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PRENYLTRANSFERASES FROM THE FLAVEDO OF *CITRUS SINENSIS*

MILTON DE LA FUENTE,* LUZ M. PÉREZ, UTA HASHAGEN, LILIANA CHAYET, CECILIA ROJAS, GLORIA PORTILLA† and OSVALDO CORI

Laboratorio de Bioquímica General, Facultad de Ciencias Químicas y Farmacológicas, Universidad de Chile; *Dep. de Fisiología y Biofísica, Facultad de Medicina, Universidad de Chile; †Departmento de Química, Facultad de Ciencias, Universidad de Chile, Casilla 233, Santiago 1, Chile

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Key Word Index—*Citrus sinensis*; Rutaceae; prenyltransferases; neryl pyrophosphate; geranyl pyrophosphate; farnesol; stereospecificity.

Abstract—A prenyltransferase activity (EC 2.5.1.1) has been partially purified from the flavedo of *Citrus sinensis* with 30–40-fold purification and 35–60% yield. The enzyme catalyses the condensation of IPP with DMAPP or GPP. The products are neryl and geranyl pyrophosphate as well as (2*E*,6*E*)- and (2*Z*,6*E*)-farnesyl pyrophosphate. The two C_{15}-products are predominant. The *E*- and *Z*-synthetase activities are partially dissociated during the purification procedure, as well as by heat or ageing. Preparations devoid of *Z*-synthetase were obtained. Mg^{2+} is required for full activity. Mn^{2+} or Co^{2+} can replace Mg^{2+}. The ratio of *E*/*Z*-products formed is different for each cation. Mg^{2+} complexes of allylic substrates or of products protect the enzyme against heat-inactivation and against inactivation by DTNB. The results are interpreted in terms of two or more prenyltransferases stereoselective for the synthesis of *E*- and *Z*-products.

INTRODUCTION

In the process of isoprenoid biosynthesis, the chain is lengthened by a condensation reaction of an allylic pyrophosphate with the olefinic C_4 of IPP,‡ catalysed by prenyltransferases (EC 2.5.1.1).§ Plant tissues contain a variety of these enzymes, differing in their specificity towards chain length [3–7] and conformation of products [8]‖. The reaction is mediated through carbocations [9] (Scheme 1) or ion pairs and is terminated by the stereospecific elimination of a proton from C_2 of IPP [10]. Elimination of the *pro-R* proton from C_2 of IPP has been described to lead to the formation of *E*-double bonds, and the opposite proton is eliminated when a *Z*-product is formed [10]. However, there are reports of the elimination of the *pro-R* proton with formation of both *E*- and *Z*-products in *Pinus* [11, 12], *Rosa* [13] and *Citrus* [11]. This has been interpreted in terms of a stereospecific synthesis of *E*-products followed by an *E*–*Z*-isomerization of the pyrophosphate [12–14], an assumption for which there is no experimental evidence [15–18] although allylic alcohols are isomerized through a redox process [16, 17, 19, 20].

In the absence of *E*–*Z*-isomerization, diastereomeric allylic pyrophosphates can be formed only by stereospecific chain lengthening. We have investigated the existence and properties of one or more stereospecific prenylsynthetases in the flavedo of *Citrus sinensis*, where both *E*- and *Z*-isomers are formed.

