A Long Chain Terpenyl Pyrophosphate Synthetase from *Micrococcus lysodeikticus*\(^*\)

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SUMMARY

A new terpene pyrophosphate synthetase (Enzyme II) distinct from the previously reported geranylgeranyl pyrophosphate synthetase (Enzyme I) has been isolated from extracts of *Micrococcus lysodeikticus* and partially purified. Enzyme II catalyzes the elongation of terpenoid allyl pyrophosphates by isopentenyl pyrophosphate to long chain products. The order of effectiveness of various allyl pyrophosphates in the elongation reaction is geranyl pyrophosphate > farnesyl pyrophosphate > geranylgeranyl pyrophosphate > dimethylallyl pyrophosphate. Isolation of doubly labeled terpene alcohol from experiments with \(^{14}\text{C}\)-isopentenyl pyrophosphate and \(^{3}\text{H}\)-farnesyl pyrophosphate and determination of \(^{14}\text{C}:/^{3}\text{H}\) ratios indicates the presence of 7 to 10 isoprene units in the elongation products with a predominance of the C\(_{28}\) and C\(_{30}\) alcohols. The same chain length distribution is estimated from the mass spectra of the acid-hydrolyzed products of Enzyme II. The incorporation of \(^{32}\text{P}\) from \(^{32}\text{P}\)-isopentenyl pyrophosphate and the acid lability of the \(^{32}\text{P}\)-labeled material indicate that the products formed are pyrophosphate derivatives. In contrast to shorter chain terpene pyrophosphates, the products formed by Enzyme II are not cleaved by bacterial alkaline phosphatase. They also have the unusual property of binding very strongly to protein.

The formation of terpene pyrophosphates containing up to 20 carbon atoms has been shown with enzyme systems isolated from bakers' yeast (1), carrot root, pig liver (2), and the yellow bacterium *Micrococcus lysodeikticus* (3). In all these cases the carbon chains of allyl pyrophosphates are lengthened by addition of isoprene units derived from isopentenyl pyrophosphate. It may be assumed that there exist also enzyme systems for the formation of longer chain terpene pyrophosphates to provide the polyisoprenoid side chains for the various benzoquinone and naphthoquinone coenzymes (4). Cell-free systems catalyzing the formation of such long chain terpene pyrophosphates have not yet been described.

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EXPERIMENTAL PROCEDURE

Materials

Spray-dried cells of *M. lysodeikticus* were obtained from the Miles Chemical Company, Elkhart, Indiana, lysozyme from Nutritional Biochemicals, pancreatic deoxyribonuclease from Calbiochem, and *Escherichia coli* alkaline phosphatase from Worthington. Hyapatite C (hydroxylapatite) and Unisil (silicic acid) were purchased from the Clarkson Chemical Company, Inc., Williamsport, Pennsylvania. Kieselguhr G was obtained from E. Merck, Germany, and Adsorbosil-1 from the Applied Science Laboratories, College Station, Pennsylvania. Farnesol was obtained from the Aldrich Chemical Company, Inc., Milwaukee. Solanesol was a gift of Dr. L. G. Crutchfield of the R. J. Reynolds Tobacco Company. Geranylinalool, kindly provided by Dr. O. Isler of Hoffman-La Roche and Company, Basel, was converted to geranylgeraniol (12). The pyrophosphate esters of the various terpene alcohols were prepared as previously described (3). \(^{3}\text{H}\)-Lithium aluminum hydride (63.3 \(\mu\text{C per \text{pmole}}\)) was purchased from New England Nuclear.

Sephadex-G-25 (fine beads) and Blue Dextran were products of Pharmacia. Redistilled hexane and benzene were used for silicic acid chromatography.

Methods

Gas-liquid chromatography was carried out as previously described (3). Mass spectra were obtained with an Associated Electronics Industry MS-G mass spectrometer. Protein concentrations were determined by optical density measurements at

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It seems well established from experiments in vivo that the isoprenoid side chains of the quinone coenzymes arise by the usual acetate-mevalonate pathway (5-10). Moreover Stoffel and Martius (11) have shown that in rat liver mitochondria synthetic geranylgeranyl pyrophosphate reacts with benzoquinone derivatives to form Q-type coenzymes with side chains containing 20 or more carbon atoms.

