AN ABSTRACT OF THE THESIS OF

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Title: TRANS-CIS ISOMERIZATION OF GERANIOL AND GERANYL PHOSPHATE BY CELL-FREE ENZYMES FROM HIGHER PLANTS

Abstract approved: Redacted for privacy

Dr. W. David Loomis

Neryl pyrophosphate, rather than geranyl pyrophosphate, is believed to be the immediate biological precursor of the cyclohexanoid monoterpenes because the cis-2, 3-double bond readily permits cyclization. Biosynthesis of neryl pyrophosphate could occur by either of two principal mechanisms: direct cis condensation of dimethylallyl pyrophosphate with isopentenyl pyrophosphate or trans-cis isomerization of geranyl pyrophosphate. Flavin-dependent enzymes that catalyze the isomerization of geraniol and geranyl phosphate to nerol and neryl phosphate, respectively, have now been isolated from peppermint and pea leaves, and carrot tops. Isomerization also occurs non-enzymatically but is greatly accelerated in the presence of the enzyme extracts.

Various factors are required in order to demonstrate

enzymatic isomerization. These factors are: (1) a flavin (FAD or FMN); (2) a thiol or sulfide (glutathione, dithiothreitol, β-mercaptoethanol, or Na_2S); and (3) light (400-500 nm wavelength). If the flavin is partially reduced chemically, light is not required. Non-enzymatic isomerization also occurs if sulfite is the sulfur compound added. When a cell-free peppermint extract is utilized, enzymatic isomerization in the light is a function of: enzyme, substrate, FAD, and dithiothreitol concentrations; temperature (at 35° the rate is twice the rate at 25°); and pH (7.5 optimum). Isomerization is also increased by anaerobic conditions. Complete reduction of the flavin inhibits isomerization. Inhibition is also observed if N-ethylmaleimide is present during the reaction, but pretreatment of the enzyme with NEM or p-hydroxymercuribenzoate, which is removed prior to incubation with substrate, causes no inhibition.

The various substrates are isomerized at different rates. Nerol appears to be isomerized twice as fast as geraniol, and the apparent equilibrium for the reaction is 67% geraniol and 33% nerol. Geranyl phosphate is isomerized at about one-half the rate of geraniol. The results are discussed in relation to postulated *in vivo* isomerization of geranyl pyrophosphate to neryl pyrophosphate.

Trans-Cis Isomerization of Geraniol and Geranyl Phosphate by Cell-Free Enzymes from Higher Plants

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

TRANS-CIS ISOMERIZATION OF GERANIOL AND GERANYL PHOSPHATE BY CELL-FREE ENZYMES FROM HIGHER PLANTS

INTRODUCTION

Plants produce a wide variety of terpenoid compounds. It was recognized by Wallach (73) in 1887 that these compounds appeared to be formed from isoprene (C_5) units and today it is assumed that all of these terpenoid compounds arise via MVA[1/], though in many cases direct evidence is still lacking (Figure 1).

Plant monoterpenes (C_{10}) are believed to be derived from the phosphorylated monoterpene GerPP, since it has been shown to be an intermediate in the biosynthesis of FPP and squalene from MVA in yeast (39, 40), animal (21, 11) and plant (7, 51, 54) systems. Indeed, the cyclohexanoid monoterpene limonene was formed by cell-free extracts from orange flavedo when GerPP was utilized as

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[1/] Abbreviations used: MVA, mevalonic acid; MVAP, phosphomevalonic acid; MVAPP, pyrophosphomevalonic acid; IPP, isopentenyl pyrophosphate; DMAPP, dimethylalyl pyrophosphate; GerP, geranyl phosphate; GerPP, geranyl pyrophosphate; NerP, neryl phosphate; NerPP, neryl pyrophosphate; LinPP, linaloyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; NADH, nicotinamide adenine dinucleotide (reduced); DTT, dithiothreitol; GSH, glutathione (reduced); GSSG, gluthathione (oxidized); CoA, coenzyme A; NEM, N-ethylmaleimide; PHMB, p-hydroxymercuribenzoate; EDTA, ethylenediamine-tetraacetate; DEAE, diethylaminoethyl cellulose; Polyclar AT, isoluble polyvinylpyrrolidone.

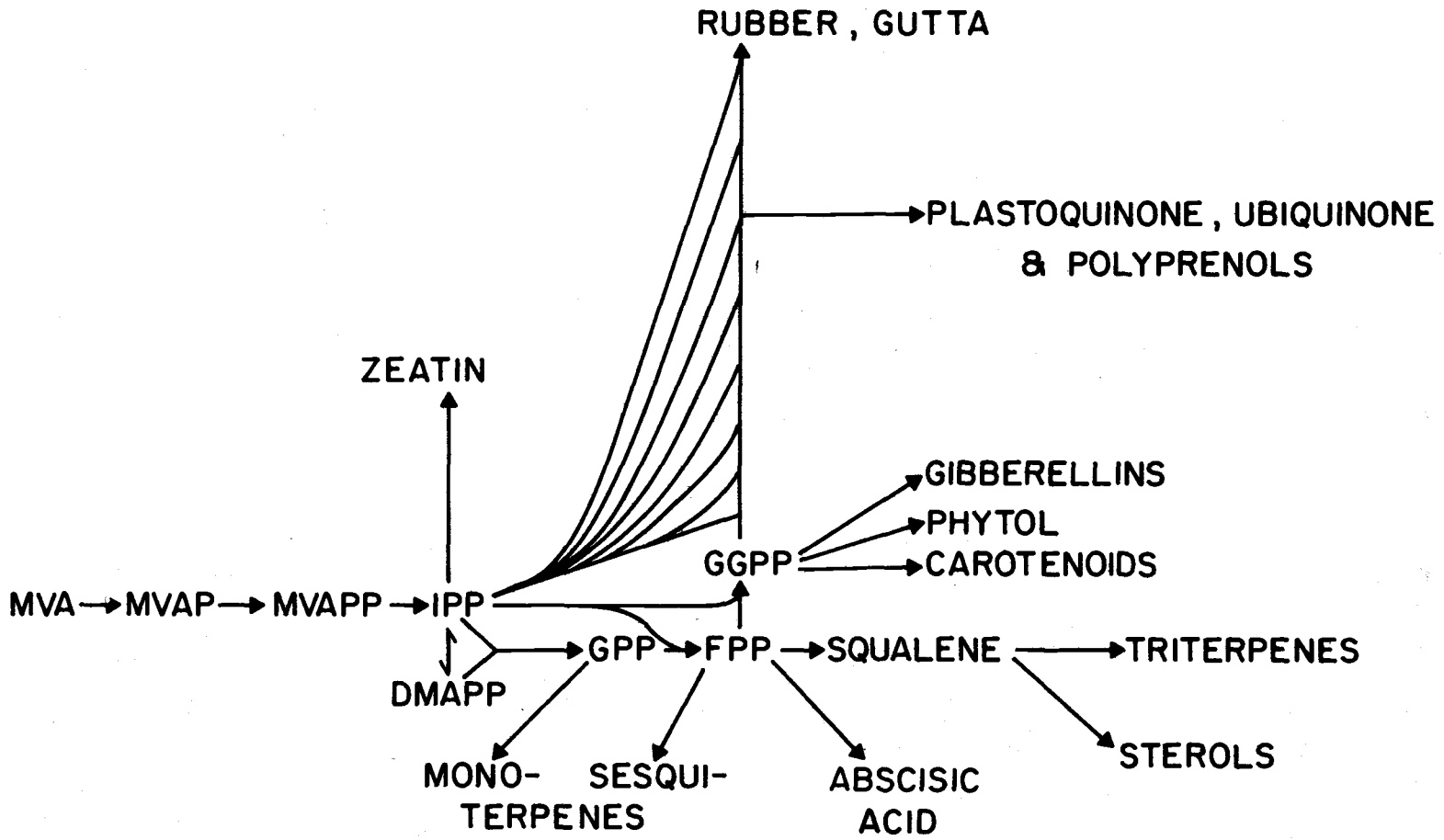

Figure 1. Isoprenoid compounds formed from mevalonic aid in plants: known and postulated pathways.

substrate (20), but incorporation was only 3% in 2 hr. Similarly, incorporation of MVA into plant monoterpenes, including geraniol, was, until recently, low (36) and evidence supporting the postulation that these monoterpenes were derived from MVA was inconclusive. In 1969, however, Francis and O'Connell (16) demonstrated that MVA could be incorporated into rose petal monoterpenes in high yield. They reported a 10.8% incorporation of label from RS-MVA-2-^{14}C into the β-D-glucosides of the acyclic monoterpenes, geraniol, nerol, and citronellol (Figure 2) within one hour. Thus, although incorporation of GerPP into plant monoterpenes has been difficult to demonstrate directly, it appears that GerPP is an intermediate in their biosynthesis. Nevertheless, the detailed biochemical steps leading to the formation of cyclic monoterpenes (Figure 2) and even of geraniol and nerol remain unknown.

Neryl pyrophosphate, rather than geranyl pyrophosphate, is believed to be the immediate biological precursor of the cyclohexanoid monoterpenes because the cis-2, 3-double bond favors cyclization (47, 70, 36) (Figure 3). Linaloyl pyrophosphate has, however, also been considered a possible precursor of these monoterpenes (36) (Figure 3). Zeitschel (77) demonstrated that acid-catalyzed cyclization occurred more readily with nerol than with geraniol and concluded from this fact that nerol had the cis- and geraniol the trans-configuration; linalool cyclized at an intermediate rate. Similarly,

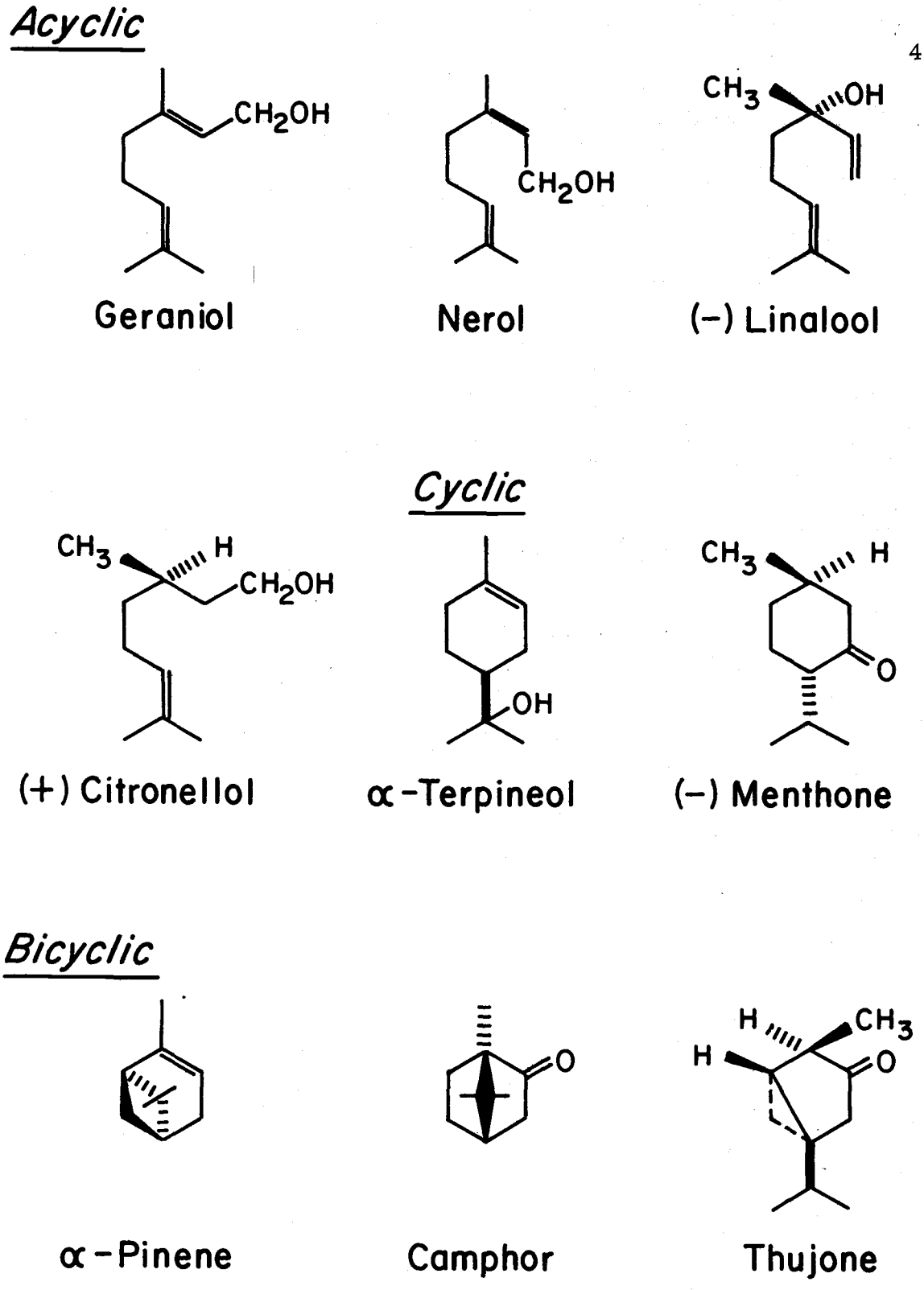

Figure 2. Structures of selected monoterpenes.

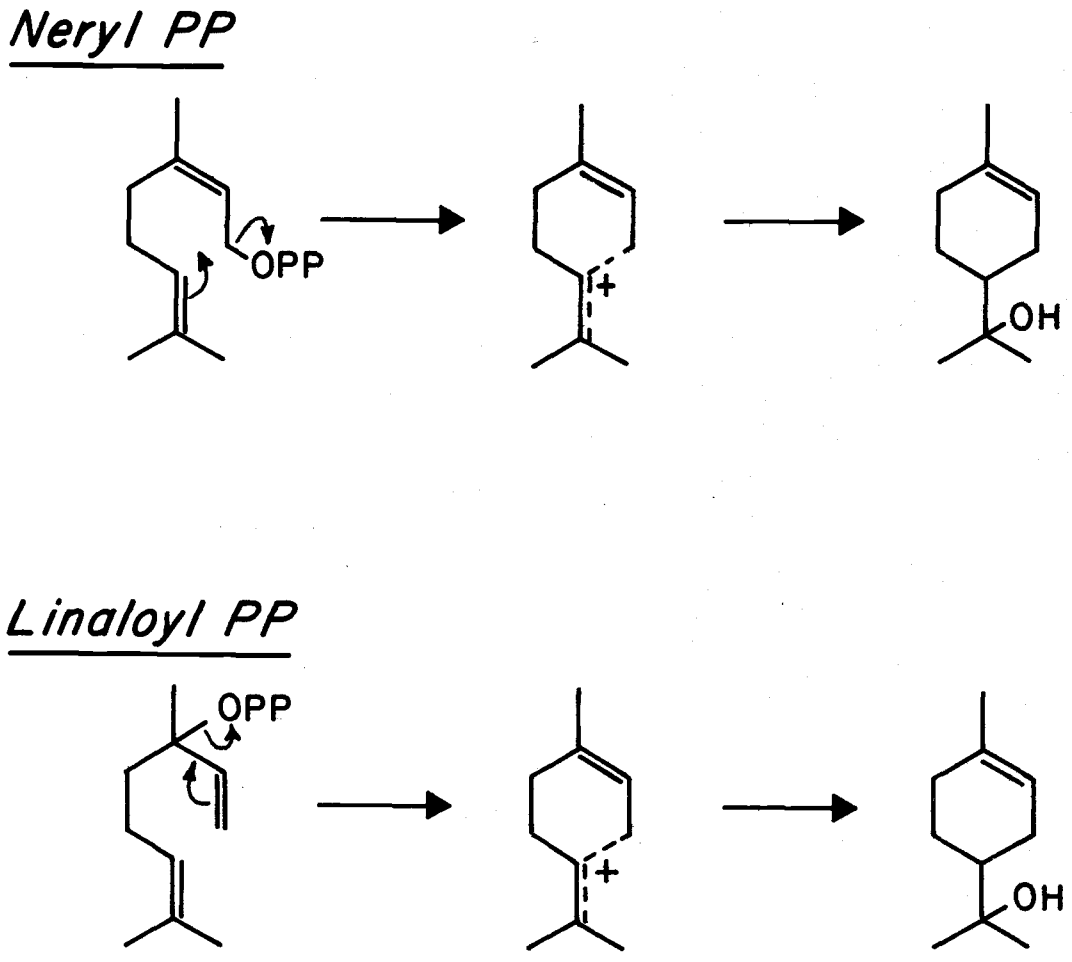

Figure 3. Proposed mechanisms for the cyclization of neryl and linaloyl pyrophosphates.

formation of the cyclohexanoid monoterpene α-terpineol has been demonstrated chemically by acid hydrolysis of NerPP, LinPP, and GerPP: four-times more α-terpineol was formed from NerPP than from LinPP and almost none was formed from GerPP (60, 71). Both steric and chemical evidence, therefore, suggests that NerPP is the more likely immediate precursor of the cyclic monoterpenes. In a biochemical study, Cori (9) reported that the bicyclic monoterpenes α- and β-pinene were formed in greater yield from NerPP than from GerPP in the presence of soluble enzymes from Pinus radiata.