RESULTS AND DISCUSSION

Partial purification of prenyltransferases

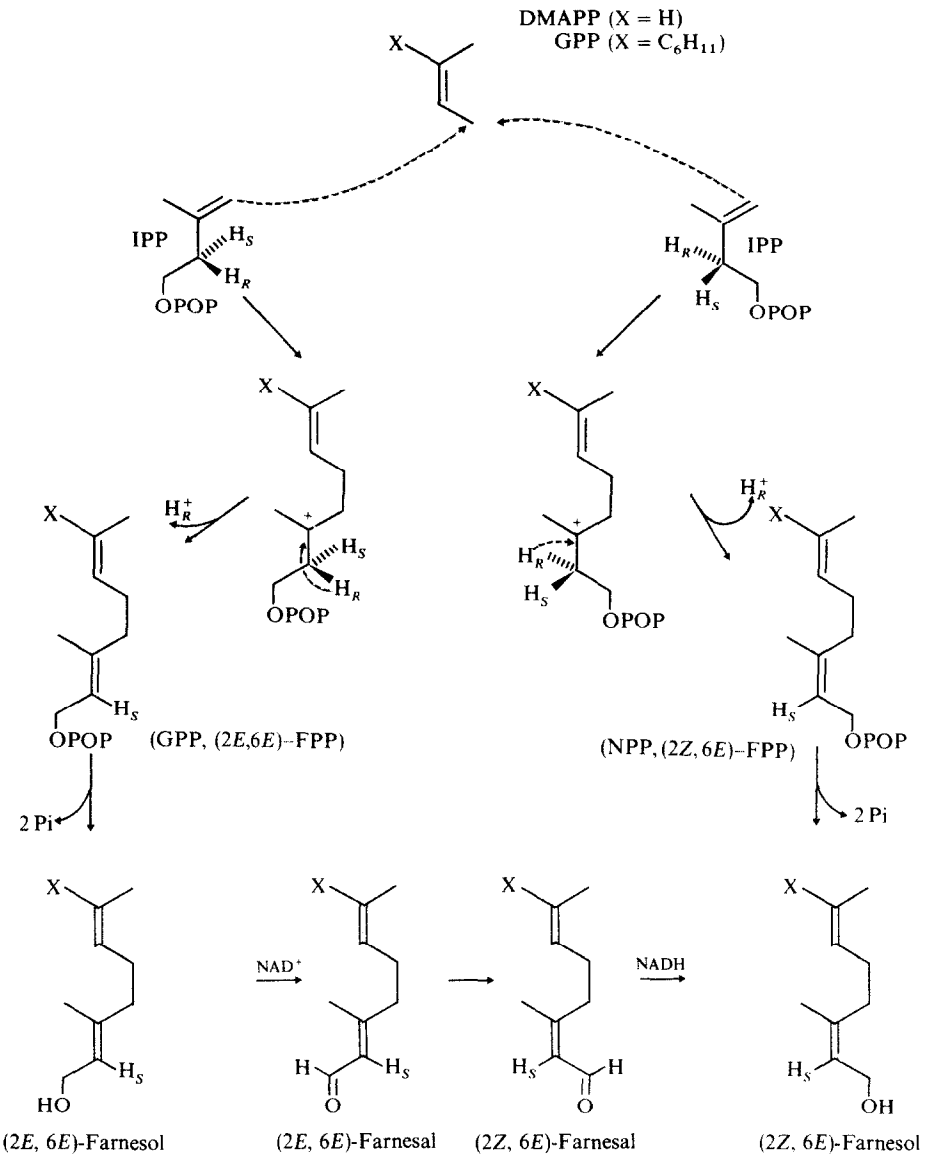
The starting material was an aqueous extract of an acetone powder from orange flavedo. It contained, in addition to prenyltransferases, all the enzymes necessary to transform mevalonic acid into GPP, NPP, (2*E*,6*E*)-FPP and (2*Z*,6*E*)-FPP. It also contained a number of acid phosphatases [21] as well as the enzyme system required for the redox isomerization of farnesols [15]. The specific activity of prenyltransferase from this acetone powder was only 50% of the activity in an aqueous extract from fresh orange flavedo. Its stability, the lower absorbency at 310 nm and the drastic reduction in essential oils made this powder an adequate starting material for the study of prenyltransferases from *C. sinensis*. The powder could be stored for at least 3 years with little or no loss of total prenyltransferase activity.