A previous report from this laboratory has described the isolation of a terpene pyrophosphate synthetase from *M. lysodeikticus* which catalyzed the formation of geranylgeranyl pyrophosphate as the main product and of smaller amounts of longer chain terpene pyrophosphates (3). Further fractionation of extracts of *M. lysodeikticus* has now yielded a second terpene synthetase which also catalyzes the condensation of allyl pyrophosphates with isopentenyl pyrophosphate but produces in the main C\(_{28}\) and C\(_{30}\) terpene pyrophosphates.

EXPERIMENTAL PROCEDURE

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Methods

Gas-liquid chromatography was carried out as previously described (3). Mass spectra were obtained with an Associated Electronics Industry MS-G mass spectrometer. Protein concentrations were determined by optical density measurements at
280 and 260 μm. For radioactive assay, samples were counted in a Packard Tri-Carb scintillation counter either in toluene scintillator solution for toluene-soluble materials or in "polyether" scintillator solution for aqueous samples.

Preparation of 1-3H-Farnesyl Pyrophosphate—Farnesol was oxidized via farnesal to farnesenic acid and the S-benzylthiouronium salt of the acid was prepared according to the directions of Bates, Gale, and Gruner (13). After several recrystallizations of the salt from 95% ethanol to remove cis,trans isomer, the S-benzylthiouronium salt of trans,farnesenic acid was obtained; m.p. 130°; reported, 13132° (13-15). The trans,farnesenic acid salt was reduced with lithium aluminum hydride-3H in ether solution (13) to give 1-3H-farnesol. Gas-liquid chromatography of the crude product on acid-washed Chromosorb W coated with diethyleneglycol succinate polyester revealed that more than 95% of the 1-3H-farnesol obtained in this fashion was the trans, trans isomer. A by-product amounting to about 20% of the total reduction products was identified as 2,5-dihydrofarnesol.1

For purification, the crude trans,trans-1-3H-farnesol was chromatographed on silicic acid with benzene-hexane as the eluting solvent. The sample of 1-3H-farnesol so obtained contained 95% trans,trans-farnesol and about 5% of 2,5-dihydrofarnesol as judged by gas-liquid chromatographic analysis. 1-3H-Farnesol was then converted into the pyrophosphate ester according to the method previously described (3). A sample of this product was digested with alkaline phosphatase and the product was identified as 2,5-dihydrofarnesol by gas-liquid chromatographic analysis. The gas-liquid chromatogram of the crude product on acid-washed Chromosorb W coated with diethyleneglycol succinate polyester revealed that more than 95% of the 1-3H-farnesol obtained in this fashion was the trans, trans isomer. A by-product amounting to about 20% of the total reduction products was identified as 2,5-dihydrofarnesol.

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Enzyme Assays—The reaction tubes contained, in a final volume of 0.5 ml, 100 μmoles of Tris buffer, pH 7.4, 5.0 μmoles of MgCl2, 0.05 μmole of 1-14C-isopentenyl pyrophosphate,2 enzyme, and one of the following nonradioactive pyrophosphates: 0.025 μmole of dimethylallyl-PP, 0.025 μmole of geranyl-PP, 0.025 μmole of farnesyl-PP, or 0.025 μmole of geranylgeranyl-PP. The reaction mixtures were incubated for 20 min at 37°. The reaction was then stopped by the addition of 0.5 ml of 50% (w/v) trichloroacetic acid and the products were hydrolyzed by heating at 90° for 10 min. After cooling in ice 0.5 ml of 5 m sodium hydroxide and 2.5 ml of 2 m potassium chloride were added, and the mixtures were extracted with two 5-ml portions of petroleum ether. After washing with water, an aliquot of the solvent layer was removed, the solvent was evaporated on a steam bath, and the sample was counted in a liquid scintillation counter. The reaction rate was found to be a linear function of enzyme concentration in all cases provided that substrate concentrations were not limiting.

Enzyme Purification—All steps were carried out at 2-5°C unless otherwise stated.

