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ISOMERIZATION OF GERANIOL AND GERANYL PHOSPHATE BY ENZYMES FROM CARROT AND PEPPERMINT

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Key Word Index—*Daucus carota*; Umbelliferae; carrot; *Mentha piperita*; labiatae; peppermint; isomerase; *trans-cis* isomerization; monoterpene; geraniol; nerol; geranyl phosphate.

Abstract—Cell-free extracts from carrot and peppermint catalyzed enzymatic *trans-cis* isomerization of geraniol and geranyl phosphate to nerol and neryl phosphate, respectively, in the presence of a flavin, a thiol or sulfide and light. Partial reduction of the flavin prior to incubation allowed the reaction to proceed in the absence of light. The reaction was reversible with the equilibrium favoring geraniol and geranyl phosphate.

INTRODUCTION

THE CYCLIZATION of NerPP to α-terpineol has been postulated to be a key reaction in the biosynthesis of the *p*-menthane series of cyclohexanoid monoterpenes.[1] Recently, Croteau *et al.*[2] demonstrated that extracts from several monoterpene producing plants, *Mentha piperita* (peppermint), *M. spicata* (spearmint) and *Daucus carota* (carrot), catalyzed this reaction.[2] Thus, the acyclic C_{10}-terpenyl pyrophosphate, NerPP, is the direct precursor of certain cyclic monoterpenes.

Although several mechanisms for the biosynthesis of NerPP have been proposed and evidence in support of each has recently been reviewed,[3] the most likely mechanisms appeared to involve either the direct *cis*-condensation of dimethylallyl pyrophosphate with isopentenyl pyrophosphate, analogous to the biosynthesis of rubber, or *trans-cis* isomerization of GerPP to yield NerPP. Francis *et al.*[4] obtained evidence in support of the *trans-cis* isomerization reaction; results obtained using stereospecifically ^{3}H-labeled mevalonate indicated that nerol in rose petals was synthesized via geraniol or a geraniol derivative, presumably GerPP.

In the present paper the isolation of cell-free enzyme systems from peppermint and carrot leaves which *catalyze* the *trans-cis* isomerization of geraniol and GerP to nerol and NerP respectively is reported. Preliminary results have been communicated recently.[5]

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Abbreviations used: GerP-geranyl phosphate; GerPP-geranyl pyrophosphate; NerP-neryl phosphate; NerPP-neryl pyrophosphate; DTT-dithiothreitol; NEM-*N*-ethylmaleimide; PHMB-*p*-hydroxymercuribenzoate. GSH-glutathione.

[1] LOOMIS, W. D. (1967) in *Terpenoids in Plants* (PRIDHAM, J. B., ed.), pp. 59–82, Academic Press, London.
[2] CROTEAU, R., BURBOTT, A. J. and LOOMIS, W. D. (1973) *Biochem. Biophys. Res. Commun.* **50,** 1006.
[3] BANTHORPE, D. V., CHARLWOOD, B. V. and FRANCIS, M. J. O. (1972) *Chem. Rev.* **72,** 115.
[4] FRANCIS, M. J. O., BANTHORPE, D. V. and LE PATOUREL, G. N. J. (1970) *Nature* **228,** 1005.
[5] SHINE, W. E. and LOOMIS, W. D. (1972) *Plant Physiol.* **49** (suppl.), 37.

Table 1. Isomerization of geraniol by carrot extract

Enzyme system	Terpene recovered (nmol)	Product (%)
Geraniol substrate		
Dark, complete	200	0·3
Light, boiled control	210	3·7
Light, complete	230	12·6
Light, no GSH	250	1·9
Light, no FAD	240	0·7
Nerol substrate		
Light, complete	170	21·1

The reaction mixture contained: 4·4 μmol K phosphate buffer; 20 nmol FAD; 44 nmol GSH; 300 nmol substrate; and 20 μl enzyme extract in a total vol. of 0·175 ml. The pH was 7·5. Light level 730 lx, 2E + 96 (density 1·00) filters, or dark. Reaction time 60 min; temp. 35°, N_2 atmosphere.