If NerPP is the immediate biological precursor of the cyclic monoterpenes, its biosynthesis from MVA could occur by either of two principal mechanisms: direct cis-condensation of IPP with DMAPP, similar to rubber biosynthesis (2), or trans-cis isomerization of GerPP. Figure 4 illustrates the second of these alternate routes. By utilizing 2-^{14}C-(4R)-4-^{3}H-MVA and 2-^{14}C-(4S)-4-^{3}H-MVA as substrates, these pathways can be distinguished: direct cis-condensation would lead to the loss of the (4R)-^{3}H while trans-condensation would result in retention of the (4R)-^{3}H and a loss of the (4S)-^{3}H (Figure 5). Francis, Banthorpe and Le Patourel (15) fed these substrates to Rosa damascena (rose) flowers and obtained results which suggested that nerol was formed via geraniol or a geraniol derivative, as suggested in Figure 4. Similarly, retention of the (4R)-^{3}H was observed for α-pinene biosynthesis by Pinus

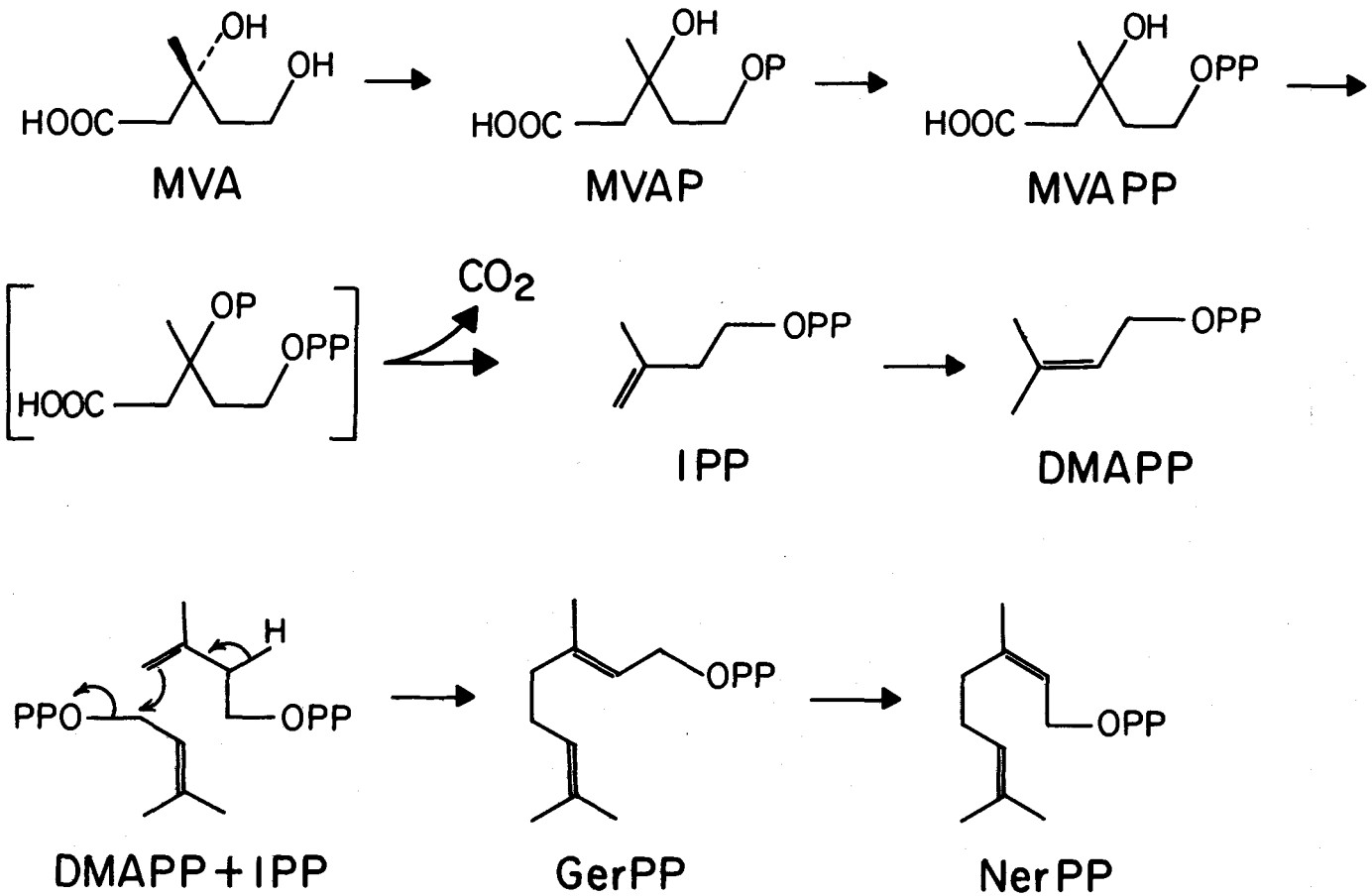

Figure 4. Formation of geranyl pyrophosphate from mevalonic acid and postulated isomerization to neryl pyrophosphate.

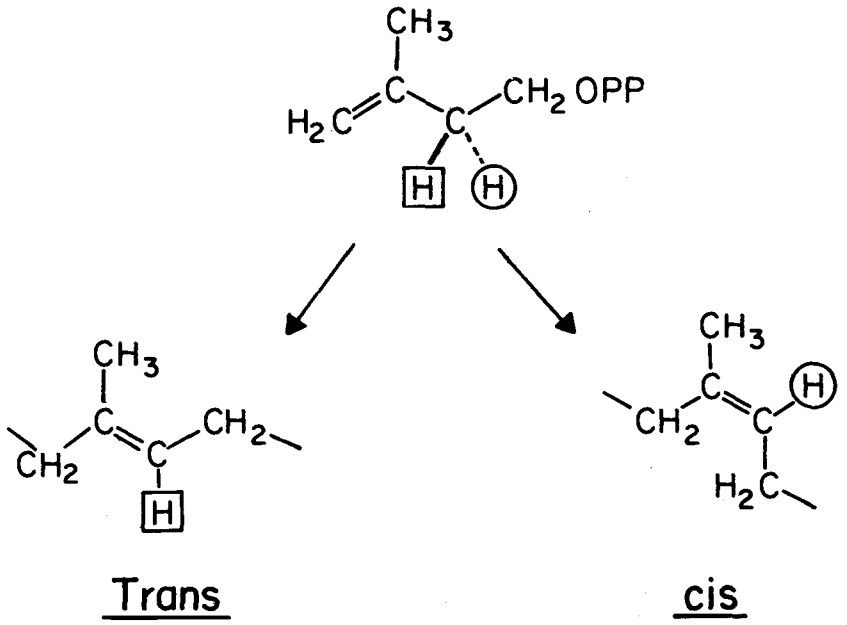

Figure 5. Stereospecific loss of hydrogen during cis- and trans-condensations.

attenuata (35), thus suggesting that both geraniol and nerol (or their pyrophosphates) were also intermediates in its biosynthesis. These results suggest that in plants such as Pinus attenuata the branch point for the formation of cyclic monoterpenes is the trans-cis isomerization of GerPP to NerPP (Figure 4).

Isomerization of GerPP itself need not occur, however. The eventual formation of cyclic monoterpenes could, for example, be initiated by the action of a phosphatase which would withdraw GerPP from the main terpene pathway (Figure 1). Then the isomerization step would follow. Indeed, small amounts of nerol are formed by acid-catalyzed isomerization of geraniol (65, 71); nerol is not the major product, however, and the acid concentrations utilized were about 0.1 M. Although this acid concentration would not be found in vivo, an enzyme could catalyze a similar reaction under physiological conditions. After isomerization, NerPP could be formed by a kinase such as geraniol kinase (41) which has been shown to phosphorylate nerol (37). Although NerPP could be formed via the phosphatase-isomerase-kinase pathway, it would, of course, be energetically more desirable if GerPP isomerization occurred directly.

Trans-cis as well as cis-trans isomerization has been reported to occur, under physiological conditions, with and without double bond migration. Double bond migration occurs when aconitate isomerase (30) and linoleate Δ^{12}-cis, Δ^{11}-trans-isomerase (28)

catalyze the interconversion of the cis and trans forms of their respective substrates. An α, β-β, γ-isomerase (10) isomerizes trans-α, β-hexadecenoyl-CoA to both cis- and trans-β, γ-hexadecenoate; when cis-β, γ-hexadecenoyl-CoA was the substrate, 40% was recovered as the α, β-isomer and 5% was recovered as trans-β, γ-hexadecenoyl-CoA. The net effect of the latter reaction sequence was the isomerization of the cis-isomer to the trans-isomer without double bond migration.

Other cis-trans isomerizations proceed without detectable double bond migration. For example, maleylacetoacetate is isomerized to the trans-isomer by an enzyme (14) which is activated by sulfhydryl reagents. Similarly, maleate isomerase (61, 67) is activated by sulfhydryl reagents. Both of these isomerizations can occur non-enzymatically in the presence of a sulfhydryl reagent but upon the addition of enzyme, isomerization is greatly stimulated. None of these enzymes was capable, however, of catalyzing trans-cis isomerization without double bond migration.

An extensively investigated trans-cis isomerization, which occurs without detectable double bond migration, is the isomerization of all-trans-retinal to form the 11-cis-retinal chromophore of the visual pigment rhodopsin. Retinene isomerase, which catalyzes the isomerization of this isoprenoid compound was first described by Hubbard (27). This isomerase was shown to catalyze both dark and light isomerization and the respective equilibrium mixtures contained

5% and 32% of the cis-isomer. Isomerization also took place in the absence of enzyme, but at a slower rate.

Clearly, trans-cis isomerization, which appears to be necessary for the formation of cyclic monoterpenes, occurs in biological systems. An investigation was undertaken to determine whether an isomerase capable of utilizing geraniol or geranyl phosphates as substrates was present in plants which are known to contain large amounts of cyclic monoterpenes.

MATERIALS AND METHODS

Sources of Materials and Chemicals

Carrot seeds (Imperator variety of Daucus carota L.) were obtained from The Chas. H. Lilly Co., Seattle, Wash. Pea seeds (Blue Bantam variety of Pisum sativum L.) were from the W. Atlee Burpee Co., Riverside, Calif. Peppermint (Black Mitcham variety of Mentha piperita L.) was propagated vegetatively from a single clone obtained from Dr. C. E. Horner of Oregon State University (3).

Perlite was obtained from the Supreme Perlite Co., North Portland, Oregon.

Gro-Lux lamps were from Sylvania Electric Products, Inc., Danvers, Mass. Wratten filters were obtained from the Eastman Kodak Co., Rochester, N. Y.

Bio-Gel P-10 (50-100 mesh), Cellex-D (DEAE-cellulose) and calcium phosphate gel were obtained from Bio-Rad Laboratories, Richmond, Calif.

Polyclar AT (insoluble polyvinylpyrrolidone) was obtained from GAF Corp., New York, N. Y.

The pressure dialyzer was from the Amicon Corp., Lexington, Mass.

Disodium ethylenedinitrilotetraacetate dihydrate

($Na_2EDTA \cdot 2H_2O$), reagent grade, was obtained from J. T. Baker Co., Phillipsburg, N. J.

Triton X-100 was from Rohm and Haas, Philadelphia, Pa.

Silica gel G was obtained from E. Merck A. G., Darmstadt, Germany.

Ethyl ether (anhydrous), analytical reagent, was obtained from Mallinckrodt Chem. Works, St. Louis, Mo.

Skellysolve-B (essentially n-hexane, with a boiling range of 60-68°) was from the Skelly Oil Co.

Dithiothreitol, "A grade", was from Calbiochem, Los Angeles, Calif. Glutathione (reduced) was obtained from P-L Biochemicals, Inc., Milwaukee, Wis. β-Mercaptoethanol was from Eastman Organic Chemicals, Rochester, N. Y. Oxidized glutathione was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

N-Ethylmaleimide was from the J. T. Baker Chemical Co., Phillipsburg, N. J. p-Hydroxymercuribenzoate, sodium salt, was obtained from the Sigma Chemical Co., St. Louis, Mo.

Flavin mononucleotide and flavin adenine dinucleotide were obtained from the Sigma Chemical Co., St. Louis, Mo.

Platinum on asbestos, 5% catalyst, was from Matheson Coleman and Bell, Div. of Matheson Co., Inc.

Calf intestinal mucosa alkaline phosphatase (3. 1. 3. 1) was obtained from P-L. Biochemicals, Inc., Milwaukee, Wis.

p-Nitrophenyl phosphate, disodium· $6H_2O$, was from Calbiochem, San Diego, Calif.

Protamine sulfate was obtained from Elanco Products Co., Inianapolis, Indiana.

Other reagents utilized were of the highest purity available, and glass distilled water was used in all preparations.

Substrates

Geraniol-^{14}C

Geraniol-^{14}C was prepared by biosynthesis from $^{14}CO_2$ utilizing Pelargonium graveolens shoot tips (3, 75). At the end of the biosynthetic period, the shoots were frozen in liquid N_2, ground in a mortar, and extracted with Skellysolve-B. The extract was decolorized with a small amount of Norit-A activated charcoal. The filtered extract was concentrated under a stream of N_2, spotted on a silica gel G thin layer plate impregnated with $AgNO_3$ (5 g $AgNO_3$, 55 g silica gel G and 100 ml H_2O), and developed with Skellysolve-B : ethyl acetate (67:33, v/v) (75). The geraniol area was scraped from the plate, and eluted from the gel with ether. It was further purified on a silica gel G TLC plate using Skellysolve-B : ethyl acetate (84:16, v/v) as the developing solvent. It was again eluted from the gel with peroxide-free ether and stored at -20°. This purified

geraniol-^{14}C contained less than 0.1% nerol-^{14}C.

Geranyl-^{14}C Phosphate

Geranyl-^{14}C phosphate was synthesized chemically from geraniol-^{14}C and di-triethylammonium phosphate (56). After extraction with ether the aqueous solution was passed through a DEAE-cellulose column, the effluent was concentrated, and LiCl was added to precipitate GerP. No significant amounts of GerPP were detected in the redissolved precipitate after paper chromatography and radioscanning.

Unlabelled Geraniol and Nerol

Geraniol was from the Aldrich Chemical Co., Inc., Milwaukee, Wis.; it contained 0.3% nerol. When higher purity geraniol was desired, purification on TLC as described above resulted in a geraniol sample containing 0.05% nerol. Nerol was a gift from Dr. E. Klein of Dragoco, Holzminden, Germany; it contained 7.9% geraniol.

Unlabelled Methyl Esters of Geranic Acid and cis-Geranic Acid

Unlabelled methyl esters of geranic acid and cis-geranic acid were separated by TLC as described above. After purification, the

trans isomer contained 0.3% of the cis isomer while the cis isomer contained less than 0.1% of the trans isomer.

MVA-2-^{14}C

RS-Mevalonic acid-2-^{14}C (DBED salt) was obtained from New England Nuclear Corp., Boston, Mass. It was used as obtained from the manufacturer, without further purification; the DBED salt was converted to the free acid, and DBED was removed with ether.

Growth of Plant Materials

Plants were grown in perlite, and nutrients were supplied daily by quarter-strength Hoagland's Number 2 solution (26). Constant growing conditions were maintained by growing the plants in a growth chamber maintained at a 25° day temperature and an 8° night temperature on a 24-hour cycle, with a 16 hour day of about 1000 ft.-c. light intensity provided by equal numbers of Sylvania Gro-Lux and Wide Spectrum Gro-Lux lamps.

Preparation of Cell-Free Enzymes and Assay Conditions

Cell-free extracts were prepared from vegetative shoot tips (growing tips and top 3 leaf pairs) of peppermint, 38 day old carrot tops (petioles and leaf blades plus a small amount of stem tissue), and young leaves from 31 day old pea plants.

All extracts were prepared with washed Polyclar AT in order to remove phenolic compounds present in the plant material (38). The polyclar AT was first flushed with a stream of N_2 to remove adsorbed oxygen; this results in better wetting of the Polyclar, less oxidation of phenolic compounds, and more effective removal of these compounds from the protein extract. The flushed Polyclar AT was suspended in sufficient buffer to give a 1:6 ratio of Polyclar (g) to buffer (ml). The ratio of Polyclar (g) to plant material (g fresh weight) was 1:1 for peppermint and 1:2 for carrot and pea. The suspending buffer contained 0.1 M potassium phosphate buffer, pH 7.0, and was also 0.01 M in potassium metabisulfite ($K_2S_2O_5$); other additions were made in individual experiments.