Spray-dried cells (20 g) of M. lysodeikticus were suspended in 400 ml of 0.05 M Tris buffer, pH 7.4. Lysozyme (100 mg) was added to the suspension, and the mixture was stirred manually at room temperature for 30 to 40 min until a thick gel formed. The viscosity of the gel was reduced by addition of 20 mg of deoxyribonuclease and stirring was continued for an additional 10 min. The resulting mixture was centrifuged at 60,000 × g for 1 hour, and the sediment was discarded. Solid ammonium sulfate was added to the supernatant layer, and the fraction precipitating between 25 and 35% saturation was collected and dissolved in 0.05 M Tris buffer, pH 7.4. The resulting solution was dialyzed against the same buffer to remove ammonium sulfate and then diluted to 100 ml. In a second ammonium sulfate step the fraction precipitating between 35 and 50% saturation was collected, dissolved in 20 ml of 0.05 M Tris buffer, pH 7.4, and then dialyzed against 0.01 M phosphate buffer, pH 6.4. The dialyzed solution containing 600 mg of protein was chromatographed on a hydroxylapatite column (35 x 2.3 cm) by elution with a linear gradient of potassium phosphate buffer, pH 6.4; 0.01 M buffer (300 ml) was placed in the mixing flask and 0.1 M buffer (300 ml) in the reservoir. Fractions of 15 ml were collected. The distribution of protein and of enzyme activity with farnesyl PP as substrate is shown in Fig. 1. The fraction containing the major portion of the two enzyme activities (Fractions 17 to 21, Enzyme I, and 26 to 30, Enzyme II) were pooled separately.

Solid ammonium sulfate was added to the pooled Fractions 17 to 21, and the material precipitating between 40 and 50% saturation was collected and then dissolved in 3 ml of 0.05 M Tris buffer, pH 7.4. This preparation (Enzyme I) retains activity for several months when stored at −20°C. Acrylamide gel electrophoresis of Enzyme I gave one major and two minor bands. Further purification of the protein was attempted by chromatography on diethylaminoethyl cellulose. After dialysis against 0.02 M phosphate buffer, pH 7.1, the protein (10 mg) was applied to a DEAE-cellulose column (15 x 1.3 cm), and eluted with a linear gradient of 0.0 to 0.6 M sodium chloride in 0.02 M phosphate buffer, pH 7.1 (total volume, 200 ml). The distribution of protein and enzyme activity was determined with farnesyl-PP as substrate. Although a single activity peak was obtained at an elution volume of 110 ml, the specific activity of the protein in this peak indicated that denaturation occurred.

For further purification of Enzyme II, solid ammonium sulfate was added to the pooled Fractions 26 to 30 (Fig. 1) and the protein precipitating between 35 and 40% saturation was collected and dissolved in 0.05 M Tris buffer, pH 7.4. At this stage the purified enzyme (Enzyme II) was frozen and stored at −70°C. After dialysis against 0.02 M phosphate buffer an aliquot of the protein (24 mg) was applied to a DEAE-cellulose column (20 ×
The protein was eluted from the column with a linear gradient of 0.0 to 0.7 M sodium chloride in 0.02 M phosphate buffer, pH 7.1 (total volume, 400 ml). Protein distribution and enzyme activity were determined with farnesyl-PP and geranyl-PP as substrates. A single relatively sharp enzyme activity peak was obtained at an elution volume of 285 ml. Dilute solutions of Enzyme II were stable on storage at 0° for several days. Acrylamide gel electrophoresis of the active fractions gave one major and two minor bands.

**Phosphatase Hydrolysis**—To solutions containing the products of the enzyme reactions 10 to 50 μg of bacterial alkaline phosphatase were added directly. The reaction tubes were then flushed with nitrogen, stoppered, and allowed to stand for 20 to 24 hours at room temperature. At the end of this period a few milliliters of 2 M KCl were added and the reaction mixture was extracted with petroleum ether. The petroleum ether extracts were washed once with water and then evaporated carefully on a steam bath. The residue was either counted for radioactivity or applied to thin layer plates for chromatography.

**Thin Layer Chromatography**—Thin layer chromatography of the terpene alcohols was performed in the two systems described by McSweeney (16). In one system, plates coated with Adsorbosil G are impregnated with paraffin oil and developed with acetone-H2O, 65:35, saturated with paraffin oil. Reference terpene alcohols were located by exposing the developed plates to iodine vapors. With few exceptions the quantities of reaction product were too small to be detected by this method. Therefore, the plates were allowed to stand in air to allow the iodine to evaporate. Sections of the chromatographic plates were then collected and the radioactive regions were determined by liquid scintillation counting. In most instances the region of interest was scraped directly from the plate into a counting vial, scintillation liquid (toluene) was added, and the sample was counted in the presence of the supporting medium. In the alternate method, scraped sections were collected on a sintered glass funnel and washed thoroughly with petroleum ether. The petroleum ether solution was collected in a scintillation liquid (toluene) was added, and the sample was counted in the presence of the supporting medium. The radioactive material was then eluted with the same buffer (0.2 M Tris, pH 7.4) with which the column had previously been washed. After passage of the void volume (V0) single drops (0.05 ml) were collected for scintillation counting.

