might suggest differences in the reactivities of farnesyl pyrophosphate and of homofarnesyl pyrophosphate in the squalene synthetase system. To this end we have calculated from the amount of the various products observed in Experiment 2 the amounts of farnesyl pyrophosphate and of homofarnesyl pyrophosphate that must have been synthesized in the enzyme preparation. The calculations were based on the 14C content of each component and on the specific activity of the 14Cl-isopentenyl pyrophosphate used, 1,043 dpm per nmole. Thus, e.g., 22,998 dpm of 14C in cholesterol was taken to be equivalent to 22,898/1043 = 21.85 nmoles of isopentenyl residues and to 21.85/6 = 3.66 nmoles of cholesterol, which needed the same molar amount of squalene as precursor and which, in turn, required the availability of 3.66 \times 2 = 7.32 nmoles of farnesyl pyrophosphate for synthesis. Homosqualene and the two "homosterols" were assumed to contain 5 and bishomosqualene was assumed to contain 4 isopentenyl residues per molecule. The calculations gave the data of Table IV, which show that 23.49 nmoles of farnesyl pyrophosphate and 19.12 nmoles of homofarnesyl pyrophosphate had to be synthesized first in order to account for the various products. The calculated and observed distributions of products synthesized from such a mixture of farnesyl and homofarnesyl pyrophosphate are shown in Table V. The data suggest an almost random combination of farnesyl and homofarnesyl pyrophosphate. In contrast, the conversion of homosqualene to homosterols, if the doubly labelled components found in the sterol fractions were identical sterols, was very uneven (cf. Table IV). In the homosqualene plus homosterol fractions of Experiment 2 only 2.53 nmoles were identifiable as sterols out of 9.96 nmoles of total products, whereas 5.55 nmoles were found in sterols and only 1.55 nmoles in squalene in the singly 14C-labelled squalene plus sterol components. This difference is also well illustrated by the finding of only small amounts of newly syn-

\begin{table}
\centering
\caption{Amounts of products synthesized in Experiment 2 calculated from their 14C content.}
\begin{tabular}{ |c|c|c|c| }
\hline
Product & Isopentenyl residues converted & Product synthesized & Farnesyl pyrophosphate equivalents & Homofarnesyl pyrophosphate equivalents \\
\hline
Squalene & 9.29 & 1.55 & 3.10 & 0.0 \\
Cholesterol & 21.95 & 3.66 & 7.32 & 0.0 \\
Desmosterol & 5.32 & 0.89 & 1.78 & 0.0 \\
Lanosterol & 5.99 & 1.00 & 2.00 & 0.0 \\
Homosqualene & 53.56 & 6.73 & 6.73 & 0.0 \\
"Homocholesterol" & 8.65 & 1.73 & 1.73 & 1.73 \\
"Homolanosterol" & 3.99 & 0.80 & 0.80 & 0.80 \\
Bishomosqualene & 19.73 & 4.93 & 9.86 & \\
\hline
Total & 108.58 & 21.29 & 23.40 & 19.12 \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{Percentage of distribution of products synthesized from farnesyl pyrophosphate and homofarnesyl pyrophosphate.}
\begin{tabular}{ |c|c|c| }
\hline
Products & % Distribution & \\
| Calculated & Observed | \\
\hline
Squalene + sterols & 30.47 & 33.4 \\
Homosqualene + homosterols & 49.40 & 45.4 \\
Bishomosqualene & 20.07 & 23.2 \\
\hline
\end{tabular}
\end{table}

\* A random association of farnesyl and homofarnesyl pyrophosphate was assumed from a mixture consisting of 55.2% of the former and 44.8% of the latter substrate.

The 14C content of the hydrocarbon and crude sterol fraction was equivalent to the utilization of 153.5 nmoles of isopentenyl pyrophosphate. The lower recovery recorded here is accounted for by the loss of radioactivity in the supernatant after precipitation of sterols with digitonin (cf. text and Table I).
thesized squalene left in the hydrocarbon fractions which were dominated by homo- and bishomosqualene (cf. Fig. 2). If homosqualene cannot be epoxidized at its isobutylidene end, as we have inferred, its rate of conversion to sterols should be much slower than that of squalene.

Acknowledgments—We thank Mr. Jean Bardenheier and Dr. Cheng Liao for their assistance with the preparation of the trans-[1-3H]3-methylpent-2-enyl pyrophosphate.

REFERENCES