Cell-free extracts were prepared by freezing the plant material in liquid nitrogen, grinding it in a mortar, mixing with Polyclar suspension, squeezing through bolting silk, passing it through a Bio-Gel P-10 column and certrifuging. A 25-65% $(NH_4)_2SO_4$ precipitate was prepared from the supernatant and the precipitate was dissolved and desalted on a Bio-Gel P-10 column. The peppermint cell-free enzyme extract was further purified.

Cell-free extract was applied to a DEAE-cellulose column. Elution with 25 mM potassium phosphate buffer, pH 7.5 was followed by a 25 mM to 90 mM potassium phosphate buffer, pH 7.5, linear gradient. Column fractions containing most of the isomerase

activity were combined and concentrated in a 10 ml Amicon pressure dialyzer fitted with a UM-10 membrane filter (approximately 28Å pore size). After concentration to a volume of 0.7 ml, the solution was removed, and the dialyzer was washed with 0.3 ml of buffer, giving a combined volume of 1.0 ml. This concentrated solution was passed through a Bio-Gel P-10 column equilibrated with 25 mM potassium phosphate buffer, pH 7.5. This enzyme preparation was used when geraniol or nerol served as substrate; when GerP served as substrate, phosphatase activity present in this enzyme preparation was removed by treatment with calcium phosphate gel equilibrated with 50 mM potassium phosphate buffer, pH 7.5. The gel-treated preparation showed a small amount of residual phosphatase activity.

Except where noted, the assay mixture included the following, in a total volume of 0.175 ml: 25 mM potassium phosphate buffer, pH 7.5; 0.114 mM FAD; 0.125 mM DTT or 0.25 mM GSH; 300 nanomoles unlabelled geraniol or nerol as substrate; and enzyme extract. All components except the substrate were mixed in 3 ml conical tubes; the tubes were then sealed with rubber serum caps and flushed 30 min with prepurified N_2 gas. The substrate was then injected, and in the case of geraniol and nerol the solvent, ether, was evaporated. If the flavin was partially reduced (by H_2 and Pt on asbestos) before addition it was injected at this point. The sealed tubes were either placed in a 35° water bath (dark) or in a light-filter

chamber (Figure 6), which was in turn placed in a 35° growth chamber. Wratten 2E (400 nm cut-off) and 96 neutral density (density 1.00, 10% transmittance) filters were inserted in the top of the filter chamber. Initial studies at higher light levels made use of other neutral density filters -- density 0.50 (32% transmittance) and density 0.20 (63% transmittance). Use of the neutral density 1.00 filter resulted in a light level at which non-enzymatic relative to enzymatic isomerization is minimized. The intensity of the filtered light was approximately 70 ft.-c. Incubation time was 60 min. At the end of the incubation, the samples were cooled, uncapped, and extracted 3 times with 1 ml portions of ether. The combined ether extracts were evaporated to about 30 µl prior to analysis by GLC. When GerP was the substrate, samples were extracted as above, and the aqueous phase was analyzed by paper chromatography followed by radio-scanning. The part of the paper containing both GerP and NerP (Rf. 0.75) was cut into small pieces, placed in a 15 ml conical tube, and then incubated with calf intestinal mucosa alkaline phosphatase for 2 hours at 25°. The free terpene alcohols were then extracted with ether and concentrated.

During some assays a central reservoir was placed inside the incubation tube (Figure 7). When used as an oxygen trap, the reservior was filled with a mixture of 0.05 mM methylene blue and 50 mM sodium ascorbate in 0.1 M potassium phosphate buffer, pH 7.0 (52).

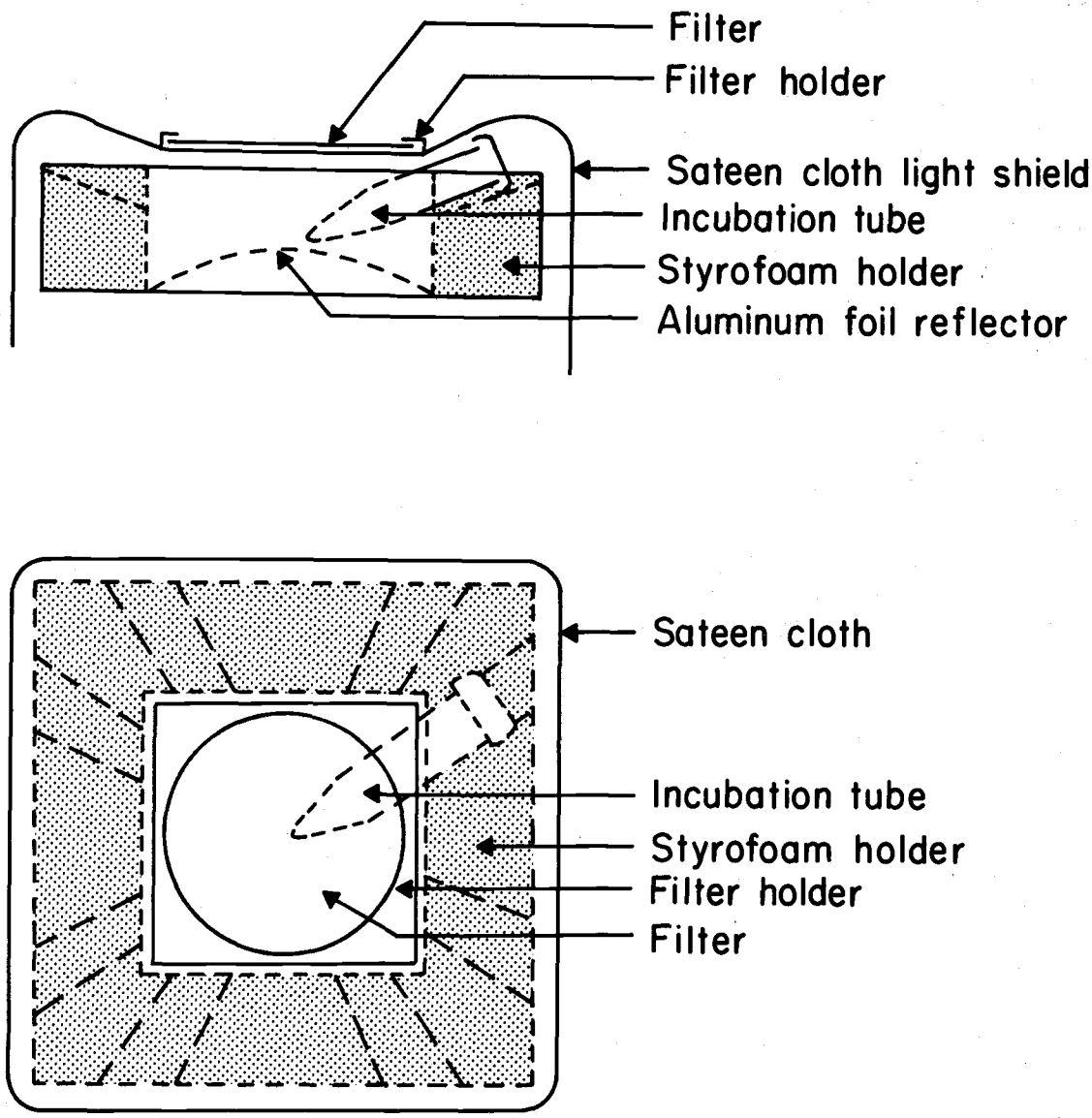

Figure 6. Side and top views of the light-filter chamber.

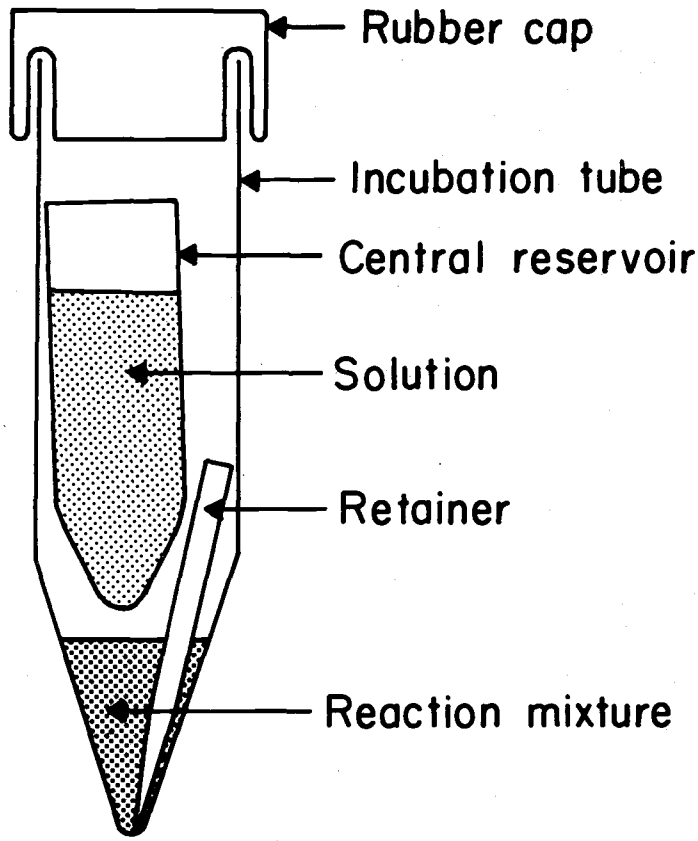

Figure 7. Position of the central reservoir in the incubation tube.

When used as a source of SO_2, the reservoir was filled with potassium phosphate buffer, pH 7.0, containing sodium dithionite.

Protein Determination

TCA Turbidimetric Method

The trichloroacetic acid (TCA) method of protein determination was used during initial purification steps in order to minimize the contribution of plant phenolic compounds (which absorb strongly in the UV). Four ml of 5% TCA were added to 1 ml of protein solution and mixed. After 5 min., the 600 nm absorbance of samples was determined with a Beckman DB spectrophotometer (32). Protein concentrations were determined by comparison with a standard curve prepared with bovine serum albumin.

UV Absorbance Method

Absorbance of protein solutions was measured at 280 and 260 nm. Where quantitative estimates were desired, protein was determined by the formula of Kalckar (34); values obtained for DEAE cellulose column fractions were compared with those obtained using the TCA turbidimetric method. Values were not compared in subsequent purification steps.

Inhibitor Studies

The effect of sulfydryl inhibitors was tested in two ways: (1) direct addition to the incubation medium or (2) preincubation with the enzyme. In the latter method enzyme extracts to be used for inhibitor studies were incubated with sulfhydryl inhibitors--NEM (4 mM) or PHMB (0.01 mM)---for 30 min. at 25°. Excess inhibitor was removed by means of a Bio-Gel P-10 column equilibrated with 25 mM potassium phosphate buffer, pH 7.5. Controls, to which no inhibitor was added, were similarly treated.

Phosphatase Assays

Samples were assayed for the presence of alkaline phosphatase by the colorimetric method involving the release of p-nitrophenol from p-nitrophenylphosphate (18).

Hydrolysis of Monoterpene Phosphates by Phosphatases

Two methods were used for assaying the hydrolysis of monoterpene phosphates. The first involved mixing the aqueous sample with calf intestinal mucosa alkaline phosphatase dissolved in Tris-hydrochloride buffer, pH 8.5; the final solution was also 0.01 M in $MgCl_2$ (33). Incubation was carried out in a 15 ml stoppered conical tube for 2 hr. at 25°. The solution was then extracted with

Skellysolve-B, concentrated, and analyzed by GLC. The second method utilized paper chromatography followed by radioscanning; the activity peak was cut into small pieces and placed in a 15 ml conical tube, the solution of calf intestinal mucosa alkaline phosphatase and $MgCl_2$ was added, the tube was stoppered, and incubated for 2 hr. at 25°. The solution was extracted with diethyl ether, concentrated, and analyzed by GLC. This second method was employed when GerP served as substrate.

Paper Chromatography

Aqueous solutions of the samples to be analyzed were spotted on Whatman no. 1 paper and developed with a solvent system of n-propanol:ammonia:water (3:1:1) by descending chromatography (4).

Radiochromatogram Scanning

Radioactive peaks were located on chromatograms by scanning with a model 7201 Packard radiochromatogram scanner. Peak areas were measured with a Disc integrator. Counting efficiency of the scanner was 23%.

Scintillation Counting

One-tenth ml of the sample and 10 ml of scintillation fluor were added to scintillation vials. Vials were counted using a Packard

model 574 liquid scintillation spectrometer. The compositions of aqueous (5) and non-aqueous fluors used for counting radioactive samples were:

Aqueous fluor (Bray's solution)

60	g	naphthalene
4	g	PPO
0.2	g	POPOP
100	ml	absolute methanol
20	ml	ethylene glycol

Make up to a final volume of 1.0 liter with p-dioxane.

Non-aqueous fluor

4	g	PPO
30	mg	POPOP

Make up to a final folume of 1.0 liter with toluene.

Gas-Liquid Chromatography and GLC-Mass Spectrometry

Unlabelled Compounds

Ether and Skellysolve-B extracts were analyzed by GLC using a Perkin-Elmer 990 gas chromatograph fitted with a flame-ionization detector. A 3.175 mm x 6.1 m stainless steel column packed with 1% phenyl diethanolamine succinate (PDEAS) and 1.5% sucrose acetate isobutyrate (SAIB) on 100-200 mesh Chromosorb G was used. Flow rate was 30 ml/min., and temperature was programmed from 140-165° at a rate of 1.5° per min. Under these conditions citronellol, neral, nerol, and geraniol peaks were clearly distinguishable from each other, although separation of adjacent peaks was not complete.

Peak areas were measured with a Disc integrator and quantitative analyses were made by comparison with pure monoterpenes. The lower limit of detection under the conditions employed was 0.025 nanomoles.

Labelled Compounds

Labelled extracts were analyzed by GLC using a Beckman Thermotrac temperature programmer fitted with a Carle Micro-detector (thermal conductivity); the effluent was counted by use of a coupled Nuclear-Chicago Biospan 4998 counter (3). The same PDEAS-SAIB column was employed. Flow rate was 30 ml/min. Analyses were conducted with the temperature programmed from 125-165° at a rate of 2° per min. The instrument was calibrated with toluene-^{14}C and peak areas were measured with a Disc integrator. The lower limit of detection under the conditions employed was approximately 1000 dpm.

GLC-Mass Spectrometry

Extracts were analyzed on an Atlas CH 7-MS coupled with a Varian 1200 GC using the column described above. Geraniol and nerol were identified by comparison with spectra obtained with standards and by comparison with published spectra (66).

RESULTS

Initial Studies with Peppermint

Monoterpenes Formed From MVA

Preparation of Cell-Free Enzymes. The Polyclar suspending buffer contained 0.1 M potassium phosphate buffer, 10 mM potassium metabisulfite ($K_2S_2O_5$), and 0.8% Triton X-100 detergent; the final buffer pH was 7.0. Metabisulfite has been found to be an unusually effective antioxidant in the isolation of plant enzymes (1).

Plant material was frozen with liquid nitrogen, ground in a mortar, and then mixed with the Polyclar suspension and allowed to thaw, squeezed through 160 mesh bolting silk, brought to 0.45 M in sucrose, and passed through a Bio-Gel P-10 column equilibrated with 0.1 M potassium phosphate buffer, pH 7.0 containing 1 mM $K_2S_2O_5$ and 0.45 M sucrose. The eluate was centrifuged 15 min. at 30,000 x g max. r.c.f.,[2/] and a 1% protamine sulfate solution (in water) was added to the resulting supernatant until it became cloudy (final concentration about 0.025% protamine). The solution was stirred gently for 5 min., then centrifuged as before. The pellet was discarded and the supernatant was brought to 25% saturation with solid

[2/] All centrifugation speeds are given as the calculated maximum relative centrifugal force.

ammonium sulfate, stirred for 5 min., allowed to stand for 10 min., and then centrifuged 15 min at 30, 000 x g. The pellet was discarded and the supernatant was brought to 65% saturation with $(NH_4)_2SO_4$, stirred, let stand, and centrifuged as before. The pellet was dissolved in 5 mM potassium phosphate buffer, pH 6. 2, containing 0. 45 M sucrose and passed through a Bio-Gel P-10 column containing the same buffer. All procedures except centrifugation were carried out in a glove box, in a chilled atmosphere under continuously flowing nitrogen. Capped centrifuge tubes were used to maintain the solution under anaeobic conditions during centrifugation. Use of Triton X-100 in the preparation of the enzyme extract was effective in disrupting membranes -- as indicated by the dark green color of the initial extract; however removal of phenolic compounds during purification was more difficult in the presence of detergent and therefore it was not used in subsequent enzyme preparations.

