Enzymatic Formation of Nerolidol in Cell-Free Extract of *Rhodotorula glutinis*¹, ²

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Enzymatic formation of nerolidol was demonstrated by incubation of [¹⁴C]farnesyl pyrophosphate with the ultracentrifugal supernatant of cell-free extract of *Rhodotorula glutinis*. Farnesol was also formed concomitantly with the formation of nerolidol and the ratios of formation of both alcohols were from 1 : 3 to 1 : 4. Divalent cation was necessary for the reaction and Mn²⁺ was much more active than Mg²⁺ for nerolidol formation. No nerolidol was formed when farnesyl monophosphate or farnesol was used instead of farnesyl pyrophosphate as a substrate. Nerolidyl pyrophosphate or nerolidyl monophosphate could not be detected as an intermediate in the reaction. Based on these observations, nerolidol was presumed to be formed not via nerolidyl pyrophosphate or nerolidyl monophosphate but via a carbonium ion intermediate which was formed by cleavage of the carbon-oxygen bond of farnesyl pyrophosphate. This reaction seems to proceed in a similar manner to the acid hydrolysis of farnesyl pyrophosphate to form nerolidol and farnesol.

Nerolidol and farnesol are the only two acyclic sesquiterpene alcohols which have so far been discovered in nature. Nerolidol has been found in essential oils of many plants since its discovery in Neroli oil. However, its formation by an enzymatic reaction has not yet been demonstrated, though the enzymatic formation of farnesol is well known. Lynen *et al. (1)*, Popják (2), and Cornforth and Popják (3) reported that nerolidol, as well as farnesol, was formed by the hydrolysis of farnesyl pyrophosphate (FPP) with acid—even with buffer of such a low pH as 3 to 4 (3)—and that the ratios of both compounds were always from 3 : 1 to 4 : 1 (2, 3). They also found that

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Abbreviations: FPP, farnesyl pyrophosphate; FP, farnesyl monophosphate.
nerolidol was formed when FPP was incubated with various phosphatases (2, 4), yeast microsomal fraction or animal liver microsomes (5), but they could not definitely conclude that it was formed enzymatically because of some ambiguities (5).

Cori and his coworkers also observed nerolidol formation in the reactions of FPP with many plant enzymes (6, 7). However, they explained its formation in terms of a non-enzymatic mechanism through a cation intermediate formed by cleavage of the C-O bond of FPP in the presence of Mn²⁺ (8). They insisted that further examination was necessary to determine whether the formation could be explained in terms of the non-enzymatic mechanism, since they considered that earlier studies had not excluded this possibility.

In the course of studies on the reaction of FPP with ultracentrifugal supernatant of cell-free extract of Rhodotorula glutinis, we found that nerolidol was formed enzymatically from FPP and that Mn²⁺ was necessary for the activity. The present paper describes the enzymatic formation of nerolidol and discusses the mechanism of the reaction.

MATERIALS AND METHODS

Chemicals—[¹⁴C]Isopentenyl pyrophosphate (specific radioactivity, 57 Ci/mol) was purchased from the Radiochemical Centre, Amersham. Alkaline phosphatase of intestinal mucosa was obtained from Sigma Chemicals. Dowex 1-X8 was a product of Bio-Rad Laboratories. All other chemicals were of analytical grade.

Preparation of Substrates—Pyrophosphates and monophosphates of geraniol, farnesol, and geranylgeraniol: Geraniol, farnesol, and geranylgeraniol were phosphorylated by the method of Cramer and Böhm (9) with some modifications according to Kandutsch et al. (10). The pyrophosphate and monophosphate of each alcohol were separated from one another by fractional crystallization and were obtained as lithium and cyclohexylammonium salts, respectively.