RESULTS

Cell-free extracts from carrot tops and peppermint shoot tips were obtained which catalyzed the *trans-cis* isomerization of geraniol and GerP in the presence of a flavin, a thiol or sulfide, and light. With geraniol as the substrate, carrot extracts catalyzed nerol formation (reported as a percentage of total terpene recovered since recovery varied slightly from assay to assay). Product formation was greatest in the presence of the cofactors FAD and GSH, and light (above 400 nm). This activity was greatly reduced in the presence of boiled enzyme (Table 1). Some enzymatic isomerization occurred in the absence of either one of the cofactors (FAD, GSH, or of light). In the presence of O_2, product formation was only 25% of that observed in N_2 (data not shown). Table 1 also shows that nerol was isomerized twice as fast as geraniol; the equilibrium, 67% geraniol and 33% nerol, was determined in the presence of the peppermint enzyme. The identity of the product, nerol, was confirmed by GC–MS. GerP also served as a substrate and was about 50% as active as geraniol (Table 2). In the assay containing boiled enzyme no hydrolysis of GerP was detected but some non-enzymatic isomerization was observed (Table 2). No phosphorylation of the substrates geraniol-[G-^{14}C] or GerP, as indicated by paper chromatography, was observed. Maximum isomerase activity with geraniol as substrate occurred at pH 7·5 and the rate of isomerization at 35° was approximately twice that at 25°. The methyl esters

Table 2. Isomerization of geranyl phosphate and geraniol by peppermint extract

Treatment	Terpene recovered (nmol)				Isomerization	
	Ner	Ger	NerP	GerP	Nerol (%)	NerP (%)
GerP						
Boiled extract	0	0	1·7	97	0	1·7
Complete	0·4	3·0	5·1	96	0·4	4·9
No GSH	0	2·6	0	119	0	0
Geraniol						
Complete	9·7	62			13·5	

The reaction mixture contained 8·8 μmol K phosphate buffer; 20 nmol FAD; 44 nmol GSH; 130 nmol substrate; and 5 μl Ca phosphate gel treated enzyme extract in a total vol. of 0·175 ml. The pH was 7·5. Light level 730 lx, 2E + 96 (density 1·00) filters; reaction time 75 min; temp. 30°, N_2 atmosphere.

TABLE 3. EFFECT OF NEM ON ISOMERIZATION OF GERANIOL BY CARROT EXTRACT

Enzyme system	Terpene recovered (nmol)	Nerol (%)
Control, boiled	280	1·5
Control, complete	250	5·1
Control, no GSH	170	0·7
NEM treated, boiled	210	1·5
NEM treated, complete	250	7·6
NEM treated, no GSH	270	0·1

The reaction mixture contained: 4·4 μmol K phosphate buffer; 20 nmol FAD; 44 nmol GSH; 300 nmol geraniol; and 25 μl enzyme extract in a total vol. of 0·175 ml. The pH was 7·5. Light level 730 lx, 2E + 96 (density 1·00) filters. Incubation time 60 min; temp. 35°, N_2 atmosphere.

of *cis*-geranic and *trans*-geranic acid were not isomerized. GerPP was not tested as a substrate because of the difficulty of preparing labeled GerPP of sufficiently high specific activity.

Both FAD and FMN served as cofactors for the isomerase. FMN was approximately 70% as effective as FAD. Near maximal enzymatic activity was observed at 0·02 mM FAD concentration; a five-fold increase in FAD level caused an increase in non-enzymatic but not enzymatic isomerization.

The thiol reagents DTT, GSH, β-mercaptoethanol or Na_2S were an absolute requirement for both the enzymatic isomerization of geraniol, using the purified peppermint isomerase, and also for its non-enzymatic isomerization. However, in contrast to the enzymatic isomerization, Na_2SO_3 would replace the thiol-compounds as a cofactor in the non-enzymatic reaction. Non-enzymatic isomerization was least in the presence of DTT (0·7% for DTT, 1·8% for GSH and 2·3% for β-mercaptoethanol at a concentration of 125 μM). Geraniol isomerization in the presence of GSSG was 25% of that with GSH and inclusion of NEM in the incubation mixture greatly inhibited isomerization. However, pretreatment of the isomerase with NEM or PHMB and removal of excess reagent on a Bio-Gel P-10 column prior to incubation caused no inhibition; indeed a slight stimulation was observed (Table 3).

The effect of light on the isomerase reaction is shown in Fig. 1. Enzymatic isomerization reached a maximum at approximately 2300 lx while non-enzymatic activity increased

TABLE 4. EFFECT OF PARTIALLY REDUCED FLAVIN ON DARK ISOMERIZATION OF GERANYL PHOSPHATE BY PEPPERMINT EXTRACT

	Terpene recovered (nmol)				Isomerization	
System	Nerol	Geraniol	NerP	GerP	Nerol (%)	NerP (%)
Oxidized flavin						
Light, complete	1·4	5·0	8·1	76	1·5	9·0
Dark, complete	0	6·2	0·4	90	0	0·4
Partially reduced flavin						
Dark, complete	0·3	6·4	2·7	85	0·3	2·9

The reaction mixture contained: 8·8 μmol K phosphate buffer; 20 nmol FAD; 88 nmol GSH; 130 nmol GerP; and 20 μl Ca phosphate gel treated enzyme extract in a total vol. of 0·175 ml. The pH was 7·5. Light level 730 lx, 2E + 96 (density 1·00) filters, or dark. Reaction time 75 min; temp. 35°, N_2 atmosphere.