Assay Conditions. The peppermint enzyme extract was assayed for ability to incorporate mevalonic acid into monoterpenes. Both ATP and Mg^{++} or Mn^{++} are required for utilization of mevalonate (55). The reaction mixture contained: Tris-maleate (10 μmoles of each) buffer, adjusted to pH 7. 4 with KOH,[3/] 1. 3 μmoles potassium phosphate buffer, pH 7. 4; 0. 9 μmoles $MnSO_4$; 1. 8 μmoles $MgSO_4$;

[3/] All Tris-maleate buffers were adjusted to pH 7. 4 with KOH.

2.6 μmoles K_2ATP; 0.08 μmole MVA-2-^{14}C (0.94 μc); and 250 μl enzyme extract in a total volume of 0.36 ml. The pH was 7.4. The 15 ml conical test tube containing the reaction mixture was stoppered and incubated for 2 hrs. at 37° in a water bath. At the end of the incubation, the sample was cooled, and extracted 3 times with one ml portions of Skellysolve-B. The combined extracts were evaporated, under N_2, to about 50 μl and analyzed by gas-liquid chromatography coupled with effluent counting. The temperature was programmed from 125°-165°.

Analysis of Products. GLC analysis of the Skellysolve-B extract showed the presence of a radioactive peak which contained 4000 dpm (0.2% incorporation) and had the same retention time as geraniol; no other radioactive peaks were detected. Synthesis of geraniol from MVA in peppermint extracts was noteworthy but the amount formed was not very great. Furthermore, the fact that no other monoterpenes, e.g. nerol or cyclic monoterpenes were formed indicated that the amount of geranyl pyrophosphate formed from MVA was too small for further metabolism. Geraniol was therefore used for subsequent studies since it had already been shown that both GerP and GerPP could be formed from geraniol by cell-free peppermint extracts (41).

Monoterpenes Formed From Geraniol

Preparation of Cell-Free Enzymes. The enzyme extract was prepared essentially as described above, but without use of sucrose or Triton X-100. The suspending buffer contained 0.1 M potassium phosphate buffer and 10 mM $K_2S_2O_5$. The 25-65% saturated $(NH_4)_2SO_4$ precipitate was dissolved in 10 mM potassium phosphate buffer, pH 7.0, containing 5 mM glutathione (GSH). This protein solution was then passed through a Bio-Gel P-10 column, equilibrated with Tris-maleate (0.1 M in each) buffer, pH 7.4.

Part of this enzyme extract was treated with calcium phosphate ($CaPO_4$) gel to remove phosphatase activity: one ml of the gel was equilibrated with 50 mM potassium phosphate buffer, pH 7.0, centrifuged at 500 x g for 2 min. and the supernatant discarded; 0.1 M potassium phosphate buffer, pH 7.0, was then mixed with the enzyme extract in a 1:1 ratio and this mixture was in turn mixed with the gel, centrifuged at 12000 x g for 2 min., and the supernatant removed; then 0.1 M potassium phosphate buffer, pH 7.4, was added to the pellet, mixed, centrifuged, and the supernatant combined with the first one since phosphatase activity was low. All solutions were kept cold by means of an ice bath. Buffer content in the combined supernatants was: Tris-maleate, 25 mM in each, and 75 mM potassium phosphate. The pH was approximately 7.3.

Assay Conditions. These peppermint enzyme extracts were assayed for ability to form other monoterpenes from geraniol. ATP and Mn^{++} are necessary for formation of GerPP from geraniol by geraniol kinase isolated from peppermint (41). The reaction mixture contained Tris-Maleate and potassium phosphate buffers, 0.9 μmole $MnSO_4$, 2.6 μmole Na_2ATP, 500,000 dpm ^{14}C-geraniol (approximately 1 μmole added as an ether solution and the ether evaporated under a stream of nitrogen), and (1) 200 μl enzyme extract, (2) 200 μl gel treated enzyme extract, or (3) 50 μl of enzyme extract and 200 μl of gel treated enzyme extract in a total volume of 0.32 ml; the pH was 7.4. Samples were incubated 2 hrs. at 35° in a water bath. After incubation, terpenes were extracted with Skellysolve-B.

A repeat assay was essentially the same. The reaction mixture contained Tris-maleate (8 μmoles of each) buffer, 7.5 μmoles potassium phosphate buffer, 0.45 μmole $MnSO_4$, 1.3 μmole Na_2ATP, 500,000 dpm ^{14}C-geraniol, 100 μl gel treated enzyme extract, and enzyme extract: (1) 12.5 μl, (2) 6.3 μl, or (3) 3.2 μl in a total volume of 0.16 ml; the pH was 7.4. Incubation time was 2 hrs. at 35°.

Analysis of Products. GLC analysis of the Skellysolve-B extracts from the first assay showed the presence of two principal new radioactive peaks (designated A and B) in the sample to which both enzyme extract and gel treated enzyme extract had been added

(Table 1). Peak A (the larger of the two) had a retention time equal to that of α-terpineol and peak B had a slightly shorter retention time. A small peak with the same retention time as nerol was also formed. When the aqueous phases from the three samples were treated with phosphatase and extracted with Skellysolve-B, scintillation counting and GLC analysis indicated the release of 17,000 dpm of geraniol from the samples to which gel treated enzyme extract had been added (Table 1). Only 3000 dpm of geraniol were released from the sample to which only enzyme extract had been added.

Table 1. Products formed from geraniol in the presence of peppermint enzyme extract.

	Enzyme Additions (µl)		Products	
Sample	Enzyme Extract	Enzyme extract treated with $CaPO_4$ gel	Hexane-Soluble[a]	Geranyl Phosphates[b]
(1)	200	-	-	3300 dpm
(2)	-	200	-	16000 dpm
(3)	50	200	+	17000 dpm

[a] Hexane-soluble products other than geraniol.

[b] Geraniol released after phosphatase treatment of aqueous phase.

The reaction mixture also included: Tris-maleate and potassium phosphate buffers; 0.9 µmole $MnSO_4$; 2.6 µmoles Na_2ATP; and 500,000 dpm of ^{14}C-geraniol in a total volume of 0.32 ml. The pH was 7.4. Incubation time 2 hr; temp. 35°.

Different ratios of enzyme extract and gel-treated enzyme extract were used in a repeat assay. GLC analysis of the Skellysolve-B extracts again indicated the presence of peak B. The sample with an intermediate amount of cell-free enzyme contained a greater amount of this compound than the other two samples (Table 2). Approximately equal amounts of nerol were also detected in all three samples (Table 2). Radioscanning of paper chromatograms of the aqueous phases indicated the presence of a large peak with an Rf corresponding to that of GerP and/or NerP (Rf 0.75) and a small peak with an Rf corresponding to that of GerPP (Rf 0.65). The second experiment (Table 2) for the first time revealed that significant amounts of nerol had been formed from geraniol. The factors necessary for this key reaction to take place were investigated.

Table 2. Formation of nerol and other hexane-soluble products from geraniol in the presence of peppermint enzyme extract.

	Enzyme Additions (μl)		Products	
Sample	Enzyme Extract	Enzyme extract treated with $CaPO_4$ gel	Nerol	Other[a]
(1)	12.5	100	+	+
(2)	6.3	100	+	++
(3)	3.2	100	+	+

[a] Hexane-soluble products other than nerol.

The reaction mixture included: 8 μmoles Tris-Maleate and 7.5 μmoles potassium phosphate buffers; 0.45 μmole $MnSO_4$; 1.3 μmoles Na_2ATP; and 500,000 dpm of ^{14}C-geraniol in a total volume of 0.16 ml. The pH was 7.4. Incubation time 2 hr; temp. 35°.

Nerol Formation From Geraniol

Preparation of Cell-Free Enzymes and Assay Conditions.

Peppermint enzyme was prepared as described previously except that: (1) the suspending buffer contained 0.1 M potassium phosphate buffer, pH 7.1, 10 mM $K_2S_2O_5$ and 1 mM Na_2EDTA; (2) no GSH was used during the preparation.

The extract was assayed for trans-cis isomerase activity. Initial assays employed various combinations of extract, flavin, and light. These studies indicated that light was necessary for isomerization. Additional experiments therefore were carried out in a light-filter chamber (Figure 6), which was in turn placed in a growth chamber, illuminated with Gro-Lux and Wide Spectrum Gro-Lux lamps in equal numbers giving a bench-top light intensity of 1000 ft.-c. A Wratten 2E (400 nm cut-off) filter was inserted in the top of the filter chamber. The 2E filter, which absorbs wavelengths shorter than 400 nm, reduced the possibility of UV-induced isomerization (24, 29).

Subsequent assays of the enzyme extract with ^{14}C-geraniol as substrate indicated that nerol was formed only in the presence of FAD or FMN, and light. Paper chromatography of the aqueous phase followed by radioscanning indicated that the amount of side-products formed when FAD was the added cofactor was less than when FMN was the cofactor. Some of these side-products were formed

enzymatically, but most were formed non-enzymatically.

To confirm the flavin and light requirement, assays were carried out in stoppered incubation tubes containing Tris-maleate and potassium phosphate buffers, pH 7.4, 250,000 dpm ^{14}C-geraniol and various combinations of enzyme extract, flavin, and GSH in a total volume of 0.175 ml. Incubation was for 2 hrs. at 35° in the light or in the dark.

Isomerization Results. The experimental results are summarized in Table 3. These experiments indicated that GSH as well as FAD and light were necessary for isomerization. Isomerization also occurred to a lesser extent in the absence of enzyme extract if GSH was present. When the assay was carried out in the presence of FAD, GSH, and boiled enzyme extract, the amount of isomerization was greater than in the absence of the extract.

The source of this heat-stable activity was investigated and the results suggested that the protamine which was added during the preparation of the enzyme extract was responsible for this activity. Table 3 shows that in the presence of the cofactors FAD and GSH, addition of bovine serum albumin (BSA) did not increase isomerization but addition of protamine did. Subsequent enzyme extracts prepared without use of protamine did not exhibit any heat stable activity.

Trans-cis isomerization of geraniol was confirmed by GLC-MS

Table 3. Effect of Flavin, GSH, light and cell-free peppermint extract on nerol formation from geraniol.

Additions		Nerol Formed	
Protein	Cofactors	Light[a/]	Dark
None	FAD + GSH	+	-
Boiled cell-free extract	FAD	-	-
Boiled cell-free extract	FAD + GSH	++	-
Cell-free extract	FMN	++	N. D.[b/]
Cell-free extract	FMN + GSH	++++	-
Cell-free extract	FAD + GSH	++++	-
Cell-free extract	GSH	-	-
BSA	FAD + GSH	+	N. D.
Protamine	FAD + GSH	+++	N. D.

[a/] The relative amount of nerol formed is indicated by the number of + signs.

[b/] N. D. Not determined.

The reaction mixture also included Tris-Maleate and potassium phosphate buffers and 250, 000 dpm of C^{14}-geraniol in a total volume of 0. 175 ml. The pH was 7. 4. Light level 700 ft. -c., 2E filter. Incubation time 2 hr; temp. 35°.

in a separate experiment. Unlabelled geraniol was incubated in the presence of peppermint enzyme extract, FAD, and GSH. The hexane extract fragmentation patterns were obtained from geraniol and nerol standards. Comparison of the patterns with each other and with published patterns (66) confirmed the identity of nerol. No other terpene patterns were detected.

These preliminary results suggested that there is indeed a specific isomerase in peppermint. Therefore, the enzymatic reaction was subsequently studied in more detail.

Detailed Investigations of the Isomerization Reaction

Peppermint, Carrot, and Pea Extracts for Assay of *trans*-*cis* Isomerase Activity

Initial Extracts. A detailed investigation of the isomerization reaction was undertaken. Potassium phosphate buffer was used in the preparation of cell-free extracts and in assays of isomerase activity since this buffer gives a low level of flavin photoreduction (76). By utilizing potassium phosphate buffer, the effect of added reductants would not be masked by photoreduction promoted by the buffer.

Extracts were prepared essentially as described before, except that no protamine sulfate was used. Also, potassium phosphate buffer was used exclusively. The suspending buffer contained 0.1 M

potassium phosphate buffer, 5 mM Na_2EDTA, and 10 mM $K_2S_2O_5$. The final buffer pH was 7.1.

Plant material was frozen with liquid nitrogen, ground in a mortar, mixed with the Polyclar suspension and allowed to thaw, squeezed through a Bio-Gel P-10 column equilibrated with 0.1 M potassium phosphate buffer, pH 7.1, containing 5 mM Na_2EDTA and 1 mM $K_2S_2O_5$. The eluate was centrifuged 15 min. at 30,000 x g and the resulting supernatant was brought to 25% saturation with solid ammonium sulfate. After gently stirring for 5 min., the solution was allowed to stand for 10 min. and then was centrifuged 15 min. at 30,000 x g. The pellet was discarded and the supernatant was brought to 65% saturation with $(NH_4)_2SO_4$, stirred, let stand, and centrifuged as before. The pellet was dissolved in 25 mM potassium phosphate buffer, pH 7.5 and desalted in a Bio-Gel P-10 column equilibrated with the same buffer. As with previous preparations, all procedures except centrifugation were carried out in a glove box, in a chilled atmosphere under continuously flowing nitrogen.

Further Purification of the Peppermint Extract. The desalted cell-free extract was applied to a DEAE-cellulose column equilibrated with 25 mM potassium phosphate buffer, pH 7.5 and eluted stepwise -- 25 mM, 75 mM, 125 mM, and 200 mM potassium phosphate buffer, pH 7.5 -- from the column. Column fractions were assayed for isomerase activity. Incubations were carried out in stoppered

conical tubes containing potassium phosphate buffer, pH 7.5, FAD, DTT, ^{14}C-geraniol, and an aliquot of individual column fractions. The stoppered tubes were placed in the light-filter chamber which was fitted with a 2E filter, and were incubated for 40 min. at 35°. DTT was used in the assay rather than GSH because non-enzymatic isomerization was less. Isomerase activity was detected only in the 75 mM column fractions. The activity and protein peaks were only partially separated but this suggested that more complete separation could be achieved by gradient elution. Details concerning gradient elution, concentration of the activity peak, and removal of phosphatase activity are given under Methods, as are the assay conditions.

Results of Peppermint Isomerase Purification

No activity was detected until after $(NH_4)_2SO_4$ precipitation. Three possible reasons are: the dilute nature of the crude extract; the presence of inhibitory substances; and the presence of EDTA. EDTA inhibition will be discussed later. Fractionation of the 25-65% $(NH_4)_2SO_4$ precipitate on DEAE-cellulose with a linear potassium phosphate gradient resulted in the elution of isomerase activity at about 50 mM potassium phosphate in a broad peak (Figure 8). Subsequent concentration of the column fractions containing most of the isomerase activity, followed by desalting on Bio-Gel P-10, resulted in an increase in apparent specific activity of between 160- and

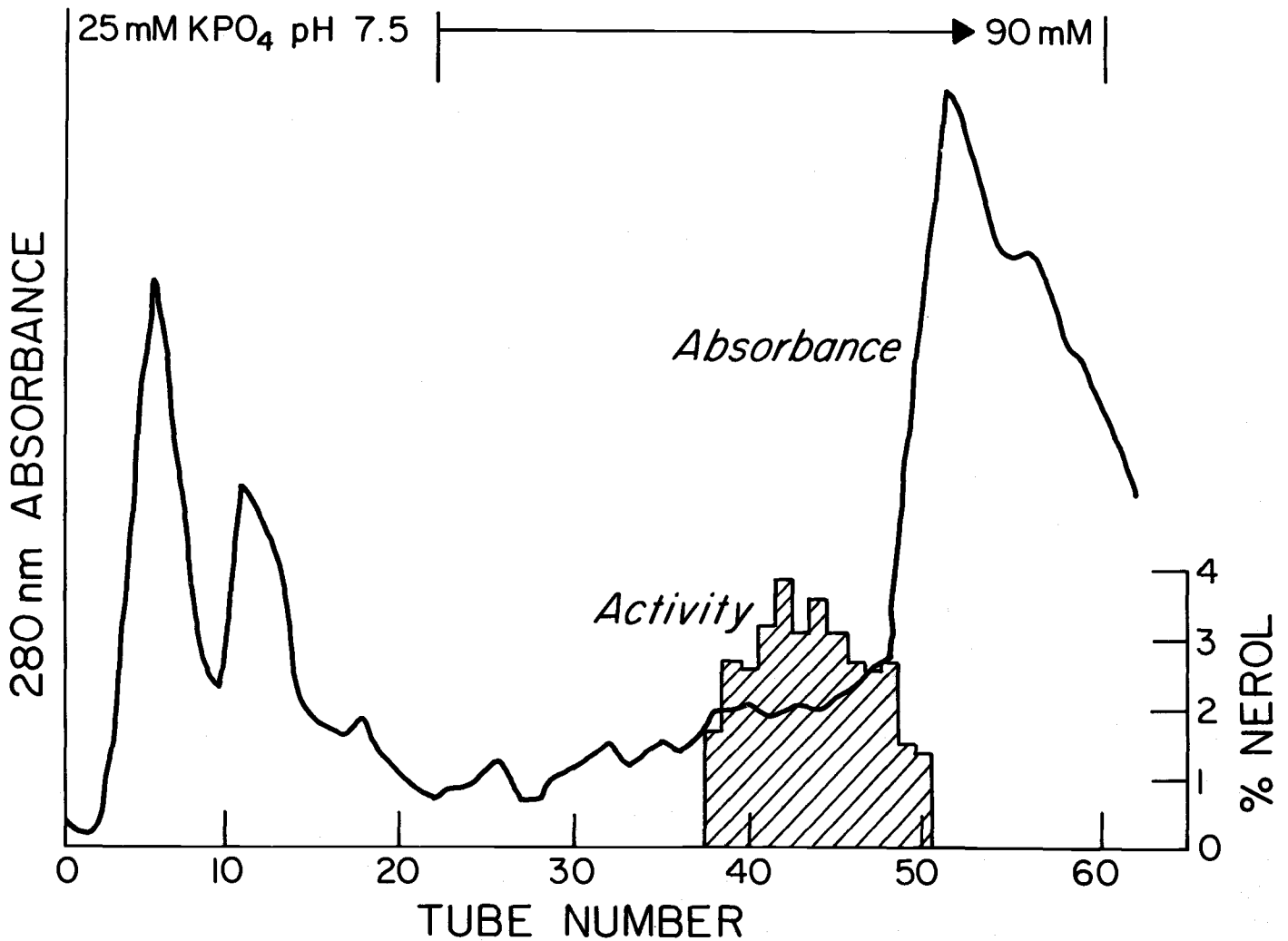

Figure 8. Elution of the isomerase activity from the DEAE-cellulose column. Volume collected per tube was 2 ml.