[¹⁴C]FPP and [¹⁴C]farnesyl monophosphate: Cell-free extracts of Saccharomyces cerevisiae (ATCC 12341) were centrifuged at 105,000 × g for 60 min and the resulting supernatant was used as enzyme. Incubation was carried out as described by Holloway and Popjak (11) with some modifications as follows. The incubation mixture contained, in a final volume of 1 ml, 10 nmol of [¹⁴C]isopentenyl pyrophosphate, 20 nmol of geranyl pyrophosphate, 5 μmol of MgCl₂, 20 μmol of KF, 4 μmol of iodoacetamide, 50 μmol of Tris-HCl buffer (pH 7.5), and the enzyme. The incubation mixture not including [¹⁴C]isopentenyl pyrophosphate was preincubated for 10 min at 37°C to allow iodoacetamide to inactivate isopentenyl pyrophosphate isomerase (1). Then the [¹⁴C]isopentenyl pyrophosphate was added to the mixture and it was incubated for a further 2 h at 37°C. After the reaction, non-polar products were extracted with petroleum ether and were discarded. Polar products including radioactive FPP and farnesyl monophosphate (FP) remaining in the residual solution were extracted with n-butanol. The extract was concentrated under reduced pressure and purified by Dowex 1-X8 ion-exchange column (1 × 10 cm) chromatography according to the method of Epstein and Rilling (12). The products were eluted with a linear gradient of increasing concentration of ammonium formate. The linear gradient was produced from 150 ml of methanol solution of 0.053 M ammonium formate in the mixing chamber and 150 ml of methanol solution of 0.43 M ammonium formate in the reservoir. The radioactive FP and FPP were eluted from the column with about 0.13 and 0.31 M ammonium formate, respectively. In order to isolate the radioactive products in the eluates, methanol was removed under reduced pressure and the products, after the addition of water, were extracted with n-butanol to separate them from ammonium formate. The radioactive FP and FPP were identified by cochromatography with authentic samples on thin-layer plates. In addition, treatment of both radioactive products with alkaline phosphatase gave rise to [¹⁴C]farnesol. The specific radioactivities of the compounds were both 57 Ci/mol.

[¹⁴C]Geranylgeranyl pyrophosphate: Cell-free extracts of spinach leaves, after being gel-filtered through a Sephadex G-50 column, were centrifuged at 105,000 × g for 60 min, and the resulting supernatant was used as enzyme. The reaction mixture contained the following constituents in a final volume of 1 ml: 20 nmol of [¹⁴C]isopentenyl pyrophosphate, 50 nmol of FPP, 5 μmol of MgCl₂, 2 μmol of MnCl₂, 30 μmol of...
KF, 4 \mu\text{mol} of iodoacetamide, and the enzyme. The reaction mixture not including \[^{14}\text{C}\]isopentenyl pyrophosphate was preincubated for 10 min at 37°C to inactivate isopentenyl pyrophosphate isomerase as described above. Then the \[^{14}\text{C}\]isopentenyl pyrophosphate was added to the mixture and it was further incubated for 2 h at 37°C. After the reaction, non-polar products were extracted with petroleum ether and were discarded. The residual solution was heated with 1 ml of \text{iso-propanol} for 30 min at 60°C according to Ogura et al. (13) to overcome the hydrophobic interaction between the products and protein. A small amount of water was added, then polar products were extracted with \text{n-butanol}. \text{n-Butanol} was removed by blowing a stream of \text{N}_2 gas over the solution, and the residue was used as \[^{14}\text{C}\]geranylgeranyl pyrophosphate. Inspection of the residue by TLC revealed that all of the radioactivity was in the same area as that of an authentic sample of geranylgeranyl pyrophosphate. In addition, treatment of the radioactive compound with alkaline phosphatase gave rise to \[^{14}\text{C}\]geranylgeraniol. Its specific radioactivity was 57 Ci/mol.