Table 1 summarizes the purification procedure. After purification by DEAE-cellulose chromatography, IPP

‡Abbreviations: IPP, isopentenyl pyrophosphate (C_5); DMAPP, 3,3′-dimethylallyl pyrophosphate (C_5); GPP, geranyl pyrophosphate ($C_{10}E$); NPP, neryl pyrophosphate ($C_{10}Z$); (2*E*,6*E*)-FPP, (2*E*,6*E*)-farnesyl pyrophosphate (C_{15}); (2*Z*,6*E*)-FPP, (2*Z*,6*E*)-farnesyl pyrophosphate (C_{15}); DTT, dithiothreitol; PCMB; *para*-chloromercuribenzoate; DTNB, 5,5′-dithiobis(nitrobenzoic acid):

§The term 'prenyltransferase' has been used in order to conform to the Enzyme Commission nomenclature. However, in the discussion of conformational and chain length specificity of the product, we will sometimes use the less ambiguous term 'synthetase'. For instance 'C_{15}-*Z*-synthetase' forms a C_{15} product with *Z*-conformation, whereas a '*Z*-transferase' would be expected to transfer a prenyl unit to a *Z*-substrate (NPP), which is not the case in the *Citrus* or *Pinus* enzymes. We will use the terms 'C_{10} or C_{15}', '*E*- or *Z*-prenylsynthetases' according to chain length and stereochemistry of the product formed.

‖*E*- and *Z*-isomers will be referred to as diastereoisomers of different 'conformation' [1, 2].



Scheme 1. Biosynthesis of diastereomeric prenols through stereospecific chain lengthening of allylic pyrophosphates or redox isomerization of prenols.

isomerase could not be detected in the enzyme preparation unless assayed under optimal conditions for this enzyme [22], in which case its activity was only 8% of the transferase activity. The purified preparations showed a single asymmetric peak on electrophoresis on cellulose acetate at pH 4.4 or 8.8 [23]. Similar amounts of four components were evident on acrylamide electrophoresis at the same pH values or through electrofocusing [24]. The highest specific activity reported for a prenyltransferase is 87 nkat/mg of protein [25]. Our partially purified preparation had a specific activity of 0.4 nkat/mg, which is comparable to the value of 0.7–3.0 nkat/mg reported for the enzymes from *Ricinus* [26], which had been purified by a factor of 700–1000. All other reports of prenyltransferase activity range between 10 and 20 nkat/mg, but the initial specific activity, as well as the amount of protein processed in *Saccharomyces* [25], avian liver [27, 28] and *Gossypium hirsutum* [8] preparations, is higher than in *C. sinensis*. The 40-fold purified enzyme was stable up to 1 year when freeze-dried and stored at −20°. Dissolved in water and stored at 4°, it had a half life of 2 weeks. Increased stability (Table 1) is attributed to the dissociative gel filtration step with ammonium sulphate, [21] which is modified from Kirkegaard's procedure [29]. It eliminates flavonoids, carotenoids and other pigments and possibly an inhibitor which would account for the yield of more than 100%. Prenyltransferase activity and absorbancy at 280 nm were neatly separated in this step by at least 0.15 column volumes. Marked seasonal and annual variations were observed in the initial specific activity of the acetone powder as well as in the purification ratio.

Table 1. Purification procedure for prenyltransferases

	Volume (ml)	Protein (mg/ml)	Specific activity (nkat/mg)	Total units (nkat)	Purification	Yield (%)	Activity ratio* C_{10}/C_{15}	Product ratio C_{15} E/C_{15} Z
Aqueous extract from acetone powder	726	1.5	0.014	15.25		100	30–100	12
$(NH_4)_2SO_4$ fraction 45/65	27	13.6	0.02	7.34	1.4	49	40–50	—
Sephadex G-100 dissociative filtration	102	2.9	0.058	17.15	4.2	113	40–100	—
Concentrated from Sephadex G-100	37	6.0	0.06	13.32	4.6	93	—	—
DEAE-cellulose	88	0.16	0.4	5.63	30.0 (30–42)†	38 (35–60)	43–125	1 (1–4)

*Activity ratio C_{10}/C_{15} is the ratio of total allylic phosphates formed from DMAPP (C_5) or from GPP (C_{10}) when used as unlabelled allylic substrate.