220-fold, depending on the method of calculation (Table 4). Calculations based on the actual μmoles of nerol formed give the larger value; calculations based on % nerol formation $(\frac{\text{Nerol x 100}}{\text{Nerol + Geraniol}})$ give the lower value. An increase in total activity was also observed (Table 4). This increase in total activity undoubtedly indicates removal of inhibitors. Similarly, the apparent decrease in specific activity after Amicon concentration could be reversed by desalting the extract on a Bio-Gel P-10 column.

Product formation could be presented in two ways: (1) actual nmoles of product recovered or (2) % product formation. Because both nerol and geraniol are volatile, losses can occur during the addition of the substrate to the reaction vessel, during the assay, and after the assay. Adsorption on the walls of the reaction vessel also causes losses. In order to minimize the effect of these losses product formation is presented in terms of % product, e.g., if geraniol is the substrate % product formation =

$$\frac{\text{recovered nerol x 100}}{\text{recovered nerol + recovered geraniol}}.$$

In Table 4 activity values are presented in terms of % nerol, as well as in terms of μmoles nerol, since values expressed in terms of % nerol formation probably reflect enzymatic activity more accurately.

If 0.25 mM DTT was added to the buffer used to dissolve the 25-65% saturated $(NH_4)_2SO_4$ precipitate, and to DEAE-cellulose

Table 4. Purification of peppermint geraniol-nerol isomerase activity.

Purification step	Apparent[a] total protein (mg)	% of original protein	Based on μmoles nerol: % original activity	Based on μmoles nerol: Specific activity μmoles nerol / mg protein	Based on % nerol: % original activity	Based on % nerol: Specific activity % nerol[b] / mg protein
Crude extract	270[c]	100	0	0	0	0
25-65% $(NH_4)_2SO_4$ ppt.	140[c]	52	100	0.013	100[d]	8.2
DEAE cellulose fractions #39-47	2.2[c/e]	0.8	240	2.0	220	1200
Amicon concentrate fractions #39-47	1.4[e]	0.5	110	1.4	71	820
Amicon concentrate P-10 desalted	0.9[e]	0.3	140	2.9	110	1400

[a] Apparent total protein: protein ± non-protein materials or compounds which are TCA precipitatable or absorb in the UV (260-280 nm).

[b] % nerol x (total protein/protein in assay), where % nerol = $\left(\frac{\text{nerol recovered in assay}}{\text{nerol + geraniol recovered}}\right) \times 100$.

[c] Protein determined turbidimetrically with trichloroacetic acid.

[d] Activity of 25-65% saturated $(NH_4)_2SO_4$ precipitate was taken as 100%.

[e] Protein content determined by UV absorption (E_{280}/E_{260}).

column buffers, a small increase in the separation of the activity peak from the adjacent protein peak could be achieved. However, an increase in enzymatically catalyzed irreversible photodecomposition of the flavin was observed -- as indicated by a decrease in the intensity of the yellow flavin color visually observed after extracting with ether in the presence of air. In addition to isomerase activity the purified extract also contained phosphatase and a small amount of geraniol kinase activity.

Other compounds and treatments were tested for their effect on the isomerization reaction. Polyclar AT has been reported to interact with riboflavin (31) and it was therefore conceivable that Polyclar was contributing to "isomerase activity". Because carrot tissue is low in phenolic compounds, it was considered possible to prepare a cell-free carrot extract with good activity without the use of Polyclar. These extracts were prepared and equal amounts of isomerase activity were found, whether or not they were prepared with Polyclar.

As indicated in Table 4 no isomerase activity was detectable until after $(NH_4)_2SO_4$ precipitation; the presence of inhibitors which were removed in subsequent steps could account for this. For example, peppermint contains numerous compounds, such as phenolics (38), which can greatly reduce enzymatic activity because they bind tightly to protein. In fact, in the presence of pyrocatechol, non-enzymatic geraniol isomerization is also inhibited. EDTA, which

is present in the early stages of purification, also causes some inhibition of isomerization.

Characteristics of the Enzymatic Reaction

As demonstrated above, flavin, DTT (or GSH), and light are required for geraniol isomerization. Therefore, various characteristics of the enzymatic reaction in the presence of these three cofactors were determined. Experimental results demonstrated that product formation is a function of substrate concentration (Figure 9). Nerol formation increases approximately with substrate concentration in the range of geraniol levels selected. Figure 9 also shows that by expressing the extent of isomerization in terms of % nerol formation instead of nmoles nerol formation, less scattering of points is observed. At a level of about 0.3 μmoles geraniol, isomerization expressed in terms of % nerol formation appears to be relatively unaffected by substrate level. If actual nerol formed is assumed to be equal to % nerol times the amount of substrate, an apparent Km of 2 mM is obtained for geraniol. However, this value may not be very meaningful because of the low solubility of geraniol in aqueous systems.

Product formation is also a function of enzyme concentration (Figure 10), but a small amount of isomerization also occurs in the absence of enzyme. Furthermore, the extent of isomerization at

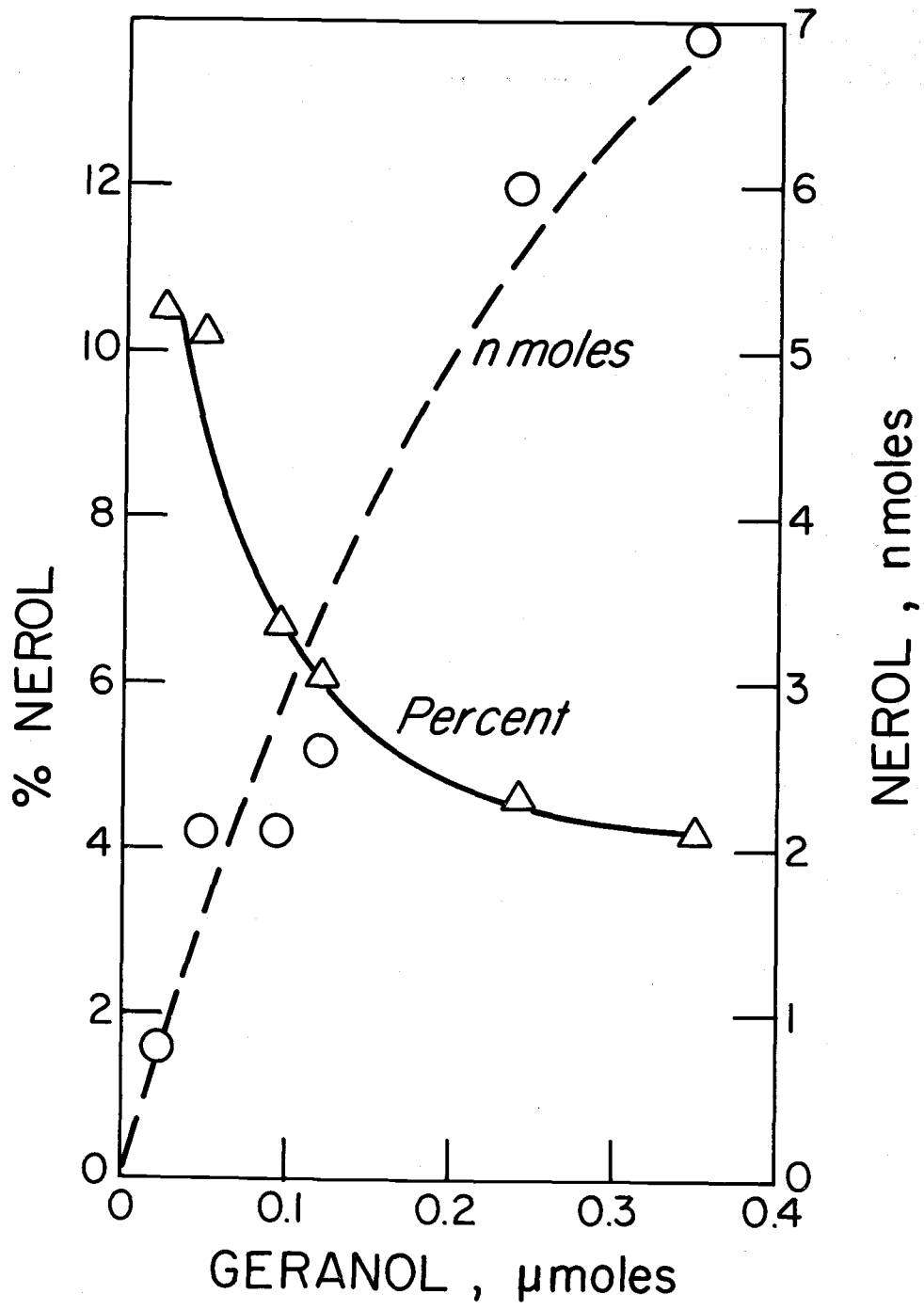

Figure 9. Effect of substrate concentration on geraniol isomerization by peppermint enzyme. The reaction mixture contained: 4.4 µmoles potassium phosphate buffer; 20 nmoles FAD; 22 nmoles dithiothreitol; and 30 µl enzyme in a total volume of 0.175 ml. The pH was 7.5. Light level 70 ft.-c., 2E + 96 (density 1.00) filters; reaction time 60 min; temp. 35°. Non-enzymatic contribution - 0.7% (1.2 nmoles) at a substrate level of 0.35 µmoles - included in values.

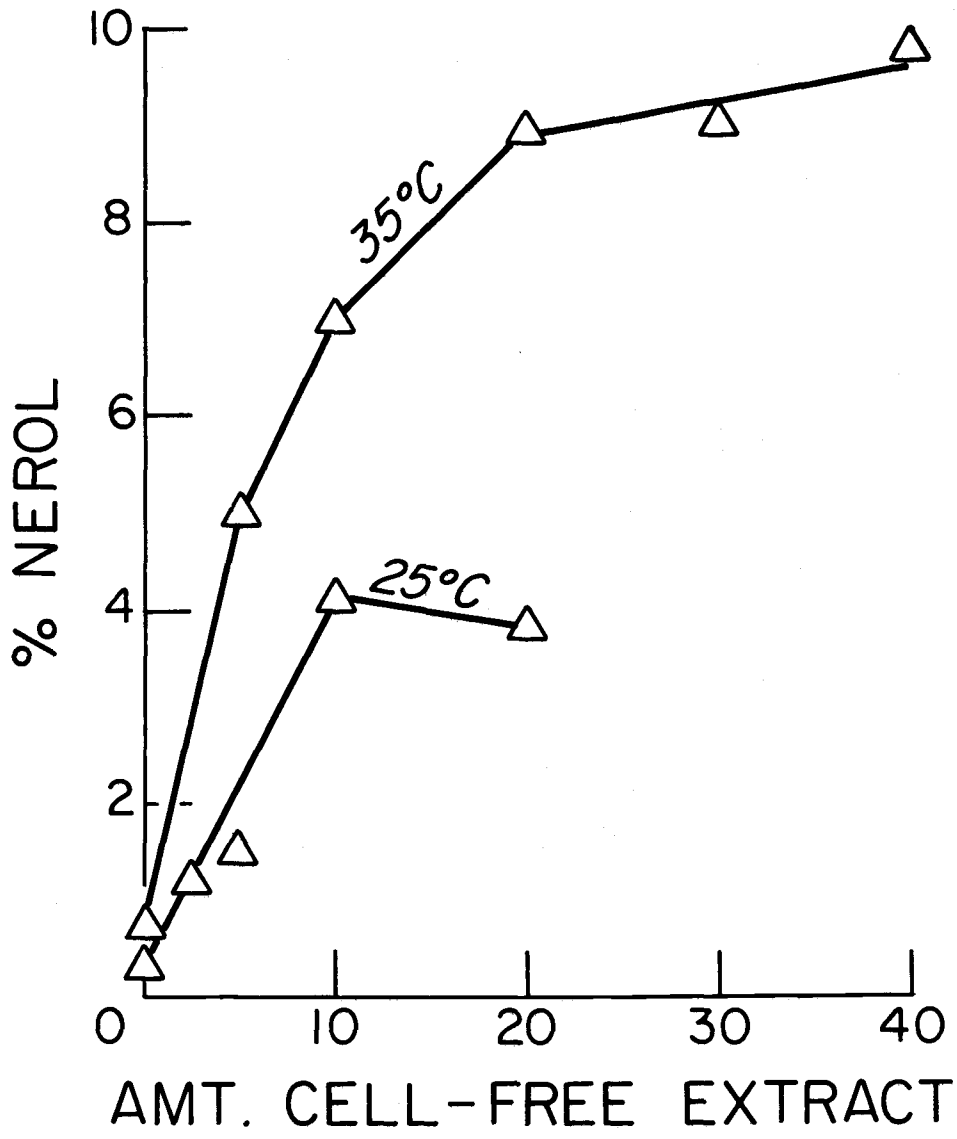

Figure 10. Effect of enzyme concentration and temperature on peppermint isomerase activity. The reaction mixture contained: 4.4 μmoles potassium phosphate buffer; 20 nmoles FAD; 22 nmoles dithiothreitol; 300 nmoles geraniol; and the indicated amounts of enzyme solution in a total volume of 0.175 ml. The pH was 7.5. Light level 70 ft.-c., 2E + 96 (density 1.00) filters. Reaction time 60 min.

35° is approximately twice that at 25° (Figure 10). Figure 10 also shows that a plateau is approached at higher enzyme concentrations; this leveling-off does not indicate that equilibrium has been reached since more nerol is formed if the incubation time is extended (5.5 nmoles or 7.8% after 1 hr. and 9.9 nmoles or 20% after 4 hr. at 35°). The pH optimum for geraniol isomerization is about 7.5 to 7.7 (Figure 11).

Various other substrates are isomerized, both non-enzymatically and enzymatically in the presence of peppermint enzyme extract. Nerol is isomerized twice as fast as geraniol, and the apparent equilibrium for the reaction is 67% geraniol and 33% nerol (Figure 12). However, these values are subject to error due to the greater evaporative losses of nerol than geraniol. GerP is isomerized about one-half as fast as geraniol (Table 5). Comparison of isomerization at pH 6.5 and pH 7.5 indicated a pH effect similar to that found for geraniol. Table 5 shows that the calcium phosphate gel treated enzyme extract contained a small amount of phosphatase activity. No GerP isomerization occurs in the absence of an added sulfur compound, e.g., GSH. Neither the methyl ester of geranic acid (trans) nor the methyl ester of cis-geranic acid is isomerized under the conditions employed.