Growth of Yeast and Preparation of Cell-Free Extracts—Rhodotorula glutinis IFO 0389 was grown on a medium containing 0.2% \((\text{NH}_4)_2\text{SO}_4\), 0.2% \(\text{KH}_2\text{PO}_4\), 0.05% \(\text{Na}_2\text{HPO}_4\), 0.025% \(\text{MgSO}_4 \cdot 7\text{H}_2\text{O}\), 0.005% \(\text{MnSO}_4 \cdot 4\text{H}_2\text{O}\), 2% glycerol, and 0.1% yeast extract (obtained from Oriental Yeast Co.) in tap water. One liter of the medium in a 3-liter Erlenmeyer flask was inoculated with 25 ml of the preculture grown on the same medium for 48 h, and then incubated for 20 h at 28°C. The cells were harvested by centrifugation (3–4 g wet cells/liter), washed with 0.1 M Tris-HCl buffer (pH 7.4) containing 1 mm dithiothreitol and suspended in the same buffer. The cells in the suspension (3–4 g wet cells/10 ml) were disrupted with a Vibrogen cell mill (Edmund Bühler) using 20 ml of glass beads (0.5 mm diameter) for 5 min at 0°C. After removal of the glass beads by filtration, the resulting supernatant was centrifuged at 15,000 \times g for 10 min. The supernatant was passed through a Sephadex G-50 column to free it from compounds of low molecular weight. The gel-filtered supernatant was centrifuged at 105,000 \times g for 1 h to free it from subcellular particulates. The resulting supernatant \((S_{105})\) was used as enzyme. Protein concentration was determined by the method of Lowry et al. (14).

Enzyme Reactions and Extraction of Products

The reaction mixture contained the following constituents in a final volume of 1 ml, unless otherwise indicated: 2 \mu\text{mol} of \text{MnCl}_2, 20 \mu\text{mol} of KF, 50 \mu\text{mol} of Tris-HCl buffer (pH 7.4), \[^{14}\text{C}\]substrate (isopentenyl pyrophosphate, \text{FPP}, \text{FP}, or geranylgeranyl pyrophosphate), and the enzyme (about 1 mg of protein). It was incubated for 3 h at 30°C.

For the analysis of non-polar products, 1 g of \text{KOH} and 3 ml of methanol were added to the reaction mixture after the reaction and it was heated for 1 h at 80°C. Then, the non-polar products were extracted with petroleum ether and the resulting extract was washed with water.

For the analysis of polar products which were obtained by the incubation with \[^{14}\text{C}\]isopentenyl pyrophosphate or \[^{14}\text{C}\]FPP, the reaction mixture after the reaction was shaken with petroleum ether without the alkali treatment described above. Then the polar products were extracted with \text{n-butanol} from the residual solution.

Analysis of the Products—Analysis of the non-polar products: The following three methods were used.

1) Normal-phase TLC: The products were separated on a precoated Silica Gel G plate (Merck) using ethyl acetate–benzene (1:4).

2) Reversed-phase TLC: The products were separated on a Kieselguhr G plate (0.25 mm thick) which had been impregnated with liquid paraffin in \text{n-hexane}, using 65% acetone saturated with liquid paraffin as the solvent according to the method of McSweeney (15).

3) Radio-gas chromatography: The products were analyzed with a Shimadzu GC-5A gas chromatograph equipped with a Shimadzu RID-2E radioisotope detector using a glass column (1 m long and 3 mm i.d.) packed with 20% \text{PEG}-20M on \text{Chromosorb WAW} (60–80 mesh) at a linearly programmed temperature change according to the method of Nishino et al. (16).

Radioactivity on the thin-layer plate was scanned with a Packard radiochromatogram scanner (Model 7201) and spots of the marker alcohols were visualized by exposure of the plate to iodine vapor. The fractions of nerolidol and farnesol were scraped from the plate into counting vials and their radioactivities were counted.
Analysis of the polar products: The following two methods were used. 1) TLC: Polar products were analyzed by TLC on a precoated plate of Silica Gel G (Merck) using iso-propanol-conc. ammonia-water (6:3:1) (12). 2) Ion-exchange column chromatography: The products were separated by chromatography through a column of Dowex 1-X8 (formate form, 200-400 mesh) and extracted from the eluates as described in the section for preparation of [14C]FPP and [14C]FP.

Measurement of Radioactivity—Radioactivity was counted with a Beckman LS-230 liquid scintillation spectrometer. Radioactivities of non-polar products extractable with petroleum ether and of polar products extractable with n-butanol were counted using toluene scintillator (0.4% 2,5-diphenyloxazole in toluene) and toluene-Triton scintillator (0.4% 2,5-diphenyloxazole in toluene-Triton X-100 (3:1), respectively.