†The figures in parentheses indicate fluctuation of purification, yield and E/Z ratio in all preparations.

Substrates and products

Prenyltransferase from *C. sinensis* utilized either DMAPP or GPP as substrates to form both C_{10}- and C_{15}-products from the former and only C_{15}-products from the latter substrate. When DMAPP was the substrate, C_{15}-products were predominant. No C_{20}-products were detected. The ratio of C_{15}/C_{10}-products did not change substantially during the purification procedure. Apparent K_m values were 0.73 μM for GPP and 4.9 μM for IPP. The accurate value for DMAPP cannot be established with certainty without separation of products, since the labelled GPP formed is in turn the substrate for the next condensation. As previously established for *P. radiata* [30], NPP was not a substrate. This, and the fact that no (6*Z*)-sesquiterpenoids were formed [15], is a difference between prenylsynthetase form *C. sinensis* and the enzyme from *G. hirsutum* which forms all four diasteromers [8] of FPP. This is also further evidence against isomerization of GPP or NPP which has been systematically ruled out in this and in all our previous enzyme preparations [19, 21], as well as in the formation of cyclic monoterpenes from GPP in *Foeniculum vulgare* [31]. Prenyltransferase from orange flavedo did not utilize the non-allylic C_{10}-analogue citronellyl pyrophosphate or the phosphomonoesters of geraniol or nerol. This agrees with the substrate specificity of prenyltransferases from other sources [32]. Prenyltransferase preparations formed four hexane-soluble allylic products from DMAPP. These were geraniol, nerol, (2*E*,6*E*)-farnesol and (2*Z*,6*E*)-farnesol, as shown by the distribution of radioactivity on GLC of the products or of their TMSi derivatives. Polar products in the aqueous phase were identified as GPP, NPP, (2*Z*,6*E*)-FPP and (2*E*,6*E*)-FPP. This was demonstrated by GLC/RC analysis of the alcohols obtained by enzymic hydrolysis of the aqueous phase or by analysis of the TMSi derivatives of these alcohols.

Formation of sesquiterpene aldehydes

The aqueous extract of an acetone powder from orange flavedo formed the two isomeric sesquiterpene aldehydes (2*E*,6*E*)-farnesal and (2*Z*,6*E*)-farnesal from GPP plus [4-^{14}C]IPP only in the presence of 0.28 mM NAD^+. We have previously described [15] the formation of sesquiterpene aldehydes by an aqueous extract from fresh orange flavedo, but the system differed from the present one in several aspects. Aldehydes were formed from GPP + IPP by the extract from fresh flavedo in the absence of added NAD^+. The sequence of product appearance was also different from the present one: whereas in the extract from acetone powder (2*E*,6*E*)-farnesol and (2*Z*,6*E*)-farnesol appeared almost simultaneously, the time required for the appearance of the 2*Z*-compound in the extract from fresh flavedo was about 18 times longer than for the 2*E*-isomer. Furthermore, the addition of 8 mM aniline, which prevented the redox isomerization of prenols by trapping aldehydes [15], did not affect the formation of (2*Z*,6*E*)-farnesol or of (2*Z*,6*E*)-FPP in the present system. Based on these differences, it may be concluded that in orange flavedo (2*Z*,6*E*)-farnesol may be formed through the two pathways depicted in Scheme 1: stereospecific synthesis followed by enzymic hydrolysis of (2*Z*,6*E*)-FPP or redox isomerization of (2*E*,6*E*)-farnesol derived from (2*E*,6*E*)-FPP. In the acetone powder extract, the removal of endogenous NAD^+ favours the former route. This view is consistent with the reported evidence of stereospecific loss of *pro-S*-[^{3}H] from C_4 of mevalonic acid in the formation of both farnesols and with the loss of 50% of ^{3}H from C_1 of farnesol in the redox process of *E–Z-interconversion of prenols* [33]. The results cited do not rule out the co-existence of the stereospecific synthesis of prenyl pyrophosphates with the redox isomerization of prenols, since they were obtained with crude extracts from *Andrographis paniculata*, where the redox interconversion of the alcohols may be predominant. It is also worth stressing that our prenyltransferase preparation forms not only the *E*- and *Z*-prenols, but also their phosphorylated derivatives.