**RESULTS**

**Enzyme Purification**—Protein recoveries and the specific activities of Enzyme I and Enzyme II at various stages of purification are shown in Table I. The two enzymes were assayed in the presence of isopentenyl-PP and one of four allyl pyrophosphates as substrates. Both Enzyme I and II show slight activity with 1-4C-isopentenyl-PP alone indicating the presence of some isopentenyl-PP-dimethylallyl-PP isomerase activity (17) in these preparations. Enzyme I, as previously reported (3), shows by far the highest specific activity with farnesyl-PP as the substrate. With dimethylallyl-PP and geranyl-PP the specific activities are only about 0.1 as high. The low activity with geranylgeranyl-PP agrees with the earlier conclusion that Enzyme I is primarily a geranylgeranyl-PP synthetase. By contrast, Enzyme II shows the greatest activity with geranyl-PP as the allylic substrate; it is somewhat less active with farnesyl-PP. Enzyme II is also quite active in elongating geranylgeranyl-PP. Since neither Enzyme I or II are pure, cross-contamination or contamination with other terpene synthetases may in part account for the over-

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**Table I**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total protein (mg)</th>
<th>Recovery %</th>
<th>Specific activity with 1-4C-isopentenyl-PP and pyrophosphates of the following alcohols:</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Crude extract...</td>
<td>4080</td>
<td>10</td>
<td>0.58 1.14 1.83 0.29 0.15</td>
</tr>
<tr>
<td>B. Second (NH4)2SO4 precipitation, 35 to 50%</td>
<td>600</td>
<td>47</td>
<td>0.20 3.00 5.86</td>
</tr>
<tr>
<td>Enzyme I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroyxalapitate column, fractions 17 to 21; (NH4)2SO4 precipitation, 40 to 50%</td>
<td>14.4</td>
<td>11</td>
<td>3.22 5.62 57.60 1.24 1.63</td>
</tr>
<tr>
<td>Peak fraction from DEAE-cellulose column...</td>
<td></td>
<td></td>
<td>44.1</td>
</tr>
<tr>
<td>Enzyme II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroyxalapitate column, fractions 26 to 30; (NH4)2SO4 precipitation, 35 to 50%</td>
<td>43</td>
<td>10</td>
<td>18.40 16.75 6.9 0.57</td>
</tr>
<tr>
<td>Peak fraction from DEAE-cellulose column...</td>
<td>1.2</td>
<td>27.54 21.71 9.9 0.32</td>
<td></td>
</tr>
</tbody>
</table>

* The specific activities for Enzyme I are calculated on the assumption that the product contains 20 carbon atoms (4 isoprene units) and the product of Enzyme II an average of 35 carbon atoms (7 isoprene units). The crude extract (A) and the ammonium sulfate fraction (B) contain both Enzyme I and II and the products will therefore be a mixture of both the shorter chain and longer chain terpene alcohols. While the composition of this mixture has not been determined directly, the extent of cleavage by phosphatase (see Table I) indicates that incubation with Fraction A produces 35 to 40% of C10 terpene and long chain terpene alcohols as the remainder. The specific activities of enzyme fractions A and B are calculated on this basis.
lap of substrate specificity. It seems clear nevertheless that Enzymes I and II are separate entities, Enzyme I acting preferentially on farnesyl-PP and Enzyme II being less selective.

Characterization of Reaction Products—The products of the reactions between 1-14C-isopentenyl-PP and either unlabeled or 1-3H-farnesyl-PP, whether catalyzed by Enzyme I or Enzyme II, yielded on trichloracetic acid hydrolysis nonpolar, petroleum ether-extractable materials. On the other hand, there is a striking difference in the response of the two products to alkaline phosphatase (Table II). The product obtained from farnesyl-PP and 14C-isopentenyl-PP with Enzyme I is hydrolyzed almost completely, whereas the product of Enzyme II appears resistant to phosphatase action. In neither case is it possible to obtain petroleum ether-soluble radioactivity by direct extraction of the enzymatic production mixture, i.e. without prior phosphatase or acid treatment.