RESULTS

Enzymatic Formation of Nerolidol from FPP—The gel-filtered S105 was incubated with [14C]FPP as a substrate in the presence of Mn2+, and the non-polar products were extracted with petroleum ether as described in "MATERIALS AND METHODS." About 40% of the radioactivity of the substrate was incorporated into the non-polar products (Table I). When the enzyme or Mn2+ was omitted from the incubation mixture, the incorporation was virtually nil. This indicates that they are indispensable for the reaction. No incorporation was observed when the heat-inactivated enzyme was used instead of the enzyme, indicating that the reaction was of an enzymatic nature.

The non-polar products formed in the enzymatic reaction in the presence of Mn2+ were analyzed by the normal- and reversed-phase TLC's. Figure 1, a and b, shows typical chromatograms, respectively. Two major components of the products coincided completely with trans-nerolidol and trans,trans-farnesol in the two chromatographies. This conclusion was confirmed by the results of radio-gas chromatography (Fig. 2). The ratios of formation of nerolidol and farnesol in these analyses were from 3:1 to 4:1. It is worth noting that the ratios in all the reactions performed were substantially in this range. This will be discussed later. Of the two minor components, one was not identified but the other was presumed to be dehydrosqualene, since the latter moved to the same position as authentic dehydrosqualene in the normal-phase TLC (Fig. 1a) as well as in the reversed-phase TLC using 95% acetone saturated with liquid paraffin (17). The activity for the formation of these two minor products virtually disappeared when the supernatant which had been obtained by twice-repeated 1-h ultracentrifugation to free it from microsomal fraction was used. This indicates that the enzyme(s) involved in the formation was in the microsomal fraction (17).

Effects of Mn2+ and Mg2+ on Nerolidol Formation—The effects of divalent metal ions on the nerolidol formation from FPP were examined (Table II). The use of Mg2+ instead of Mn2+ in
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**Fig. 1.** Normal- and reversed-phase thin-layer radio-chromatograms of the non-polar products. The enzyme reaction was carried out using \[^{14}C\]FPP as a substrate as described in "MATERIALS AND METHODS." The non-polar products extracted from the reaction mixture with petroleum ether were mixed with authentic samples (A, B, C, and D) and subjected to normal-(a) and reversed-phase (b) TLC's as described in "MATERIALS AND METHODS." The radioactivities of the chromatograms were scanned with a Packard radiochromatogram scanner, then the spots of the authentic samples were visualized by exposure of the plates to iodine vapor. A, trans, trans-farnesol; B, cis, trans-farnesol; C, trans-nerolidol; and D, dehydro-squalene.

**Fig. 2.** Radio-gas chromatogram of the non-polar products. The enzyme reaction was carried out as described in Fig. 1. The non-polar products extracted from the reaction mixture with petroleum ether were mixed with authentic samples, \(-linalool\) (A), geraniol (B), trans-nerolidol (C), cis, trans-farnesol (D), trans, trans-farnesol (E), all-trans-geranylinalool (F) and all-trans-geranylgeraniol (G)—. The chromatography was carried out with a Shimadzu GC-5A gas chromatograph equipped with a Shimadzu RID-2E radioisotope detector using a glass column (1 m long and 3 mm i. d.) packed with 20% PEG-20M adsorbed on Chromosorb WAW at a linearly programmed temperature change (4°C/min from 140 to 245°C) under nitrogen (50 ml/min).

The reaction mixture markedly decreased the formation, demonstrating the requirement for Mn\(^{2+}\) for the reaction. However, the coexistence of Mg\(^{2+}\) with Mn\(^{2+}\) did not modify the effect of Mn\(^{2+}\).

The effects of divalent metal ions will be discussed in the subsequent section.

*Is FP or Farnesol an Intermediate in Nerolidol Formation?*—In order to examine whether or not
the nerolidol formation from FPP proceeds via FP or farnesol, the reaction was performed using FP instead of FPP (Table III). Virtually no nerolidol was formed, irrespective of the presence of Mn²⁺ or Mg²⁺. However, farnesol was produced in the presence of Mn²⁺ as well as Mg²⁺.