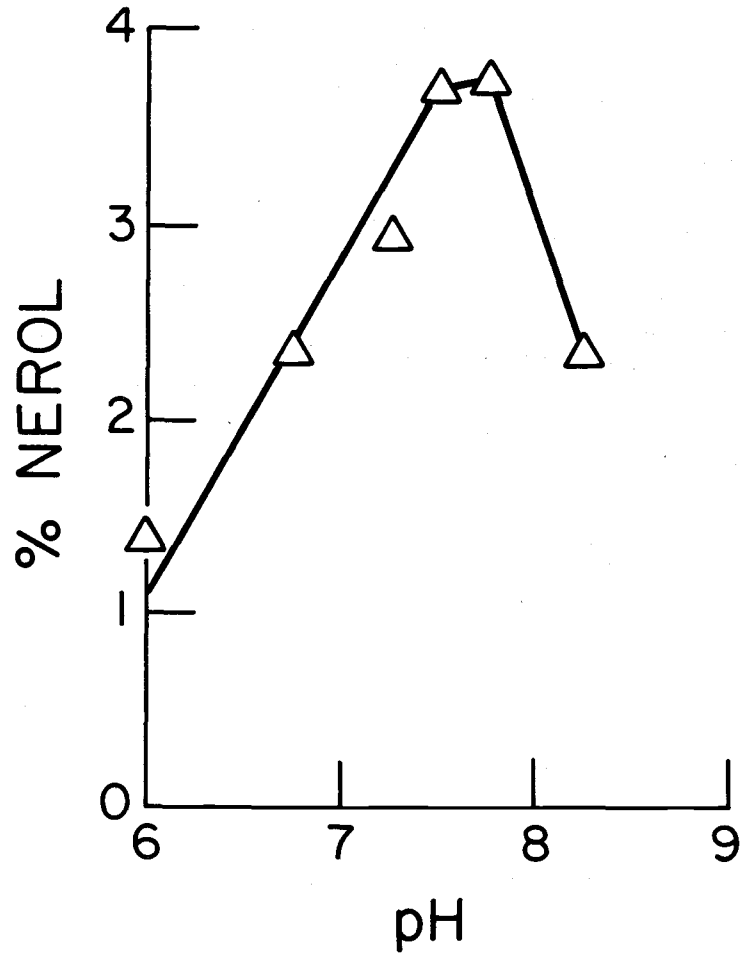

Figure 11. Effect of pH on peppermint isomerase activity. The reaction mixture contained: 8.8 μmoles potassium phosphate buffer; 20 nmoles FAD; 22 nmoles dithiothreitol; 300 nmoles geraniol; and 25 μl of enzyme in a total volume of 0.175 ml. Light level 70 ft.-c., 2E + 96 (density 1.00) filters. Reaction time 60 min; temp. 35°. Non-enzymatic contribution (0.7% at pH 7.5) included in values indicated.

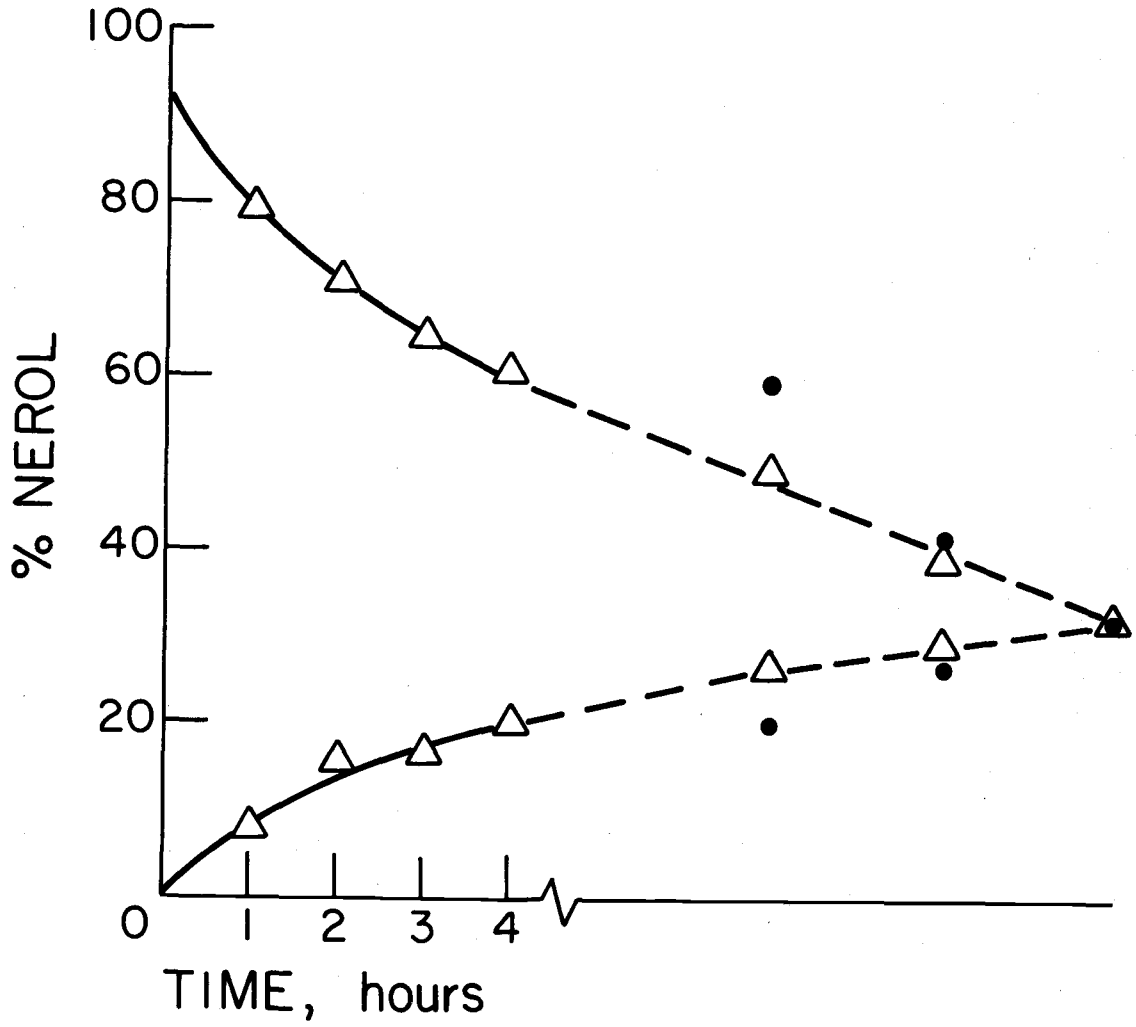

Figure 12. Time course and equilibrium for the isomerization reaction. The reaction mixture contained: 4.4 μmole potassium phosphate buffer; 20 nmole FAD; 33 nmole DTT; 100 nmole substrate; 10 μl peppermint enzyme in a total volume of 0.175 ml. The pH was 7.5. Light level 70 ft.-c., 2E + 96 (density 1.00) filters; temp. 35°. For the five points on the right side of the figure, the substrate contained the indicated % nerol (black dots) and samples were incubated for 3 hr. Values include non-enzymatic contribution, at 1 hr : 1.0% for geraniol and 2.0% for nerol.

Table 5. Isomerization of geranyl phosphate and geraniol by peppermint extract.

Treatment	nmoles recovery				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 μmoles potassium phosphate buffer; 20 nmoles FAD; 44 nmoles GSH; 130 nmoles substrate; and 5 μl calcium phosphate gel treated enzyme extract in a total volume of 0.175 ml. The pH was 7.5. Light level 70 ft.-c., 2E + 96 (density 1.00) filters; reaction time 75 min; temp. 30°.

Flavin Effect. Both FAD and FMN are effective cofactors for the isomerization reaction. FMN appears to be about 70% as effective as FAD in the presence of enzyme extract. As already mentioned, fewer hexane insoluble side-products are formed when FAD is the cofactor. Therefore, FAD was selected for use in all assays. Enzymatic isomerization of geraniol in the light is dependent on FAD concentration (Figure 13). Non-enzymatic nerol formation at the highest FAD level was never greater than 0.7% at the dithiothreitol level used.

Effect of Sulfur-Containing Compounds. A sulfur-containing compound is also necessary for isomerization to occur. Many

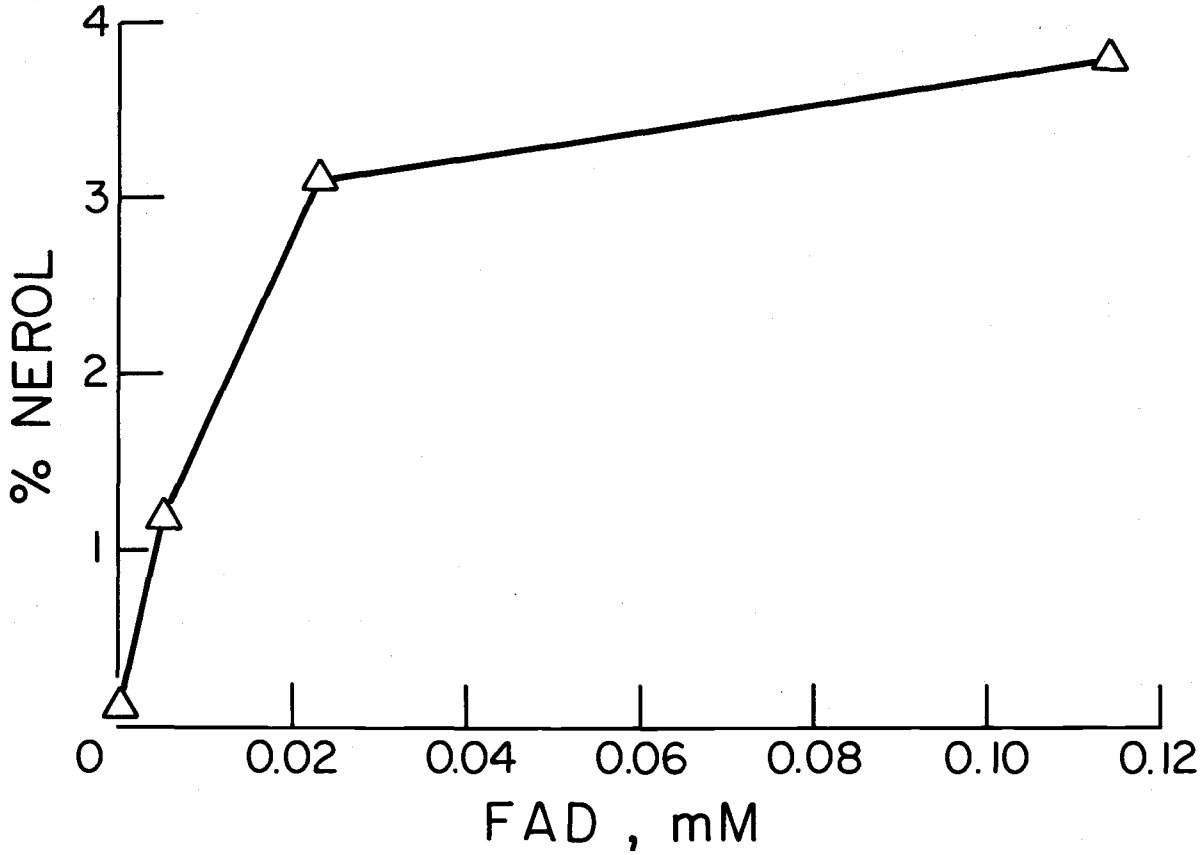

Figure 13. Effect of FAD concentration on geraniol isomerization by peppermint enzyme. The reaction mixture contained: 8.8 μmoles potassium phosphate buffer; 22 nmoles DTT; 300 nmoles geraniol; and 25 μl enzyme in a total volume of 0.175 ml. The pH was 7.5. Light level 70 ft.-c., 2E + 96 (density 1.00) filters; reaction time 60 min; temp. 35°. Non-enzymatic contribution - 0.7% at a FAD level of 0.114 mM - included in values.

compounds can serve this function: β-mercaptoethanol; DTT; GSH; Na_2S; 5,5'-dithio-bis-(2-nitrobenzoic acid), also known as Ellman's reagent or DTNB; and Na_2SO_3. The relative effectiveness of some of the sulfur compounds appears to depend on concentration. As illustrated in Table 6, DTT is more effective than GSH at a 50 μM concentration, but at a 250 μM concentration these sulfhydryl reagents are equally effective. At higher concentration, Na_2S is as effective as these two sulfhydryl reagents (Table 6). On the other hand, mercaptoethanol was found to be about twice as effective as DTT at both concentrations. Nevertheless, DTT rather than GSH or mercaptoethanol was selected for use in assays because in its presence less non-enzymatic isomerization (0.7% vs. 1.8% for GSH and 2.3% for mercaptoethanol at the 125 μM level) and a higher ratio of enzymatic to non-enzymatic isomerization was observed. Figure 14 shows that enzymatic isomerization is dependent on DTT concentration; excess DTT inhibits isomerization.

The oxidation state of the sulfur compound is also important. Effects of oxidized glutathione (GSSG) vs reduced glutathione (GSH) on the isomerization of geraniol are shown in Table 7. The results suggest that GSH is the active form. Activity with GSH in the air was comparable to the anaerobic activity with GSSG observed here. The sulfhydryl inhibitor NEM, when present during the reaction, inhibited isomerization. On the other hand, pretreatment of the enzyme with

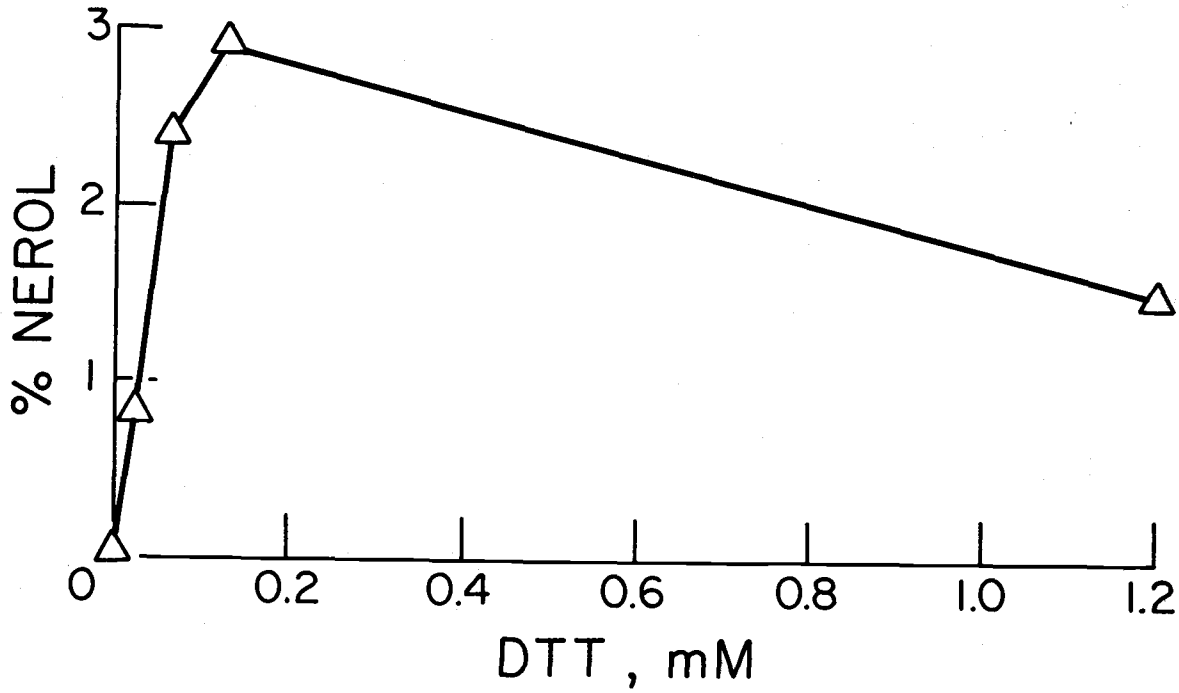

Figure 14. Effect of dithiothreitol concentration on geraniol isomerization by peppermint enzyme. The reaction mixture contained: 4.4 μmoles potassium phosphate buffer; 20 nmoles FAD; 300 nmoles geraniol; and 10 μl enzyme in a total volume of 0.175 ml. The pH was 7.5. Light level 70 ft.-c., 2E + 96 (density 1.00) filters; reaction time 60 min; temp. 35°. Non-enzymatic contribution - 0.4% at a DTT level of 0.125 mM - included in values.

Table 6. Effect of sulfur-containing compounds on geraniol isomerization in the presence of peppermint enzyme.

Concentration of sulfur compound (μM)	% Nerol Formation				
	None	Mercaptoethanol	DTT	GSH	Na_2S
0	0.3				
50		2.7	1.1	0.4	N.D.[a/]
250		6.8	3.2	3.7	3.5

[a/] N.D. Not determined

The reaction mixture contained: 4.4 μmoles potassium phosphate buffer; 20 nmoles FAD; 300 nmoles geraniol; and 10 μl enzyme extract in a total volume of 0.175 ml. The pH was 7.5. Light level 70 ft. c., 2E + 96 (density 1.00) filters. Incubation time 60 min; temp. 35°.