When the material formed by Enzyme I and rendered petroleum ether soluble by the action of phosphatase was chromatographed on thin layer, the results shown in Fig. 2 (upper) were obtained. The radioactive product had the same mobility as geranylgeraniol. However, this result is not conclusive since the chromatographic system does not separate geranylgeraniol from the isomeric geranyllinalool or from shorter and longer chain terpene alcohols. On the other hand, chromatography in the reverse phase (Fig. 3, upper) effectively resolves related allylic alcohols. In this system also the radioactive (doubly labeled) product from Enzyme I has the RF of geranylgeraniol, in agreement with the results of silicic acid chromatography reported earlier (3). The faster moving farnesol peak (singly labeled) in the same chromatogram is derived from unreacted substrate.

After acid treatment, the radioactive product from incubations with Enzyme I moved on thin layer at the same rate as a mixture of geranylgeraniol and geranyllinalool. Acid hydrolysis, therefore, leads to the expected mixture of isomeric terpene alcohols.

As shown in Table II, phosphatase treatment did not render the product of Enzyme II petroleum ether-soluble even though acid hydrolysis converted the same product to a petroleum ether-soluble material which showed the mobility of a long chain terpene alcohol on thin layer chromatography. In the standard system (Fig. 2, lower), the product of acid hydrolysis moved slightly faster than geranyllinalool or geranylgeraniol while in the reverse phase system it failed to move from the origin (Fig. 3, upper). This is also true for solanesol (C20) while geranylgeraniol and geranyllinalool both move well ahead of the origin. These observations suggest that the terpene chain of the product formed by Enzyme II is longer than 20 carbon atoms. In some cases, radioactive material was also observed to move slightly ahead of the origin, suggesting the presence of terpene alcohols of a size intermediate between C20 and the longer chain alcohols at the origin. Since separations in the reverse system are strongly influenced by the type of alcohol function and since the enzymatic product had been hydrolyzed with acid, conditions known to produce isomeric terpene alcohols, the chain length of the unknown products relative to solanesol cannot be estimated from these experiments.

**Table II**

<table>
<thead>
<tr>
<th>Radioactivity in nonpolar lipid after phosphatase hydrolysis</th>
<th>Additional radioactivity in nonpolar lipid after trichloracetic acid hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpm</td>
<td>%</td>
</tr>
<tr>
<td>Crude extract</td>
<td>410</td>
</tr>
<tr>
<td>Enzyme I</td>
<td>242</td>
</tr>
<tr>
<td>Enzyme II</td>
<td>48</td>
</tr>
</tbody>
</table>

**FIG. 2.** Thin layer chromatography of terpene alcohols. Upper, the product formed by Enzyme I from 1-14C-isopentenyl-PP and farnesyl-PP under the normal assay conditions was treated with alkaline phosphatase and the petroleum ether-soluble radioactive material applied to Adsorbosil-1 as described in the text. Lower, the products formed by Enzyme II from the same substrates were hydrolyzed with trichloracetic acid and extracted with petroleum ether. For comparison, the RF values of standard terpene alcohols are indicated: A, farnesol; B, geranylgeraniol; C, solanesol; D, geranyllinalool.
FIG. 3. Reverse phase thin layer chromatography of terpene alcohols. Experimental conditions were the same as in Fig. 2 except that the substrates were 1-14C-isopentenyl-PP and 1-3H-farnesyl-PP. The petroleum ether extracts containing radioactivity were applied to paraffin-coated Kieselguhr G as described in the text. Upper, products of Enzyme I including farnesol from unreacted substrate; lower, products of Enzyme II. Solid lines represent 3H and dashed lines 14C activity. For comparison, the Rf values of standard terpene alcohols are indicated: A, geranyllinalool; B, geranylgeraniol; and C, farnesol.

Fig. 4. Sephadex G-25 chromatography of mixtures after incubation of enzyme and labeled substrate. Curve A (X—-X). Enzyme I (1.17 mg) incubated with farnesyl-PP (0.024 μmole) and 1-14C-isopentenyl-PP (0.019 μmole) for 1.5 hours under the usual assay conditions. Curve B (O——O), Enzyme II (1.22 mg) incubated with farnesyl-PP (0.048 μmole) and 1-14C-isopentenyl-PP (0.048 μmole) for 1.5 hours under the usual conditions. Curve C (O——O), Enzyme II (1.22 mg) incubated for 3 hours with 1-14C-isopentenyl-PP alone (0.048 μmole) under the usual conditions.