The formation of farnesol from FP seemed to be catalyzed by phosphomonoesterase, a kind of phosphatase, which hydrolyzes not only isoprenyl monophosphate but also isoprenyl pyrophosphate to yield isoprenol and orthophosphate. The fact that nearly three times as much farnesol as that in the presence of Mn²⁺ was formed in the presence of Mg²⁺ seems to be explainable in terms of the metal-ion specificity of the phosphatase. Moreover, it can be concluded that no nerolidol was formed via farnesol since nerolidol was not formed in spite of the formation of large amounts of farnesol in this experiment. For further confirmation, the reaction was carried out using [¹⁴C]farnesol as substrate. No ¹⁴C-incorporation into nerolidol was observed.

From these results, it was concluded that the nerolidol formation from FPP in the presence of Mn²⁺ did not proceed via FP or farnesol as an intermediate.

Is Nerolidyl Pyrophosphate or Nerolidyl Monophosphate an Intermediate in Nerolidol Formation?

Another possibility is that nerolidol is formed by the elimination of pyrophosphate from nerolidyl pyrophosphate (or by the elimination of orthophosphate from nerolidyl monophosphate) which is produced as an intermediate from the substrate.

TABLE III. ¹⁴C-Incorporation from [¹⁴C]FP into nerolidol and farnesol. The reaction mixture contained, in a final volume of 1.0 ml, [¹⁴C]FP (20,000 dpm), 20 μmol of KF, 50 μmol of Tris-HCl buffer, pH 7.4, the enzyme (1 mg of protein), and additions as indicated (MgCl₂, 5 μmol; MnCl₂, 2 μmol). The preparation of the "Boiled enzyme" and the extraction of non-polar products were carried out as described in Table II.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Radioactivity (dpm)</th>
<th>Nerolidol</th>
<th>Farnesol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn²⁺</td>
<td>60</td>
<td>1,690</td>
<td></td>
</tr>
<tr>
<td>Mn²⁺, Mg²⁺</td>
<td>50</td>
<td>3,850</td>
<td></td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>50</td>
<td>5,190</td>
<td></td>
</tr>
<tr>
<td>(Boiled enzyme)ᵃ, Mn²⁺</td>
<td>40</td>
<td>180</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ Boiled enzyme was used instead of the enzyme.

FPP. In order to examine whether or not nerolidyl pyrophosphate or nerolidyl monophosphate is formed as an intermediate, the reaction was carried out using [¹⁴C]isopentenyl pyrophosphate and Mg²⁺ as substrate and metal ion, respectively. After non-polar products had been extracted with petroleum ether, polar products were subjected to Dowex 1-X8 column chromatography. Analysis of the non-polar products revealed that the major products were farnesol and nerolidol; compounds such as linalool, geraniol, geranylinalool, and geranylgeraniol were not detected. Figure 3 shows the elution pattern of polar products in the Dowex 1-X8 chromatography. The radioactive polar products corresponding to peaks, A, B, C, D, E, and F were analyzed by normal-phase TLC as described in "MATERIALS AND METHODS." Compounds B, C, E, and F were identified as geranylgeranyl monophosphate, FP, presqualene pyrophosphate, geranylgeranyl pyrophosphate, and FPP, respectively. Compound A seemed to be presqualene monophosphate, but this was not confirmed.

Attempts were made to detect nerolidyl pyrophosphate in the fraction between presqualene pyrophosphate and geranylgeranyl pyrophosphate in the eluates from the Dowex 1-X8 column (I8). However, no nerolidyl pyrophosphate could be detected in the fraction. Similar attempts to detect nerolidyl monophosphate were unsuccessful.

When the reaction was carried out with Mn²⁺ as the metal ion, the alkaline phosphatase treatment of the reaction products from which non-polar products had been removed by extraction with petroleum ether also revealed the presence of farnesol but not of nerolidol. Moreover, neither nerolidyl pyrophosphate nor nerolidyl monophosphate could be detected in the polar products which had been obtained by extraction with n-butanol, though FPP and small amounts of FP were found in the products.
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**Fig. 3.** Ion-exchange chromatogram of the polar products formed from $^{[14]C}$isopentenyl pyrophosphate. The reaction was carried out using $^{[14]C}$isopentenyl pyrophosphate as a substrate as described in "MATERIALS AND METHODS" except for the use of MgCl$_2$ (5 µmol) instead of MnCl$_2$. The non-polar products were extracted with petroleum ether, then the polar products were extracted from the water layer with n-butanol and applied to a column of Dowex 1-X8 (formate form, 1 x 10 cm). The products were eluted with a linear gradient of ammonium formate concentration in methanol as described in "MATERIALS AND METHODS." Five-milliliter fractions were collected and their radioactivities were counted. The identification of each product was carried out as described in the text.