TABLE 5. LIGHT VS. DARK ISOMERIZATION OF GERANIOL BY PEPPERMINT EXTRACT

Enzyme system	Terpene recovered (nmol)		Isomerization
	Nerol	Geraniol	Nerol (%)
Oxidized flavin			
Light, boiled control	8·0	200	3·8
Light, complete	37	170	18·3
Partially reduced flavin			
Dark, complete	18	190	8·8
Dark, No GSH	0	200	0

The reaction mixture contained: 4·4 μmol K phosphate buffer; 20 nmol FAD; 88 nmol GSH; 250 nmol geraniol; and 10 μl of peppermint extract in a total vol. of 0·175 ml. The pH was 7·5. Light level 730 lx, 2E + 96 (density 1·00) filters, or dark. Reaction time 75 min; temp. 35°. N_2 atmosphere.

linearly with light intensity. Maximum activity occurred at wavelengths between 400 and 500 nm. In order to minimize non-enzymatic isomerization a light intensity of 730 lx was selected. If the flavin was partially reduced (by hydrogen with platinum catalysis) prior to incubation, isomerization of geraniol and GerP occurred even in the absence of light (Tables 4 and 5). Under these conditions, dark isomerization approached 50% of that observed in the light reaction and GSH was still a required cofactor (Table 5). Dark isomerization was markedly less sensitive to pH changes than was the light reaction. Almost no isomerization was observed in the presence of fully reduced flavin in either the presence or absence of light.

DISCUSSION

Recently Croteau *et al.*,[2] reported the existence in peppermint, spearmint and carrot cell-free extracts, of enzymes which catalyze the formation of the cyclic monoterpene, α-terpineol, from NerPP. The results reported herein demonstrate that peppermint and carrot tissues also contain enzyme systems which convert geraniol and GerP to the corresponding *cis*-isomers and which convert nerol to the *trans*-isomer; the equilibrium, determined in the presence of the isomerase, was 67% geraniol and 33% nerol. Thus, tissues which form cyclohexanoid monoterpenes can transform the C_{10}-product of the isoprenoid pathway, geraniol, to nerol, the acyclic precursor of the cyclic monoterpenes. Since GerPP was not available for testing, there is no evidence from the present study to suggest whether the *in vivo* pathway involves direct *trans-cis*-isomerization of GerPP or hydrolysis of the pyrophosphate followed by isomerization of the monophosphate (or the free alcohol) and subsequent rephosphorylation of the isomerization product to form NerPP. Kinases which phosphorylate geraniol and nerol to their monophosphate and pyrophosphate derivatives have been isolated from mint leaves.[6,7]

The evidence in support of various isomerization mechanisms has been reviewed recently.[3] Subsequent reports have dealt with one of these mechanisms which involves a terpene aldehyde[8,9] with catalysis by SH groups.[9] In the present study formation of aldehydes or other monoterpene alcohols such as linalool or citronellol was not observed. Fur-

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[8] CHAYET, L., PONT-LEZICA, R., GEORGE-NASCIMENTO, C. and CORI, O. (1973) *Phytochemistry* **12**, 95.
[9] DUNPHY, P. J. as cited by CHAYET *et al.*[8]

thermore, a direct isomerization of GerP was also observed, thus eliminating the possibility of an aldehyde intermediate; in assays containing none of the cofactors known to be necessary for geraniol kinase activity [6,7] ten times as much NerP was formed as free nerol (Table 2). Additionally, only 3% of the substrate was recovered as the free alcohol, thus indicating minimal phosphatase activity in the enzyme preparation. Some GerP was non-enzymatically isomerized to NerP in the boiled control although no free alcohols were detected, again illustrated that a direct *trans-cis* isomerization of GerP to NerP had occurred. Since the rate of isomerization of GerP was less than that of geraniol, additional experiments with GerPP are needed to determine whether the presence of the second phosphate group causes a further decrease in isomerase activity.