Table 7. Effect of oxidized and reduced glutathione on isomerization of geraniol by peppermint extract.

Treatment	Nanomoles Recovered		% Nerol
	Geraniol	Nerol	
No GSH or GSSG	220	0	0
40 nmoles GSH	240	15	5.8
40 nmoles GSSG	220	4	1.9
40 nmoles GSSG + NEM	220	2	0.7

The reaction mixture contained: 4.4 μmoles potassium phosphate buffer; 20 nmoles FAD; 300 nmoles geraniol; and 25 μl of enzyme solution in a total volume of 0.175 ml. The pH was 7.5. Light level 70 ft. c., 2E + 96 (density 1.00) filters; reaction time 60 min; temp. 35°.

NEM or PHMB, which was then removed prior to incubation with the substrate, caused no inhibition; in fact, NEM pretreatment caused a slight stimulation. Table 7 also shows that no detectable isomerization occurred in the absence of GSH or GSSG.

As previously mentioned, oxygen inhibits isomerization. The 30 min. period of flushing incubation tubes with N_2 prior to incubation appeared to be sufficient to lower the oxygen concentration to a level which did not cause inhibition of geraniol isomerization, as use of the central reservoir filled with methylene blue and ascorbate as an oxygen trap did not further increase the degree of isomerization.

Isomerization also occurs in the presence of dithionite or sulfite and as with the other sulfur-compounds, the presence of flavin is also required. Sulfite can be generated from dithionite *via* SO_2; therefore if a dithionite solution is added to the central reservoir, as illustrated in Figure 7, and SO_2 allowed to diffuse into the incubation solution, geraniol isomerization occurs. Dithionite can also be added directly to the incubation solution; Figure 15 shows that low levels of dithionite greatly increased isomerization while higher dithionite concentrations caused marked inhibition, similar to that seen with the highest DTT level (Figure 14). Inhibition at these higher levels could be reversed by air. Other studies indicated that sulfite (Na_2SO_3)-promoted isomerization was not increased by enzyme--in the presence of 200 μM sulfite, geraniol isomerization in the presence of

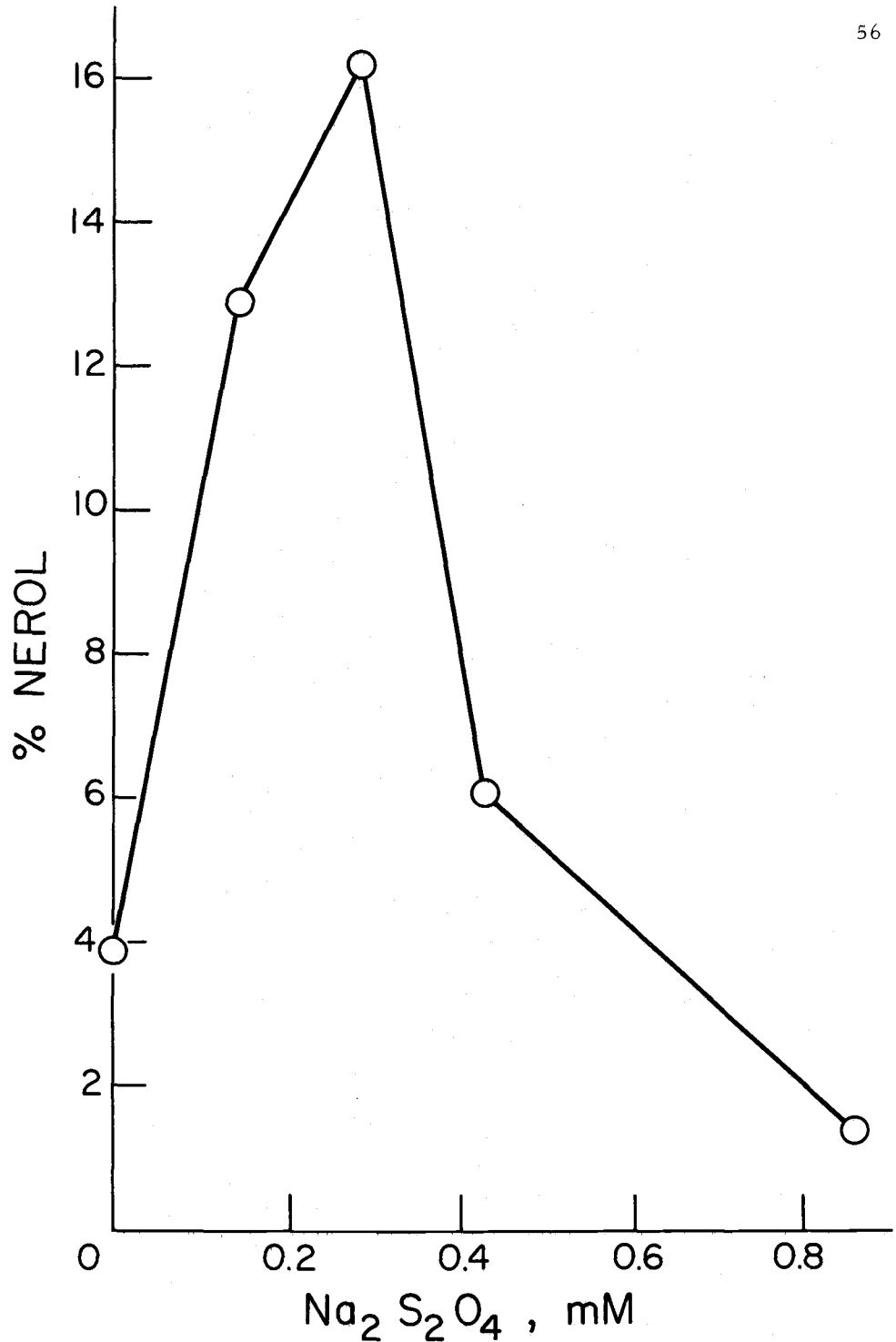

Figure 15. Effect of dithionite concentration on geraniol isomerization. The reaction mixture contained: 4.4 μmoles potassium phosphate buffer; 20 nmoles FAD; 5 nmoles dithiothreitol; 300 nmoles geraniol; and 20 μl peppermint cell-free extract in a total volume of 0.175 ml. The pH was 7.5. Light level 70 ft.-c., 2E + 96 (density 1.00) filters. Reaction time 60 min; temp. 35°. In addition, the central reservoir contained a potassium phosphate buffer solution containing dithionite and methylene blue. The indicated amounts of dithionite were added directly to the reaction mixture.

peppermint enzyme (2.8%) was equal to that of the boiled control (2.8%) -- in contrast to the other sulfur-compounds discussed. At a 250 μM concentration, sulfite and mercaptoethanol are about equally effective in promoting non-enzymatic isomerization.

Effect of Light. Investigation of the effect of light is illustrated in Figure 16. This figure shows that non-enzymatic isomerization increases linearly with light level. Enzymatic isomerization, however, increases much more rapidly with light level and appears to reach a maximum at about 225 ft.-c.

At the highest light level employed, apparent inhibition of enzymatic isomerization was observed (Figure 16). Other experiments indicated that inhibition at this light level was not observed if a higher concentration of DTT (2.75×10^{-4}M) was used in the assays. An increased rate of DTT oxidation and/or irreversible photobleaching of FAD at higher light levels may account for these observations. Additional studies showed the presence of activity in the enzyme extract which caused irreversible photobleaching of the flavin; this photobleaching activity could be inhibited by boiling, or pretreatment with the sulfhydryl inhibitor NEM. Enzymatic studies at the highest light level indicated that longer wavelengths of light were less effective in promoting the isomerization reaction. Thus at wavelengths longer than 500 nm (Wratten 2E and 12 filters), nerol formation was only 1%, whereas at wavelengths longer than

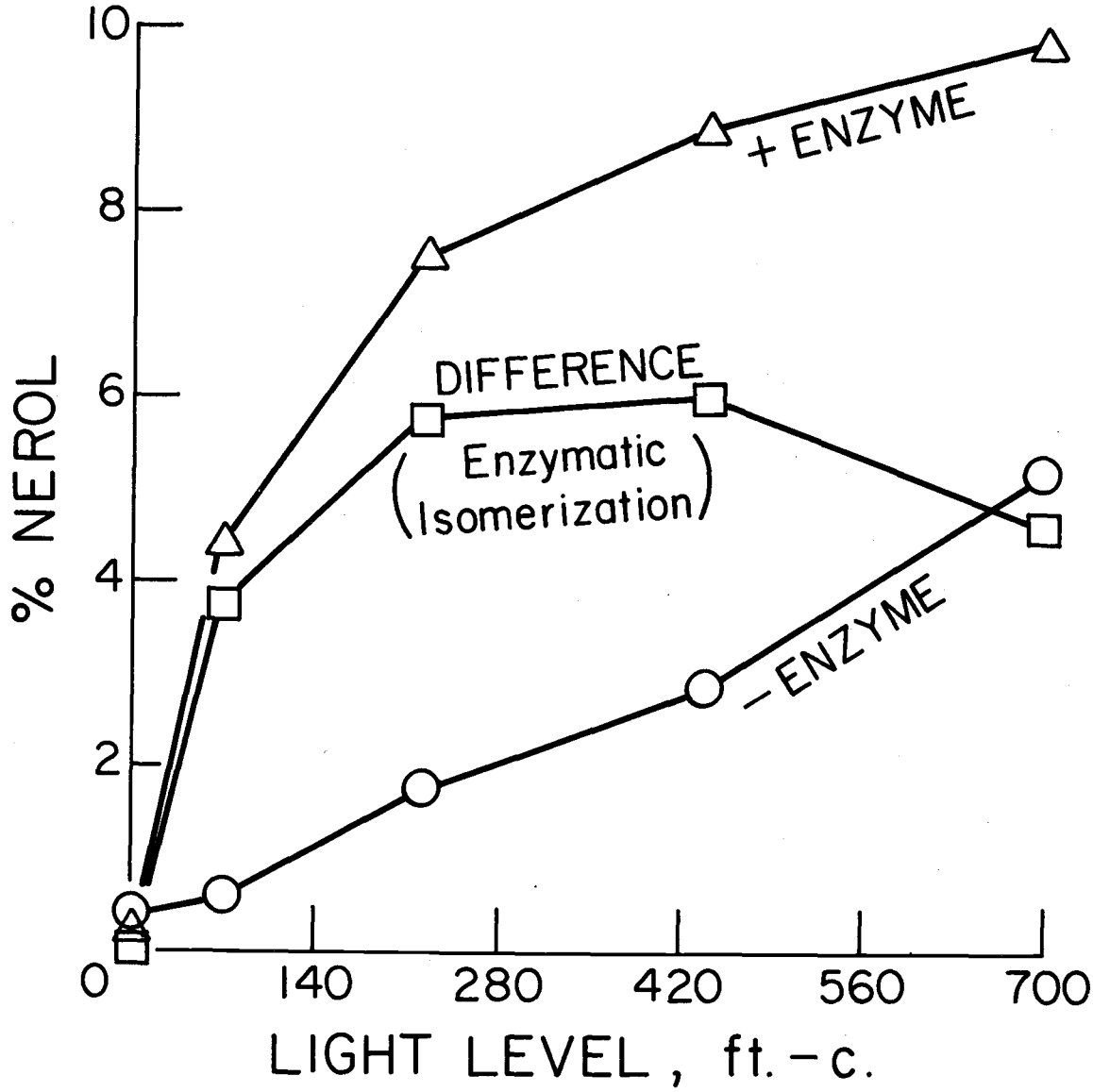

Figure 16. Effect of light on geraniol isomerization by peppermint enzyme. The reaction mixture contained: 8.8 μmoles potassium phosphate buffer; 20 nmoles FAD; 22 nmoles dithiothreitol; 300 nmoles geraniol; and 25 μl enzyme in a total volume of 0.175 ml. The pH was 7.5. Reaction time 60 min; temp. 35°.

400 nm (2E filter) 15% nerol was obtained. The effective wavelengths for the reaction, as indicated in these studies, are therefore in the range of 400-500 nm. A light level of 70 ft.-c. (2E + N.D. 1.00 filters, Figure 16) was selected for assays in order to maximize the ratio of enzymatic to non-enzymatic isomerization.

Isomerization in the Absence of Light. If the flavin is partially reduced (some color remaining) prior to injection into the reaction mixture, rapid isomerization of GerP and geraniol takes place in the absence of light. GerP is isomerized much more rapidly in the presence of partially reduced flavin than in the presence of oxidized flavin (Table 8). Table 9 shows that it has been possible to achieve about half as much geraniol isomerization in the dark as in the light. No dark isomerization takes place in the absence of the sulfur compound (e. g. GSH). Although no attempt was made to optimize enzyme concentrations, a 20-40% increase in dark isomerization of GerP and geraniol is observed in the presence of peppermint enzyme extract as compared with the boiled control (Tables 10, 11). Comparison of the amount of dark and light isomerization in the presence of enzyme at pH 6.5 and pH 7.5 indicated that there is much less pH effect in the dark reaction than in the light reaction -- where oxidized flavin was utilized. These preliminary results concerning dark isomerization may be a first step in elucidating the reaction mechanism of this isomerization.

Table 8. Effect of partially reduced flavin on dark isomerization of geranyl phosphate by peppermint extract.

System	nmoles Recovery				Isomerization	
	Ner	Ger	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 μmoles potassium phosphate buffer; 20 nmoles FAD; 88 nmoles GSH; 130 nmoles GerP; and 20 μl calcium phosphate gel treated enzyme extract in a total volume of 0.175 ml. The pH was 7.5. Light level 70 ft.-c., 2E + 96 (density 1.00) filters, or dark. Reaction time 75 min; temp. 35°.

Table 9. Light versus dark isomerization of geraniol by peppermint extract.

Enzyme System	nmoles Recovery		Isomerization
	Ner	Ger	% 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 μmoles potassium phosphate buffer; 20 nmoles FAD; 88 nmoles GSH; 250 nmoles geraniol; and 10 μl of peppermint extract in a total volume of 0.175 ml. The pH was 7.5. Light level 70 ft.-c., 2E + 96 (density 1.00) filters, or dark. Reaction time 75 min; temp. 35°.

Table 10. Enzymatic versus non-enzymatic dark isomerization of geranyl phosphate.

	nmoles Recovery				Isomerization	
Enzyme System	Ner	Ger	NerP	GerP	% Nerol	% NerP
Boiled control	0	0	5.1	112	0	4.4
Complete	1.7	12	6.8	103	1.4	5.5

The reaction mixture contained: 4.4 μmoles potassium phosphate buffer; 20 nmoles partially reduced FAD; 88 nmoles GSH; 130 nmoles GerP; and 20 μl of calcium phosphate gel treated peppermint enzyme extract in a total volume of 0.175 ml. The pH was 7.5. Dark; reaction time 75 min; temp. 35°.

Table 11. Enzymatic versus non-enzymatic dark isomerization of geraniol.

	nmoles Recovery		Isomerization
Enzyme System	Ner	Ger	% Nerol
Boiled control	10	110	8.2
Complete	18	130	11.8

The reaction mixture contained: 4.4 μmoles potassium phosphate buffer; 20 nmoles partially reduced FAD; 88 nmoles GSH; 180 nmoles geraniol; and 10 μl of peppermint extract in a total volume of 0.175 ml. The pH was 7.5. Dark; reaction time 75 min; temp. 35°.

Isomerase Activity in Carrot and Pea Extracts

The cell-free enzyme extract obtained from carrot tops also contained isomerase activity (Table 12). Activity per gram fresh weight was about twice that found in peppermint leaves. Cofactors necessary for the reaction were the same as those described for peppermint. Some activity was observed if either GSH or FAD was omitted, but almost no activity was detected if both were omitted. Pretreatment of the enzyme extract with NEM again caused an apparent increase in isomerase activity (Table 13).

Table 12. Isomerization of geraniol by carrot extract.

Enzyme system	Terpene recovered (nmoles)	% 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 μmoles potassium phosphate buffer; 20 nmoles FAD; 44 nmoles GSH; 300 nmoles substrate; and 20 μl enzyme extract in a total volume of 0.175 ml. The pH was 7.5. Light level 70 ft.-c., 2E + 96 (density 1.00) filters, or dark. Reaction time 60 min; temp. 35°.

Table 13. Effect of NEM on isomerization of geraniol by carrot extract.

Enzyme system	Terpene recovered (nmoles)	% 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 μmoles potassium phosphate buffer; 20 nmoles FAD; 44 nmoles GSH; 300 nmoles geraniol; and 25 μl enzyme extract in a total volume of 0.175 ml. The pH was 7.5. Light level 70 ft.-c., 2E + 96 (density 1.00) filters. Incubation time 60 min; temp. 35°.

Isomerase activity was also detected in extracts from pea leaves (Table 14). The amount of activity, on a fresh weight basis, was about the same as observed in carrot tops.