Similar binding experiments were conducted with farnesyl-PP and 32P-isopentenyl-PP. When Enzyme II was incubated with 1-14C-isopentenyl-PP alone, a small amount of 14C was bound to protein (Fig. 4, Curve C) and this may result from contaminating dimethylallyl-PP-isopentenyl-PP isomerase activity which will furnish the allylic substrate needed for chain elongation. Protein-bound 14C (Fig. 4, Curve B) could not be separated from protein by rechromatography on Sephadex G-25 after the complex had been treated with 0.5% Triton X-100 or with 4.0 M urea. Prior incubation in 1.67 M NaOH for 45 min at room temperature and subsequent chromatography at pH 13 also failed to separate 14C from the protein. Equally ineffective were attempts to remove radioactive material from protein by extraction with collidine (18).

Similar binding experiments were conducted with farnesyl-PP and 32P-isopentenyl-PP. When Enzyme II (0.062 mg) was incubated with 0.048 μmole each of farnesyl-PP and 32P-isopentenyl-PP under conditions similar to those used above, 3,870 cpm out of a total of 29,400 cpm were found to emerge from Sephadex G-25 with the protein peak. This is a substantial percentage considering the fact that the elongation product retains only 1 terminal pyrophosphate residue whereas several (probably 4 or 5) isopentenyl PP molecules are consumed in the condensation process. When farnesyl-PP was omitted in a control experiment, no significant radioactivity emerged in the protein area. Iodoacetamide was added in these experiments in order to minimize isomerase activity (17). These results suggest that in the reaction catalyzed by Enzyme II the product is also a phosphate (or pyrophosphate) ester. The nature of the 32P bound to protein was further investigated by the following experiment. The 32P-protein complex obtained with the aid of Sephadex G-25 was heated at 60° for 60 min in 0.1 N HCl and the products were chromatographed on Whatman No. 3MM paper in butanol-formic acid-water, 40:10:16. Radioactive peaks for orthophosphate and inorganic pyrophosphate were obtained in a ratio of 1:8. Acid treatment of inorganic pyrophosphate itself under the same conditions led to 16% hydrolysis. The 32P in the protein complex therefore has the same acid stability as inorganic pyrophosphate. Since the ester bond of allylic pyro-
phosphates is cleaved under these conditions the above observations suggest that the protein-associated 32P is due to the presence of a terpene pyrophosphate.

The experiments discussed so far may be summarized as follows: Enzyme I, in confirmation of earlier findings (3), produces a geranylgeranyl derivative which may be presumed to be the pyrophosphate ester. The enzyme product is cleaved by phosphatase to free geranylgeraniol and by acid to isomeric C20 terpene alcohols. No binding occurs between the product and Enzyme I. The product of Enzyme II appears to be a derivative of a longer chain terpene alcohol, judging both from the chromatographic behavior and from the ability of the enzyme to elongate geranylgeranyl-PP. Product formed by Enzyme II from either 14C- or 3H-isopentenyl-PP is tightly bound to protein. This strong binding may explain why the product, though undergoing the acid cleavage typical of allylic pyrophosphates, fails to be hydrolyzed by phosphatase.

**Ratio Experiments**—To obtain an estimate of the number of isoprenoid units added to the allyl pyrophosphate in the reaction catalyzed by Enzyme II, product was prepared by incubation of enzyme with l-3H-farnesyl-PP and l-14C-isopentenyl-PP. The doubly labeled terpenoid alcohol derived from alkaline phosphatase treatment of the product of Enzyme I served as a standard. This was assumed to be geranylgeraniol containing 14C and 3H in a ratio corresponding to the condensation of farnesyl-PP with an authentic sample developed in parallel with the phosphatase product.

**Isotope ratios in reaction products formed from 1-14C-isopentenyl-PP and 3H-farnesyl-PP**

The enzymatic products were hydrolyzed either enzymatically or chemically as described below. The free alcohols were separated by thin layer chromatography. In Experiments 2 to 5 the reaction product was isolated as the protein complex by passage over Sephadex G-25 as described in the text. In all other experiments separation of the enzymatic product from the reaction mixture prior to hydrolysis was not attempted. In some cases the solutions containing reaction products were treated with alkaline phosphatase (Experiments 1 to 6) in order to cleave contaminating C20 pyrophosphates. The solutions were then extracted with petroleum ether for isolation of the terpene alcohols. By comparison the major product at the origin of a reverse phase chromatographic plate after hydrolysis with phosphatase or acid. This impurity could have lowered the 14C:3H ratio in some instances. However, some of the lower ratios were obtained even when the excess substrate (3H-farnesyl-PP) was removed before hydrolysis by passage of the product through Sephadex G-25 (Experiment 2, Table III). A more likely explanation for the variable ratios is that the chain length specificity of Enzyme II is not absolute and that unrecognized factors alter the specificity of the enzyme with the production of variable amounts of homologous terpene chains.

