ENZYMATIC CYCLIZATION OF NERYL PYROPHOSPHATE TO α-TERPINEOL BY CELL-FREE EXTRACTS FROM PEPPERMINT

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Summary. A cell-free system prepared from peppermint (Mentha piperita L.) shoot tips catalyzed the cyclization of neryl pyrophosphate to α-terpineol. Cyclization could be demonstrated in the absence of added cofactors, but addition of NaF inhibited competing phosphatase/pyrophosphatase activity, resulting in much higher levels of α-terpineol formation. Under certain conditions cyclization was stimulated by Mg^{++}. Similar enzyme preparations were obtained from spearmint (Mentha spicata L.) leaves and carrot (Daucus carota L.) storage organ. The cyclization of neryl pyrophosphate to α-terpineol appears to be a key reaction in the biosynthesis of cyclohexanoid monoterpenes.

The biosynthesis of cyclic monoterpenes is thought to involve the transformation of mevalonic acid to an acyclic C_{10} terpenyl pyrophosphate, followed by cyclization, with elimination of the pyrophosphate moiety (1). Although geranyl (3,7-dimethyl-octa-2(trans), 6-dienyl) pyrophosphate is a well established intermediate in terpenoid biosynthesis, several investigators (1,2) have proposed the geometric isomer, neryl (3,7-dimethyl-octa-2(cis), 6-dienyl) pyrophosphate, as the most likely immediate precursor of cyclohexanoid monoterpenes, because its cis-configuration favors cyclization. Support for this hypothesis is found in non-enzymatic solvolysis experiments (3-6), in which phosphorylated nerol derivatives were shown to yield primarily cyclic compounds [in non-aqueous systems predominantly limonene and terpinolene, in aqueous systems predominantly α-terpineol (p-menth-1-en-8-ol)], while the corresponding geraniol derivatives yielded primarily acyclic compounds. Furthermore, the conversion of neryl pyrophosphate, in low yields, to cyclic monoterpene hydrocarbons (pinenes, limonene) has been demonstrated in cell-free systems from Pinus (7) and Citrus (8). Based on both chemical and genetic considerations we postulated earlier (1) the cyclization of neryl pyrophosphate to α-terpineol as a key reaction in the biosynthesis of the p-menthane series of monoterpenes.

We have recently isolated a cell-free enzyme systen from peppermint that catalyzes the isomerization of geraniol or geranyl phosphate to nerol or

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neryl phosphate, respectively, (trans-cis isomerization) (9). In this communication we describe a cell-free enzyme system from peppermint which catalyzes the cyclization of neryl pyrophosphate to α-terpineol.

EXPERIMENTAL

Nerol (99+%) was a gift from Dr. C.H. Manley, Givaudan Corp., Clifton, N.J. Geraniol (95%) was a gift from Dr. E. Klein, Dragoco, Holzminden, Germany. RS-α-terpineol was a product of Eastman Organic Chemicals, Rochester, N.Y. The phosphates and pyrophosphates of nerol and geraniol were synthesized and purified by standard procedures (10). Insoluble polyvinylpolypyrrolidone (PVP or PVPP; trade name, Polyclar AT) was donated by GAF Corp., New York, N.Y. Amberlite XAD-4 (polystyrene) resin was by courtesy of Rohm and Haas Company, Philadelphia, Pa., and was washed thoroughly with methanol, followed by H_2O, before use (11).

Peppermint plants were Mentha piperita L., cv. Black Mitcham, propagated vegetatively from the clone used previously, under conditions already described (12). Extracts of peppermint plants were made from 20-30g of vegetative shoot tips (growing tip and top two leaf pairs). The fresh tissue was frozen and ground with liquid N_2 in a mortar, and then slurried with an equal weight of purified Polyclar AT (13) pre-soaked in 0.1 M potassium phosphate buffer (pH 7.2) containing 0.01 M potassium metabisulfite (7 ml buffer per g Polyclar AT). Liquid was extracted from the resulting paste using an Oster Juice Extractor (yield was 3-4 ml per g fresh tissue). The extract was immediately placed on a Biogel P-10 column (10 ml bed volume per g tissue) and the high molecular weight fraction eluted with 0.1 M potassium phosphate buffer (pH 7.2). The P-10 eluate was filtered through an XAD-4 column (1.5 cm diam. × 9 cm) and eluted with 0.1 M Tris-HCl buffer (pH 7.3). Filtration through XAD-4 quantitatively removes endogenous monoterpenes [as determined by extraction with CH_2Cl_2 followed by gas liquid chromatography (GLC) at maximum sensitivity] and permits the use of unlabeled substrates. The XAD-4 eluate was centrifuged at 3000 × g max. r.c.f. for 5 minutes and the pellet discarded. The 3000 × g supernatant was centrifuged at 30,000 × g max. r.c.f. for 20 minutes and the pellet suspended in appropriate buffer (1 ml buffer per 6 g fresh tissue weight) for subsequent assay.