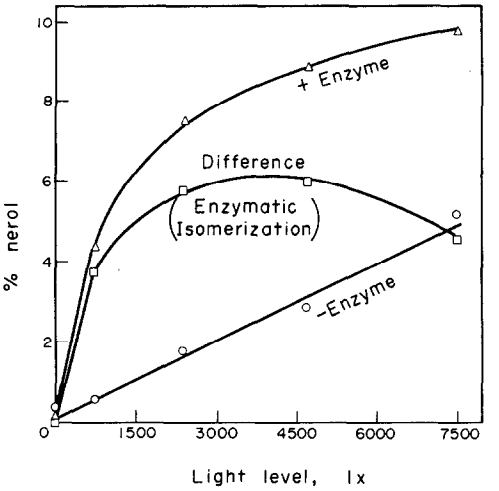

FIG. 1. EFFECT OF LIGHT ON GERANIOL ISOMERIZATION BY PEPPERMINT ENZYME.
The reaction mixture contained: 8·8 μmol K phosphate buffer; 20 nmol FAD; 22 nmol dithiothreitol; 300 nmol geraniol; and 25 μl enzyme in a total vol. of 0·175 ml. The pH was 7·5. Reaction time 60 min; temp. 35°, N_2 atmosphere.

Posthuma and Berends[10] first demonstrated the importance of flavins in catalyzing non-enzymatic photoisomerization reactions. Similarly, both all-*trans*-retinol and 13-*cis*-retinol were shown to undergo a flavin-sensitized photoisomerization,[11] whereas the dark isomerization of all-*trans*-retinal, but not of all-*trans*-retinol, was catalyzed by dihydroflavin and other nucleophiles.[12] The present results demonstrate that geraniol isomerization was also photoinducible (Fig. 1); in the absence of light, isomerization occurred if the flavin coenzyme was partially reduced prior to incubation (Table 5). Extensive reduction of the flavin, however, greatly inhibited isomerization, thus suggesting that the flavin semiquinone was involved, either directly or indirectly, in geraniol isomerization.

In contrast to previous reports, a thiol or sulfide was also essential in order to demonstrate both the non-enzymatic and enzymatic isomerization of geraniol and GerP. The thiol was not acting simply as a reducing agent since the thiol-compound could not be replaced by sodium ascorbate, NADH, EDTA (a photoreductant) or partially reduced flavin.

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[11] GORDON-WALKER, A. and RADDA, G. K. (1967) *Nature* **215,** 1483.
[12] FUTTERMAN, S. and ROLLINS, M. H. (1973) *J. Biol. Chem.* **248,** 7773.

This thiol could function to stabilize an active flavin state,[13] either by direct interaction with the flavin, or by freeing a sulfhydryl group(s) on the isomerase which would in turn interact with the flavin. Although thiol-compounds were essential for geraniol isomerization, preincubation of the enzyme with sulfhydryl blocking agents followed by removal of excess reagent prior to the assay did not inhibit the reaction (Table 3). This may indicate that an active sulfhydryl group of the isomerase is masked and only reacts with blocking agents under the conditions of assay.[14,15] Indeed, if NEM was added during assays isomerization was virtually eliminated.

The reversible *trans-cis* isomerization reaction reported here may well function as a branch point in the isoprenoid pathway, allowing a diversion of GerPP into the *p*-menthane series of cyclohexanoid monoterpenes. Thus the isomerase, in conjunction with NerPP cyclase, could regulate the biosynthesis of cyclic monoterpenes and higher terpenes from their common precursor. Only C_{10}-compounds have been tested as isomerase substrates and further studies will be necessary to determine whether a similar isomerase is involved in the biosynthesis of *cis*-bonds in other isoprenoid compounds such as gossypol precursors[16,17] and absicic acid.[18]

EXPERIMENTAL

Plant material. Peppermint plants (*Mentha piperita* L. cv. Black Mitcham) were propagated vegetatively from a clone used previously.[19] Carrot (*Daucus carota* L. cv. Imperator) plants were grown from seed. All plants were grown with 25° day and 8° night on a 24-hr cycle, with a 16-hr day at 11000 lx provided by equal numbers of Sylvania Gro-Lux and wide spectrum Gro-Lux fluorescent lamps.