The minor components isolated in Experiments 4 and 7 (Table III) with RF values of 0.03 to 0.15 in the reverse phase system had low 14C:3H ratios (0.95 to 0.94) corresponding to a C25 terpene alcohol. By comparison the major product at the origin (RF 0.0 to 0.03) from the same experiments had ratios of 1.08 and 2.09, respectively.

Additional evidence that Enzyme II, at the present stage of purity, catalyzes the formation of a relatively broad spectrum of long chain terpene alcohol derivatives is given in the next section.

**Mass Spectrometry**—In order to obtain terpene alcohol in sufficient quantity for analysis by mass spectrometry, a large scale incubation was carried out with 6.1 mg of Enzyme II and 10 times the amounts of reagents used in the standard assay procedure (final volume of 5.0 ml). After incubation for 2 hours at 38°, the protein-product complex was separated from substrate by means of Sephadex G-25 as previously described. The free alcohol was obtained from this complex by the usual trichloroacetic acid hydrolysis and extracted with petroleum ether.

Despite the attempts to remove extraneous material by the indicated procedures, the initial scans obtained on injection of

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Table III

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Terpene alcohol derived from</th>
<th>14C</th>
<th>3H</th>
<th>14C:3H</th>
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a RF = 0.00 to 0.03.
b RF = 0.03 to 0.15.
the hydrolysis product into the mass spectrometer gave evidence for the presence of a number of impurities and no recognizably pattern was observed. Perhaps these impurities are lipid materials extracted from the enzyme itself. As scanning continued, however, the less volatile products became more prominent and a more distinguishable pattern emerged (Fig. 5).

The base peak in the mass spectrum given by the unknown product as well as by standard samples of geranylgeraniol, geranyllinalool, and squalene is at m/e = 69. Von Sydow (23) has examined the fragmentation pattern of monoterpene alcohols and has found the base peak at m/e = 69 to be characteristic of acyclic alcohols bearing a terminal isopropylidene grouping. A comparison of the other major ion peaks shows good correspondence between the reference isoprenoid alcohols and the product of Enzyme II. The mass spectra observed here are, therefore, consistent with the spectra of monoterpenoid alcohols reported by Von Sydow. Furthermore the close correspondence of base peaks, major ion peaks, and characteristic metastable peaks indicates that the enzymatic product is closely related in general structure to the reference isoprenoid alcohols.

Von Sydow further reported (23) that the hydroxyl groups of monoterpene alcohols are always revealed by peaks at m/e = M - 18. The spectra of the higher terpenoid alcohols also show a clear pattern of molecular ion peaks followed by peaks at M - 18 of greater intensity corresponding to the loss of water. The salient feature of the spectrum of the enzymatic product in Fig. 5 is the presence of ion peaks at 630 and 562 mass units followed by peaks of greater intensity at 612 (630 - 18) and 544 (562 - 18) mass units. Judging from the behavior of the reference compounds, this is the pattern expected from terpenoid alcohols consisting of 8 (molecular weight 562) and 9 (molecular weight 630) isoprenoid units. In addition there is a small ion peak at m/e = 680 which would correspond to the expected M - 18 ion peak for a terpenoid alcohol 10 isoprenoid units long.

From Fig. 5, it can be seen that the m/e ions resulting from the C40 and C45 alcohols (562 and 612) show nearly equal intensities while considerably smaller amounts of the m/e ions resulting from the C40 alcohol (680) are recorded. It is, however, impossible to determine precisely the chain length distribution since the pattern in Fig. 5 represents the spectrum obtained on the fifth scan. The more volatile alcohols of lesser chain length would have vaporized to a significant extent before this pattern developed. The following analysis does allow us to estimate the relative chain length distribution with the conclusion that the C45 and C40 alcohols are the major products.