In the enzyme assays, cork-stoppered conical centrifuge tubes containing 0.25 ml of the pellet suspension and 0.2 μmoles of substrate, plus appropriate additions as indicated, in a final volume of 0.5 ml, were incubated at 30° in a water bath for 2 hours, unless otherwise indicated. Reactions were stopped

by chilling the reaction mixture and extracting with 50 µl CH_2Cl_2 (after thoroughly mixing, the phases were separated by centrifugation). The CH_2Cl_2-soluble reaction products were then analyzed by GLC using a 20 ft × 1/8 in (6 m × 3.175 mm) stainless steel column packed with Triton X-305 on 100-120 mesh Chromosorb G and operated at 185° (Perkin-Elmer 990 gas chromatograph with flame ionization detector). At no time was there evidence of any decomposition or rearrangement of the reaction products during this assay procedure. Reaction products from several incubations were pooled for analysis by combined GLC-mass spectrometry (GLC-MS) (14). A boiled-enzyme control was run for each assay, and all reported results, except those in Table 1, are corrected for the indicated non-enzymatic reactions.

RESULTS AND DISCUSSION

Incubation of the 30,000 × g pellet suspension with neryl pyrophosphate gave rise to several products as shown by GLC analysis. Combined GLC-MS indicated that the major reaction products were α-terpineol and nerol (the latter liberated by endogenous phosphatases and/or pyrophosphatases). Smaller quantities of geraniol [from cis-trans isomerization (9)], an unidentified oxygenated monoterpene, and terpinen-4-ol (tentative) were also formed. In addition to neryl pyrophosphate, neryl phosphate and nerol could also function as substrates for cyclization, although they were less effective (50% and 25% respectively). Geraniol, geranyl phosphate and geranyl pyrophosphate were not cyclized when tested under the same conditions. The 30,000 × g supernatant fraction was ineffective in converting neryl pyrophosphate to α-terpineol. Initial attempts to solubilize the cyclase with Triton X-100 were not very encouraging, so preliminary studies of the enzyme were carried out with the suspended 30,000 × g pellet.

NaF was an effective inhibitor of competing phosphatase/pyrophosphatase activity, a level of 50 mM F^- increasing the yield of α-terpineol from 2.5 nmoles to 10 nmoles. NaCl was less than 10% as effective as NaF in increasing α-terpineol production, indicating that ionic strength was not responsible for the stimulation observed. Although the cyclase itself must function as a pyrophosphatase, the activity of the cyclase was not appreciably affected by F^- concentrations as high as 100 mM. Therefore, NaF was added routinely in most assays.

The effect of pH on production of α-terpineol and nerol from neryl pyrophosphate is shown in Fig. 1. The effect of pH on an impure enzyme system such as this is undoubtedly complex. Below pH 5, non-enzymatic acid-catalyzed cyclization of neryl pyrophosphate to α-terpineol was significant.

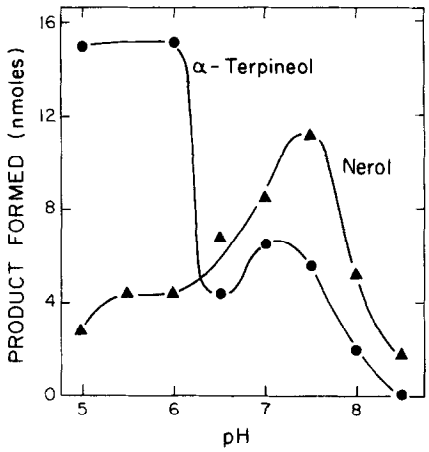

Fig. 1.