Cell-free extracts were prepared from vegetative shoot tips (growing tip and top 2 leaf pairs) of peppermint or 38-day-old carrot tops (petioles and leaf blades plus small amounts of stem) by freezing the fr. tissue, 20 g of peppermint or 40 g of carrot, in liquid N_2 and grinding in a mortar. The resultant powder, still frozen, was slurried with 20 g acid washed Polyclar AT (insol. PVP)[20] previously flushed with N_2 to remove O_2 and subsequently soaked with 120 ml of extracting medium. This medium contained 0·1 M K phosphate buffer, pH 7; 5 mM Na_2EDTA; 10 mM K metabisulfite ($K_2S_2O_5$) and 2 mM β-mercaptoethanol. The thawed slurry was squeezed through 200 mesh bolting silk and passed through a 200 ml Bio-Gel P-10 (100–200 mesh) column equilibrated with 0·1 M K phosphate buffer, pH 7, containing 1 mM $K_2S_2O_5$. The column eluate (70 ml) was centrifuged for 15 min at 30000 *g* (max. r.c.f.); the supernatant was brought to 25% saturation with solid $(NH_4)_2SO_4$ and centrifuged as before. The supernatant was then brought to 65% saturation and centrifuged; the ppt. was dissolved in 25 mM K phosphate buffer, pH 7·5, and desalted on a Bio-Gel P-10 column. The peppermint extract was further purified by DEAE-cellulose chromatography, using a linear gradient of 25–90 mM K phosphate buffer, pH 7·5, followed by concn of the active fraction (eluted with 50 mM buffer) in an Amicon pressure dialyzer fitted with a UM-10 (10000 MW exclusion limit) membrane and was desalted on a Bio-Gel P-10 column. When GerP served as the substrate the desalted Amicon concentrate was treated with Ca phosphate gel[21] equilibrated with 50 mM K phosphate buffer, pH 7·5; after stirring at 4° for 5 min the slurry was centrifuged and the pellet discarded. This gel-treated enzyme preparation showed a small amount of residual phosphatase activity.

Substrates. Geraniol from the Aldrich Chemical Co., Milwaukee, Wisc., was purified by TLC.[22] It was spotted on a silica gel G TLC plate impregnated with $AgNO_3$ (5 g $AgNO_3$, 55 g silica gel G, 100 ml H_2O), and developed with Skellysolve-B-EtOAc (67:33). Geraniol was eluted with Et_2O and contained 0·05% nerol. Nerol (92·1%) was a gift from Dr. E. Klein, Dragoco, Holzminden, Germany. Geraniol-[G-^{14}C] was prepared biosynthetically by allowing *Pelargonium graveolens* L'Her (rose geranium) cuttings to fix $^{14}CO_2$; the geraniol-[G-^{14}C] was isolated

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by TLC from a Skellysolve-B extract of the leaves that had been treated with activated charcoal and then filtered. Purification of the labeled geraniol by $AgNO_3$-TLC resulted in geraniol-[G-^{14}C], 2·2 μCi/μmol, containing less than 0·1% nerol. Geranyl-[G-^{14}C] phosphate was synthesized chemically[23] using this purified geraniol-[G-^{14}C] diluted with the unlabeled geraniol to give a sp. act. of 0·2 μCi/μmol. The GerP was then precipitated from the mixture with LiCl and purified by PC using PrOH-NH_4OH-H_2O (3:1:1)[24] as a solvent. With this solvent the monophosphate and pyrophosphate were clearly separated, the monophosphate having the higher R_f.

Assay procedures were carried out anaerobically in 3 ml conical tubes capped with rubber stoppers. Assays in the light utilized Gro-Lux wide spectrum fluorescent lamps and Wratten 96 neutral density and 2E filters; the 96 filter had a density of 1·00 and 10% transmittance and the 2E filter absorbs wavelengths shorter than 400 nm. The intensity of the filtered light was 730 lx. After incubation, samples were chilled in ice and extracted 3× with 1 ml portions of Et_2O. The pooled extracts were concentrated under a stream of N_2 and analyzed by GLC on an FID instrument using dual 6·1 m × 3·2 mm stainless steel columns packed with 1% phenyldiethanolamine succinate and 1·5% sucrose acetate isobutyrate (PDEAS–SAIB) on 100–200 mesh Chromosorb G, and programmed from 140–150°. Under these conditions linalool, citronellol, neral, nerol and geraniol peaks were clearly distinguishable although adjacent peaks partially overlapped. Peak areas were measured with a Disc Integrator and quantitative data were obtained for geraniol and nerol by calibration with authentic standards. When geranyl-[G-^{14}C] phosphate served as substrate, the aq. phase was chromatographed on paper as above, the chromatogram was scanned in a radiochromatogram scanner, the GerP/NerP peak was treated with calf intestinal mucosa alkaline phosphatase and the Et_2O extract of this soln was analyzed by GLC as described above. The lower limit of detection under the conditions employed was 0·025 nmol. Reaction products were confirmed by GC–MS[25] utilizing the column described above; geraniol and nerol were identified by comparison with spectra obtained with standards and by comparison with published spectra.[26] Utilizing geraniol-[G-^{14}C] as a substrate, nerol formation from geraniol was also confirmed by GC-RC analysis on the same column using a thermal conductivity detector coupled to radioactivity detector.

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