Careful examination of the earliest analyzable scans (2 and 3) failed to show the pattern of m/e peaks at 426 and 408 corresponding to M+ and M - 18 expected for a C40 isoprenoid alcohol. Early scans, however, did show m/e peaks of M+ and M - 18 for a C35 isoprenoid alcohol. In the spectrum of scan 2 the intensity of the ions was slightly greater than the intensity of the corresponding m/e peaks at 562 and 544 derived from the C40 alcohol. In scan 3 the intensities of these sets of ion peaks were about equal and in scan 4 the m/e peaks at 562 and 544 were more intense. As can be seen from scan 5 (Fig. 5), the m/e = 494 and 476 peaks ascribable to the C35 alcohol are relatively weak while those derived from the C40 alcohol at m/e = 562 and 544 are prominent. The expected pattern of m/e at 630 and 612 corresponding to M+ and M - 18 for the C45 alcohol first appears in scan 3, and increases in intensity with time until in scan 5 this pattern is about equal to the m/e peaks of C40 alcohol at 562 and 544. Finally, the m/e peak corresponding to M - 18 for a C35 alcohol appears as a low intensity peak in the last spectrum obtained. The over-all trend, therefore, is readily interpreted in terms of a series of isoprenoid alcohols and on the basis of the volatility expected for alcohols of varying chain length.

**DISCUSSION**

Several lines of evidence indicate that Enzyme II represents a hitherto unnoticed activity differing in function from the previously described geranylgeranyl-PP synthetase (Enzyme I). The two enzymatic reactions differ in the following respects: (a) relative activities of the various allyl pyrophosphates as substrates; (b) the effect of alkaline phosphatase on the products of the two enzymes; (c) the binding of product of Enzyme II to the protein which is not seen with Enzyme I and its product; (d) chromatographic properties of the terpene alcohols obtained after acid hydrolysis; and (e) molecular weights of the two products as indicated by isotope ratios.

Enzyme I described in our earlier report had about one-half the specific activity with geranyl-PP as with farnesyl-PP (3). The corresponding enzyme described in the present investigation was much less active with geranyl-PP relative to farnesyl-PP. Conceivably the earlier preparation of Enzyme I was contaminated.
with Enzyme II, the existence of which had not been recognized at that time.

The observation that Enzyme II catalyzes the elongation of geranylgeranyl-PP was the first clue that this enzyme produces a terpene alcohol derivative of chain length greater than C₉. Apparently the specificity of the enzyme is relatively broad, the products ranging in size from C₁₆ to C₄₀ in proportions that appear to vary with different enzyme preparations.

Alkaline phosphatase hydrolyzes the product of Enzyme I to the free alcohol almost completely. By contrast the product or products of Enzyme II are resistant to alkaline phosphatase. This stability may be due either to the inability of the phosphatase to act on a pyrophosphate derivative of very long and therefore strongly hydrophobic terpene alcohols, or to the fact that the strong binding to protein may protect the pyrophosphate derivative against enzymatic hydrolysis. Further attempts to free the long chain terpene pyrophosphate from associated protein are under way.

The experiments described in this paper are an example of a strong interaction between protein and reaction products but they do not indicate the nature of the binding forces involved. It is clear however that binding to protein occurs only as a result of the enzyme-catalyzed condensation between isopentenyl-PP and allyl pyrophosphate. Furthermore, since the protein-bound product contains ³²P when ³²P-isopentenyl-PP is the substrate and since it releases ³²P-pyrophosphate on hydrolysis with dilute acid, labile pyrophosphate linkages must be present in the complex. Presumably, therefore, the product is an allylic pyrophosphate bound to or absorbed to the enzyme.²

Both mass spectrometric analysis and experiments with two isotopic labels indicate that Enzyme II catalyzes the formation of a family of terpene alcohol derivatives ranging in chain length from 35 to 45 carbon atoms. Thus the sample of terpene alcohol analyzed in the mass spectrometer gave parent ion peaks and MO - 18 peaks corresponding to C₃₅, Go, G₅, and C₆ alcohols as major products and the C₆₀ alcohol a trace component. This is borne out by the range of isotope ratios which correspond to a content of 7 to 8 isoprene units in the condensation product.

It is tempting to assume that the function of the second terpene synthetase described in this paper is to provide the polyisoprenoid moiety of the bacterial quinone coenzyme. In M. lysodeikticus this has been shown to be a vitamin K₅ derivative with a C₉ side chain (24). The chain length specificity of Enzyme II is therefore appropriate for this biosynthetic function even though under conditions in vitro the C₉ chain is not the sole or even the major elongation product. It will be of interest to examine more highly purified preparations of terpene synthetases I and II from M. lysodeikticus for variations in chain length specificity as a function of environmental changes.

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A Long Chain Terpenyl Pyrophosphate Synthetase from Micrococcus lysodeikticus

C. M. Allen, W. Alworth, A. Macrae and Konrad Bloch


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