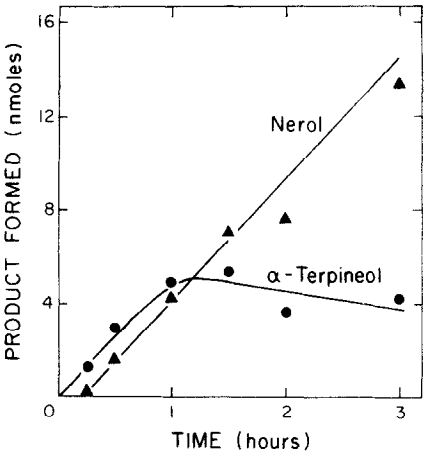

Fig. 2.

Figure 1. Effect of pH on Formation of α-Terpineol and Nerol from Neryl Pyrophosphate.

The reaction mixture contained: (Potassium) Tris-maleate, 25 μmoles each, pH as indicated; NaF, 15 μmoles; pellet suspension, 0.25 ml; neryl pyrophosphate, 200 nmoles; total volume, 0.5 ml. Incubated at 30° for 120 minutes.

Figure 2. Time Course of α-Terpineol and Nerol Production from Neryl Pyrophosphate.

The reaction mixture contained: Tris-HCl, 50 μmoles, pH 7.3; NaF, 15 μmoles; pellet suspension, 0.25 ml; neryl pyrophosphate, 200 nmoles; total volume, 0.5 ml. Incubated at 30°.

The high cyclization activity at pH 5 to 6 reflects, in part, reduced phosphatase/pyrophosphatase activity, as indicated by the corresponding low yields of nerol. The lower peak at pH 7 to 7.5 is apparently real, as stimulation of both cyclase and phosphatase activities was repeatedly observed in this pH range.

A time-course of α-terpineol and nerol production is shown in Fig. 2. While nerol production was linear with time for 3 hours, α-terpineol production ceased after 1 to 1½ hours, and there was even a slight apparent loss of α-terpineol after this time. This observation is probably due in part to instability of the cyclase in these preparations. When pellet suspensions were stored frozen overnight most of the cyclase activity was lost, while the phosphatase/pyrophosphatase activity was retained. The effects of substrate concentration and enzyme concentration on α-terpineol production are shown in Fig. 3. At higher enzyme levels, phosphatases overcome F^- inhibition and again compete effectively for the substrate. High substrate concentrations appear to be inhibitory.

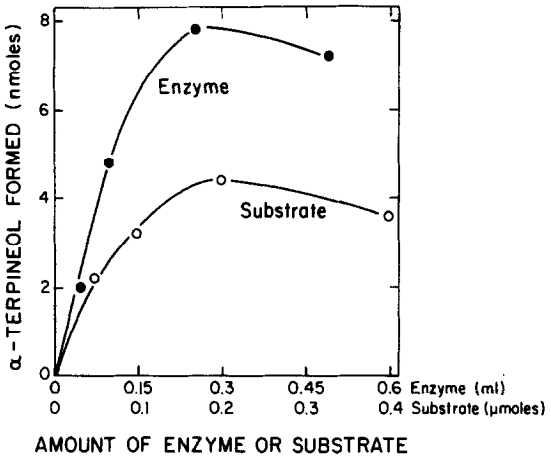

Figure 3. Effect of Enzyme and Substrate Concentration on α-Terpineol Formation from Neryl Pyrophosphate.

The reaction mixture contained: Tris-HCl, 50 μmoles, pH 7.3; NaF, 12 μmoles; pellet suspension, 0.25 ml, or as indicated; neryl pyrophosphate, 200 nmoles, or as indicated; total volume, 0.5 ml. Incubated at 30° for 90 minutes.

Eight cations (Mg^{++}, Mn^{++}, Zn^{++}, Ca^{++}, Cu^{++}, Co^{++}, Fe^{++} and K^{+}) at levels from 0.2 mM to 20 mM were examined for their effect on α-terpineol production. Most of these cations were inhibitory; however Mg^{++}, between 10 mM and 20 mM, approximately doubled the amount of α-terpineol formed. The effects of Mg^{++} and EDTA on α-terpineol and nerol biosynthesis from neryl pyrophosphate are shown in Table 1. It will be seen that 4 mM Mg^{++} had little or no effect when added by itself. EDTA added alone had no effect on cyclization but greatly enhanced the hydrolysis of neryl pyrophosphate to nerol. In the presence of both EDTA and 4 mM Mg^{++}, both cyclization and hydrolysis were enhanced. Tentatively, it would appear from these data that the cyclase is activated by Mg^{++}, and that competing phosphatase/pyrophosphatase activity may be slightly inhibited by Mg^{++} but is inhibited to a greater extent by unidentified endogenous cations. It is hoped that current efforts to solubilize and purify the cyclase will allow a definitive answer to the question of cation effects.