The experimental results presented above are consistent with the postulated pathway for the biosynthesis of NerPP (Figure 4). Initial experimental results indicated that geraniol could be formed enzymatically from MVA and that nerol and possibly cyclic monoterpenes were formed from geraniol. Utilizing geraniol and GerP as substrates the isomerization reaction was studied in more detail.

Cell-free enzyme extracts, isolated from peppermint shoot tips, as well as pea leaves and carrot tops, increased the rate of geraniol isomerization and this enhancement was not observed in

boiled controls. This isomerase activity was precipitated by saturated $(NH_4)_2SO_4$, eluted from Bio-Gel P-10 after one void volume, and was retained by an Amicon pressure dialyzer fitted with a UM-10 (10,000 mol. wt. exclusion limit) filter. Isomerase activity was dependent on flavin as well as a sulfur-containing compound.

Table 14. Isomerization of geraniol by pea extract.

Enzyme system	Terpene recovered (nmoles)	% Product
Boiled control	160	5.5
Complete	200	10.9

The reaction mixture contained: 4.4 μmoles potassium phosphate buffer; 20 nmoles FAD; 44 nmoles GSH; 300 nmoles geraniol; and 18 μl enzyme extract in a total volume of 0.175 ml. The pH was 7.5. Reaction time 60 min; temp. 35°. Light level 70 ft.- c., 2E + 96 (density 1.00) filters.

In the absence of added flavin, enzymatic isomerization was observed to occur in the presence of carrot extract but not in the presence of peppermint extract. This difference suggests that the flavin of the carrot enzyme is less easily dissociated during the purification procedure than that of the peppermint enzyme. The peppermint isomerase showed a small preference for FAD over FMN, as indicated by the observation that geraniol isomerization was 30% greater with FAD than when FMN was the added coenzyme.

The amount of isomerization was also observed to vary with substrate. Nerol was isomerized twice as fast as geraniol, the

apparent equilibrium being 67% geraniol and 33% nerol. Geraniol was isomerized twice as fast as GerP. Since GerPP is a common precursor of sterols and higher terpenes (Figure 1), it is probably the natural substrate for the isomerase. However, it was not tested as a substrate because only a small quantity of geraniol-^{14}C of low specific activity was produced biosynthetically and yields of the pyrophosphate obtained by chemical synthesis are low. The results obtained with GerP in this study demonstrate that GerP is a substrate for the isomerase and suggest that GerPP as well as GerP could serve as a substrate. In contrast, neither methyl geranate nor the methyl ester of cis-geranic acid were isomerized.

The requirement for a sulfur-containing compound could not be replaced by reducing agents such as NADH. Furthermore, GSSG also served as an effective cofactor. Pretreatment of the enzyme extract with NEM or PHMB did not reduce isomerase activity.

Of great significance was the observation that the light requirement--originally thought to be absolutely necessary for geraniol, nerol, or GerP isomerization--could be replaced by partial reduction of the flavin. Even under these conditions, however, there was still an absolute requirement for a sulfur-containing compound. Moreover, preliminary results also indicated enhancement of dark isomerization in the presence of the isomerase.

DISCUSSION

The importance of flavins in promoting isomerization reactions was first demonstrated by Posthuma and Berends (57). They observed that riboflavin and a number of other compounds sensitized the photoisomerization of both cis- and trans-stilbene-4-carboxylic acid. Isomerization was proposed to proceed by way of the flavin triplet state, involving triplet-triplet energy transfer. Similarly, Gordon-Walker and Radda (22) reported the flavin-sensitized photoisomerization of both all-trans-retinol and 13-cis-retinol. They also suggested that the flavin triplet was involved. Dihydroflavin has been reported to catalyze the isomerization of all-trans-retinal (17); and the photobleaching of rhodopsin, which involves the cis-trans isomerization of 11-cis-retinal, is also increased in the presence of flavin (23).

Geraniol isomerization also was observed to be photoinduced. Isomerization increased with increasing light intensity up to 225 ft.-c. Light of wavelengths longer than 500 nm was much less effective than 400-500 nm light, consistent with involvement of the 450 nm flavin absorption band. Inhibition of the light reaction was observed, however, if the flavin was extensively reduced with dithionite, DTT, or NADH.

Shiga and Piette (62, 63) observed that production of the flavin triplet itself also depends on light intensity, wavelength, and the state

of flavin reduction. Triplet yield, as indicated by electron paramagnetic resonance (EPR), was reported to be directly related to the flavin absorption spectrum in the visible region and no triplet signal was observed with 528, 560, or 595 nm light (62). Furthermore, when FMN was partially or fully reduced with hydrosulfite, no triplet signal was observed (62). As will be discussed later, it is significant however, that the presence of semiquinone-free-radicals indirectly increased the half-life of the FMN triplet signal, probably by interacting with other flavin molecules in the triplet state (63). It therefore seems likely that if triplet state flavin participates in geraniol isomerization, the presence of some flavin molecules in the semiquinone state may increase isomerization by increasing triplet life-time, thus allowing more time for energy transfer between the flavin triplet and geraniol. Thus, the primary function of light may be the formation of the flavin triplet state; a secondary function would be the formation of semiquinone-radicals which would in turn stabilize the triplet state of other flavin molecules.

These semiquinone-radicals can be formed by anaerobic photoreduction, either directly or by comproportionation ($FlH_2 + Fl \rightarrow 2\,\dot{F}lH$). Photoreduction can take place intramolecularly and in the pH range 4-10 can involve a triplet mechanism (6, 59). Photoreduction can also take place intermolecularly with hydrogen donors such as EDTA (45, 59). Reduction of riboflavin by

dihydrolipoic acid, however, is not affected by visible light (19).

The observation that geraniol isomerization also occurred in the absence of light--if the flavin was partially reduced prior to incubation--poses a perplexing problem: if the flavin triplet state is involved in isomerization how is it produced in the dark? Since partial reduction of the flavin was necessary in order for extensive isomerization to occur, one of the reduced flavin states must be involved.

Excited electronic states can be generated in the absence of light in the process of oxidation or reduction. For example, oxidation of the phenanthrene radical-anion results in the production of phenanthrene in the triplet state (42). Electronic-excited states have also been detected--by chemiluminescence--in a system containing riboflavin, Cu^{++}, H_2O_2, and β-mercaptoethanol (72). The latter system differs from the isomerase system in two important respects: first, there is an absolute requirement for H_2O_2 or O_2 and secondly, ascorbic acid can be substituted for mercaptoethanol (72). These two examples--the phenanthrene triplet and chemiluminescence--suggest that the flavin triplet could be produced in the dark but the mechanism involved is not clear. Since a large amount of dark isomerization is observed only after partial reduction of the flavin, it is possible that oxidation of reduced flavin by trace amounts of oxygen could result in the

production of flavin triplets.

On the other hand, the flavin-semiquinone itself, not the flavin-triplet, could be responsible for geraniol isomerization. One possible mechanism for this reaction would be the temporary formation of a C-N bond between C-2 of geraniol and N-5 of the semiquinone, one electron for the bond being donated by each. The second electron from the double bond would reside on C-3 of geraniol, thus forming a tertiary radical. Alternately, an excited semiquinone state -- the doublet state -- could be involved in the isomerization reaction. The observation that neither geranic acid nor cis-geranic acid were isomerized may aid in determining the reaction mechanism.

The sulfur-containing compound, which was also found to be essential for isomerization, is not acting simply as a reducing agent. Sodium ascorbate, NADH, reduced flavin, or EDTA could not replace the requirement for a sulfur compound. Furthermore, the sulfur compound was necessary both in the light reaction (Figure 14) and in the dark reaction (Table 9). Therefore, it must serve some other function.

One possible function could be the stabilization of the flavin in a particularly reactive state. Sulfite and β-mercaptoethanol have been shown to have very different effects on the flavoprotein adenylyl sulfate reductase as indicated by the EPR signal detected (46). A 10:1 excess of sulfite (moles per mole enzyme-bound FAD) was

necessary in order to produce an EPR radical signal corresponding to 0.5% of the flavin. If a 1:2 ratio of β-mercaptoethanol to bound FAD was used instead of sulfite, a radical signal corresponding to 12% of the flavin was observed, and the signal was similar to that which one would expect from a flavin semiquinone (46). It appears, therefore, that the sulfur compounds necessary for isomerization may be increasing the amount of semiquinone present. Other information suggests that this stabilization might be achieved by an association between the flavin and the sulfur compound. Muller and Massey (49) have shown that sulfite binds to the N-5 position of flavins and Draper and Ingraham (12) demonstrated that disulfides (GSSG or cystine) have a strong affinity for flavin-semiquinones. Significantly, mercaptans do not bind to oxidized flavins in the absence of light (49) and disulfides have almost no affinity for either fully oxidized or fully reduced FMN (12).

The studies described above suggest that the sulfur compounds may function in one of two ways. First, they may stabilize a flavin-semiquinone. If the triplet mechanism is involved in isomerization these semiquinones could: (1) extend triplet life-time as previously discussed; (2) extend triplet life-time because there is proportionately less triplet quenching by oxidized flavin (69); and (3) be a substrate for triplet formation in the dark. Second, if isomerization does not involve the triplet state, the sulfur compounds might function in the

association-dissociation reaction between geraniol and the flavin-semiquinone. In the non-enzymatic system the sulfur compounds would act directly but in the enzymatic system they could also reduce an essential disulfide group or activate a cysteine residue of the enzyme.

Although sulfur compounds are essential for isomerization, inhibition of isomerase activity with sulfhydryl-blocking reagents could not be demonstrated. Neither NEM (which forms a stable reaction product) nor PHMB pretreatment caused a decrease in enzymatic isomerization. In fact, NEM preincubation caused an apparent increase in enzymatic activity (Table 13). This stimulation was perhaps due to inhibition of other interfering enzymes present in the extract. On the other hand, if an excess of NEM was present during the reaction period, no isomerization occurred. Apparently, NEM reacted with the sulfur-containing cofactor (e. g. GSH) and possibly with the isomerase itself. It was also demonstrated that oxidized glutathione was an effective sulfur compound in the light reaction, although it was less effective than GSH. However, photo-reduced flavin probably reduces GSSG to GSH, the latter being the active form. This reduction was suggested by the observation that when both NEM--which does not react with disulfides--and GSSG were added to the reaction mixture, isomerization was inhibited as compared with GSSG alone (Table 7).

Furthermore, it was not possible to inhibit the isomerase by preincubation with NEM or PHMB. It is possible, however, that the enzyme contains a cysteine or cystine group which is necessary for enzymatic activity. For example, it was not possible to demonstrate enzymatic activity when sulfite was the sulfur compound added. This suggests that sulfite does not activate the isomerase. Sulfite is also known to react with disulfides (8). Numerous examples of masked cysteine or cystine groups have been reported. The sulfhydryl titer of the flavoprotein glutathione reductase, for example, increased by one after treatment with sulfite (44) and it was suggested that sulfite ruptured an active center disulfide. Similarly, when glutathione reductase was preincubated with 5 mM NEM, no decrease in activity was observed but preincubation with NEM plus NADPH--which reduces the disulfide--resulted in 100% inactivation (74). Thus only after the disulfide was reduced by NADPH treatment was NEM inhibition observed. Likewise, two sulfhydryl groups of rhodopsin were masked by associated phospholipid and two others reacted with PHMB only after bleaching by light (78). These examples of masked cystine and cysteine groups suggest that the active center of geraniol isomerase could contain one of these groups even though enzymatic activity could not be inhibited by preincubation with NEM or PHMB.

A number of reactions could be contributing to the enzymatic activity peak observed between pH 7.5 and 7.7. For example,

anaerobic reduction of riboflavin by dihydrolipoic acid increases with increasing pH since the reducing species is the sulfur anion (19). That the reductive step contributes to the pH dependence in the pH range 6.5 to 7.5 is also suggested by the observation that there is less pH effect in this range for dark isomerization. Binding of flavin to the apoenzyme could also be dependent on the state of flavin reduction. Finally, an increased degree of flavin phosphate ionization with increasing pH could contribute to the pH effect by increasing flavin binding to apoenzyme (68).

Other reactions may decrease the extent of isomerization above pH 7.7. For example, the pK_a of the N-5 hydrogen of the neutral FMN-semiquinone is 8.6 (48)--the semiquinone-anion being formed by the dissociation. Therefore, the decrease in isomerase activity above pH 7.7 may indicate that it is the neutral-semiquinone that is the active flavin cofactor. Alternately, the isomerase itself could stabilize a certain reactive species of the flavin and this stabilization could be pH dependent. For example, a positively charged amino acid residue with a pK of 8.2 has been postulated to stabilize the anion radical form of the flavin coenzyme of D-amino acid oxidase (43). This type of stabilization could also account for the observed increase in non-enzymatic isomerization in the presence of protamine, which contains a large percentage of basic amino acids. Finally, trace amounts of oxygen could be contributing to the decrease in

activity above pH 7.7. The semiquinone anion reacts much more rapidly with oxygen than the neutral semiquinone does (13).

Since pH also affects triplet life-time, isomerization dependence on pH is to be expected, if the flavin triplet is involved. The half-life of the FMN triplet, for example, has been reported to be greatest at pH 7 (62).

Various substances present in the initial enzyme extract, as well as oxygen, inhibited isomerization. Oxygen is known to be an efficient triplet quencher as well as an inhibitor of isomerization reactions (23). Initial studies showed that geraniol and/or nerol were oxidized to water soluble products in the presence of oxygen, and it was observed that more of these side-products were formed with FMN than with FAD. Other studies have shown similar results: in the presence of oxygen and light, oxidation of GSH was sensitized by FMN but not by FAD (64); indolacetic acid was destroyed more rapidly in the presence of oxygen and light when FMN, rather than FAD, was the cofactor added (50); and D-amino acid oxidase was inactivated when incubated aerobically in the light with FMN but not when incubated with FAD (53). Oxygen may also inhibit by oxidizing reduced flavin, since the latter was found to be necessary for dark isomerization.

Inhibitors which were present during early stages of enzyme purification could decrease geraniol isomerization by interacting

with the flavin. For example, EDTA, which was present during the initial stages of enzyme purification, has been observed to cause an increased rate of flavin semiquinone oxidation by oxygen (13). Alternately, excessive photoreduction of the flavin may occur in the presence of EDTA and light. That this type of inhibition could occur was suggested by an 85% inhibition of isomerization after the addition of 1.4 mM NADH. EDTA could also inhibit by chelating metal cofactors but none of the other results suggested a metal requirement.

Substances present in the peppermint leaf could also cause inhibition. Paramagnetic metal ions could cause inhibition of isomerization by quenching the flavin triplet (57). Also, phenolic compounds are abundant in peppermint leaves and it was demonstrated that pyrocatechol inhibited geraniol isomerization in the light or dark. This inhibition could be the result of triplet quenching (58, 69) or competitive inhibition involving geraniol and nerol. Phenolic compounds could also cause general tanning of the enzyme.

This study establishes that both geraniol and GerP can be isomerized enzymatically to form the corresponding cis isomers, nerol and NerP, and that nerol can be isomerized to geraniol. These isomerizations were shown to occur only in the presence of flavin and certain sulfur-containing compounds. Isomerization occurred: (1) in the absence of light if the flavin was partially reduced chemically prior to incubation, or (2) in the presence of light. Although

isomerization was also observed to occur non-enzymatically, non-enzymatic isomerization as well as a requirement for a thiol containing compound have been reported for other isomerase systems (14, 27, 61, 67).

Geraniol isomerization may require flavin in the triplet state, as has been suggested for other flavin-dependent isomerizations (22, 57). Substances which quench the triplet state, such as oxygen and pyrocatechol, inhibited geraniol isomerization. Formation of the triplet state in the absence of light is postulated to depend on the oxidation of reduced flavin. Alternate isomerization mechanisms could involve the doublet-semiquinone state, or simply the unexcited flavin-semiquinone itself.

The significance of the results is best illustrated by referring to Figures 1 and 4. As postulated in these schemes, cyclic monoterpenes are formed from MVA via GerPP and NerPP. Trans-cis isomerization of GerPP to NerPP is a key reaction of the postulated pathway; this branch point may determine whether higher terpenes or cyclic monoterpenes are formed. This study does not establish but strongly suggests that GerPP is also a substrate for the isomerase which would permit the direct biosynthesis of NerPP from GerPP.

The results presented in this study should aid in elucidating the reaction mechanism, and the function of the isomerase in the reaction. Furthermore, the isomerase would be expected to be

present in peppermint and carrot plants, which form large amounts of cyclic monoterpenes, but further study will be necessary in order to determine whether cis bonds in other isoprenoid compounds, such as gossypol precursors (25) and abscisic acid, are formed by a similar enzyme. If this is the case such trans-cis isomerases would presumably occur universally in higher plants.

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