These results substantially exclude the possibility that nerolidol was formed from FPP via nerolidyl pyrophosphate or nerolidyl monophosphate.

**Time Course of the Reaction and pH Optimum**

—Figure 4 shows time courses of the formations of nerolidol and farnesol from FPP. Nerolidol and farnesol were produced linearly for at least 2 h. The ratios of the formation of the two compounds remained constant (about 2.7 : 1) during this period.

The activity of nerolidol formation showed a broad pH optimum in the range from 6.5 to 7.0. The ratios of the formation of nerolidol and farnesol were nearly constant independently of pH value in the pH range of 6.0 to 8.0.

**Product in the Reaction Using Geranylgeranyl Pyrophosphate as Substrate**—For further investigation of nerolidol formation, the reaction was carried out using $^{[14]C}$FPP or $^{[14]C}$geranylgeranyl pyrophosphate as a substrate. $^{14}C$-Incorporation into non-polar products were 27 and 16% from
the two substrates, respectively, showing that FPP was a better substrate than geranylgeranyl pyrophosphate. The analysis of the reaction products by normal-phase TLC showed that the ratio of \(^{14}\)C-incorporations into nerolidol and farnesol from FPP was about 3:1, whereas that into geranylinalool and geranyleranoliol from geranylgeranyl pyrophosphate was about 2:3. Determination of the radioactivities incorporated into tertiary alcohol and primary alcohol as products in the two cases showed that the incorporation into nerolidol from FPP was 20\%, whereas that into geranylinalool from geranylgeranyl pyrophosphate was 6.5\%. This means that FPP was a better substrate than geranylgeranyl pyrophosphate if the two reactions were catalyzed by a single enzyme, though the possibility that the two reactions are catalyzed by different enzymes cannot be excluded.

**DISCUSSION**

We found an enzyme activity catalyzing the formation of nerolidol and farnesol from FPP in the ultracentrifugal supernatant of cell-free extracts of *Rhodotorula glutinis*. The present study seems to be the first to demonstrate the enzymatic formation of nerolidol, as described in the previous section.

Some experiments were carried out to elucidate the mechanism of nerolidol and farnesol formations.

The possibility that nerolidol was formed by the hydrolysis of FPP catalyzed by phosphatase was essentially excluded. This possibility is clearly unlikely on the basis of the reaction mechanism of phosphatase elucidated by Cohn (19), and by Stein and Koshland (20). They extensively studied the mechanism of the phosphatase reaction and observed that, in the alkaline and acid phosphatase reactions in \(\text{H}_2\text{H}_{18}\text{O}\), \(^{18}\text{O}\) was not found in the produced alcohol. They explained this observation in terms of the cleavage of the C-O-P bond between the O and P atoms in the ester. On this basis, only farnesol should have been formed in the hydrolytic reaction of FPP with phosphatase.

The second possibility, that geranylpyrophosphate, which was demonstrated to be an intermediate for cyclonerodiol synthesis in the recent study by Cane (21), is formed by some mechanism and then nerolidol is formed therefrom, catalyzed by phosphatase, also seems unlikely in view of the following observation: inspection by Dowex 1-X8 column chromatography of the polar products formed enzymatically from isopentenyl pyrophosphate failed to detect nerolidyl pyrophosphate or nerolidyl monophosphate (Fig. 3). In addition, a separate experiment showed that no nerolidol was present in the reaction mixture from which non-polar products had been removed by extraction with petroleum ether followed by treatment with phosphatase, indicating that nerolidyl pyrophosphate or nerolidyl monophosphate had not been formed in the reaction. Further, polar products were extracted with \(n\)-butanol from the reaction mixture, and attempts were made to detect nerolidyl pyrophosphate or nerolidyl monophosphate (which should not be decomposed under these conditions) in the \(n\)-butanol extract (18). However, it could not be detected. In addition, the possibility that phosphatase acts only on the once-formed nerolidyl pyrophosphate or nerolidyl monophosphate but not on FPP seems unlikely, because the substrate specificity of the enzyme is not high. The linear time courses of nerolidol and farnesol formations also tend to rule out the second possibility (Fig. 4): if it is assumed that nerolidol was formed via nerolidyl pyrophosphate or nerolidyl monophosphate, the observed linearity of nerolidol formation cannot be explained.