In a final experiment, ^{14}C-neryl pyrophosphate was employed as substrate, and the resulting product was isolated by thin-layer chromatography and diluted with carrier RS-(±)-α-terpineol. Resolution of the optical isomers via the brucine salt of α-terpinyl hydrogen phthalate (15) suggested that the enzymatic cyclization product is S-(—)-α-terpineol. However this identification must be regarded as tentative in view of the low count levels available and low yields obtained.

Enzyme extracts that catalyze the cyclization of neryl pyrophosphate to

Table 1. Effect of Mg^{++} and EDTA on α-Terpineol and Nerol Formation from Neryl Pyrophosphate.

Conditions	Product formed (nmoles) α-terpineol	nerol
Enzyme and substrate only	2.8	12.2
With 4 mM Mg^{++}	3.4	12.8
With 10 mM Mg^{++}	6.2	10.6
With 2 mM EDTA	3.2	20.4
With 2 mM EDTA* + 4 mM Mg^{++}	6.0	17.8
Enzyme (boiled) and substrate	0.5	0.2

The reaction mixture contained: Tris-HCl, 50 μmoles, pH 7.3; pellet suspension, 0.25 ml; neryl pyrophosphate, 200 nmoles; $MgCl_2$ and EDTA, as indicated; total volume, 0.5 ml. Incubated at 30° for 90 minutes. NaF was omitted in order to avoid complications due to complexing of Mg^{++}.

*2 minute preincubation.

α-terpineol have also been prepared from spearmint (Mentha spicata L.) shoot tips and carrot (Daucus carota L.) storage organ, suggesting that this cyclase may be widespread in essential oil-producing plant tissues. α-Terpineol is widespread in essential oils (16), including carrot (17) and peppermint (18) oils, occasionally in high concentration, frequently as a minor component. In peppermint, α-terpineol is reported to comprise about 0.1% of the essential oil (18). These observations are consistent with the postulated active metabolic role of α-terpineol as a precursor of other cyclic monoterpenes. Results described in this communication provide for the first time direct evidence for the enzymatic cyclization of neryl pyrophosphate to α-terpineol.

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REFERENCES

1. Loomis, W.D., in Terpenoids in Plants, J.B. Pridham, Ed. (Academic Press, London, 1967) p. 59.
2. Valenzuela, P., Beytía, E., Cori, O. and Yudelevich, A., Arch. Biochem. Biophys. 113, 536 (1966).

3. Cramer, F., and Rittersdorf, W., Tetrahedron 23, 3015 (1967).
4. Valenzuela, P., and Cori, O., Tetrahedron Lett., 3089 (1967).
5. Rittersdorf, W., and Cramer, F., Tetrahedron 24, 43 (1968).
6. Haley, R.C., Miller, J.A. and Wood, H.C.S., J. Chem. Soc. (C), 264 (1969).
7. Cori, O., Arch. Biochem. Biophys. 135, 416 (1969).
8. George-Nascimento, C., and Cori, O., Phytochemistry 10, 1803 (1971).
9. Shine, W.E., and Loomis, W.D., In preparation.
10. Edmond, J., Popják, G., Wong, S. and Williams, V.P., J. Biol. Chem. 246, 6254 (1971).
11. Rohm and Haas Company, Preliminary Technical Notes, Amberlite XAD-4, 1971.
12. Battaile, J., Burbott, A.J., and Loomis, W.D., Phytochemistry 7, 1159 (1968).
13. Loomis, W.D., and Battaile, J., Phytochemistry 5, 423 (1966).
14. Croteau, R., and Loomis, W.D., Phytochemistry 11, 1055 (1972).
15. Fuller, A.T., and Kenyon, J., J. Chem. Soc. 125, 2304 (1924).
16. Gildemeister, E., and Hoffmann, F., Die ätherischen Öle (4th Edition), W. Treibs, Ed., Vol. IIIb (Akademie-Verlag, Berlin, 1962) p. 55.
17. Buttery, R.G., Seifert, R.M., Guadagni, D.G., Black, D.R., and Ling, L.C., J. Agr. Food Chem. 16, 1009 (1968).
18. Lawrence, B.M., Hogg, J.W. and Terhune, S.J., J. Agr. Food Chem. (In press).