All the observations seem to support the validity of the following mechanism for nerolidol formation. As described in the earlier section, it is known that the phosphate ester bond is cleaved between the C and O atoms upon the acid hydrolysis of FPP to yield allylic cations which undergo an allylic rearrangement. When a water molecule adds to these cations, nerolidol and farnesol are produced in a molar ratio 3:1 to 4:1 (2, 3). Most of the ratios of enzymatic formations of nerolidol and farnesol found in the present study were slightly lower than the above ratios (2.7:1 to 3:1). This seems to be due to the catalysis of farnesol formation by phosphatase(s) present in the enzyme preparation. No study was performed on the intracellular concentration and specificity of the phosphatase(s). Thus, these phenomena can be explained if it is assumed that the C-O bond in the FPP molecule is cleaved and the reaction proceeds in a manner similar to that
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in the acid hydrolysis to form nerolidol and farnesol. Some reactions have been reported which can be considered to proceed via a carbonium ion produced by cleavage of the C-O bond in isoprenoid biosyntheses. Rilling and Poulter and their associates demonstrated that the prenyltransferase reaction proceeds via a carbonium ion intermediate produced by C-O bond cleavage in the process of ionization-condensation-elimination but not of displacement-elimination (22–26). According to their explanation of the FPP synthetase reaction, the double bond between 3C and 4C of the isopentenyl pyrophosphate molecule is added nucleophilically to the cation intermediate produced by cleavage of the C-O bond in geranyl pyrophosphate followed by elimination of H⁺ attached to 2C in the isopentenyl pyrophosphate molecule to yield FPP. For the reaction of squalene synthesis, they had proposed a mechanism in which squalene was produced by rearrangement of a cyclopropylcarbinyl cation formed by cleavage of the C-O bond of the presqualene pyrophosphate molecule, the precursor of squalene (27, 28). However, no detailed evidence seems to have been obtained for this. In our previous paper (17, 29), we reported the formation of dehydrodesqualene with yeast microsomal fraction. When NADPH was present in the reaction system, squalene was formed instead of dehydrodesqualene. We proposed a mechanism by which dehydrodesqualene is formed from a cation intermediate produced through a carbonium ion by cleavage of the C-O bond of the presqualene pyrophosphate molecule.

These considerations appear to support the validity of the proposed mechanism for nerolidol formation.

The effects of divalent metal ions on the activity of nerolidol formation are noteworthy. As described in the previous section, Cori et al., in their study on the effects of metal ions on the solvolysis of FPP, reported that Mn²⁺ cleaved the C-O bond of the FPP molecule, while Mg²⁺ rather cleaved the O-P bond of the compound (8). As already discussed briefly, Brems and Rilling also obtained similar results in their study on the solvolysis of geranyl pyrophosphate and reported that linalool and geraniol were formed in a ratio of 5:1 (30). For the formation of dehydrodesqualene, we also proposed a mechanism in which the compound was formed by rearrangement of a carbonium ion produced by cleavage of the C-O bond of the presqualene pyrophosphate molecule. Mn²⁺ was much more active than Mg²⁺ for dehydrodesqualene formation as well (17, 29). The higher activity of Mn²⁺ than of Mg²⁺ in nerolidol formation observed in the present study suggests that Mn²⁺ coordinates to the pyrophosphate part of FPP to make cleavage of the C-O bond easier. Such a big difference between the two metal ions in their action towards some biosynthetic activities, suggesting a significant contribution to the regulation of biosynthesis, seems remarkable.

The enzyme catalyzing nerolidol synthesis seems to be a new enzyme which should be named nerolidol synthetase on the basis of the properties so far examined. However, if the enzyme is to be named from the viewpoint of the reaction mechanism, the name "farnesyl pyrophosphate solvolylase" might be preferable. Further studies are in progress.

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REFERENCES