Changes in product ratio

The ratio of *E*/*Z*-products varied largely from one acetone powder to another, generally being of the order of

12 in the extract (Table 1). There were important seasonal variations. In winter, prenyltransferase was highest and formed both diastereoisomers. In spring, the acetone powder practically did not contain any *Z*-prenylsynthetase and the total activity was very low. The *E*/*Z* ratio in the DEAE-cellulose fraction differed from the ratio in the extract and was between one and four (Fig. 1). The *Z*-prenylsynthetase was very unstable in all late fractions.

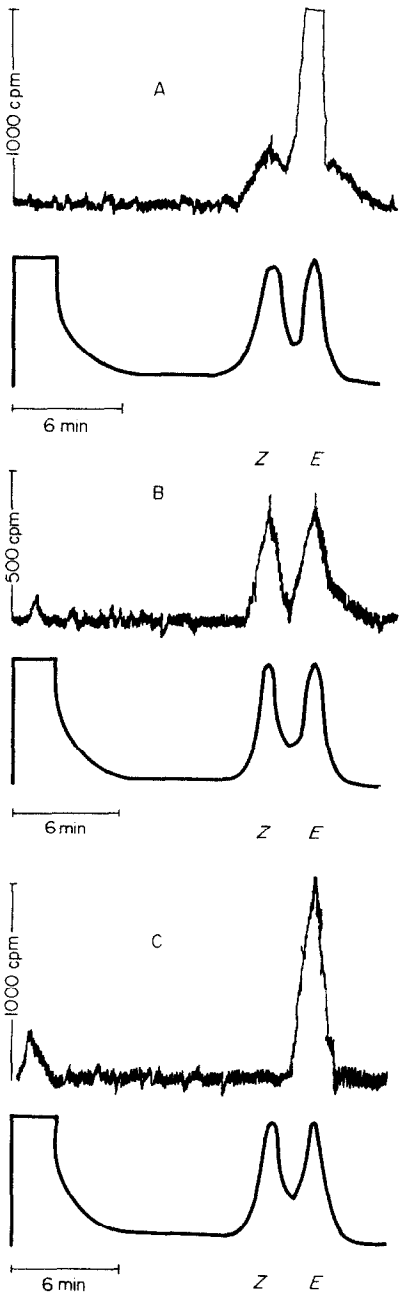


Fig. 1. Analysis of C_{15} pyrophosphates formed by prenyltransferase. A, crude extract; B, DEAE-cellulose fraction; C, DEAE-cellulose fraction heated at 60° or aged at 4°. Upper trace: radioactivity; lower trace: carrier peaks. *Z*: (2*Z*,6*E*)-Farnesol; *E*: (2*E*,6*E*)-farnesol. The unrearranged prenols for GLC/RC analysis were obtained from the phosphorylated products of prenyltransferase by enzymic hydrolysis (prenylphosphates).

We have been unable to purify it further or to obtain preparations of *Z*-prenylsynthetase devoid of *E*-activity, although the opposite dissociation has been achieved frequently.

Prenyltransferase had a pH optimum of 7.5 with half maximum activities at 6.8 and 7.8. No change in product ratio was observed at different pH values. Mg^{2+} was required for maximum activity. Mn^{2+} and Co^{2+} had 32 and 50% of the activity of Mg^{2+}. Optimum activities were observed at concentrations of 2 mM Mg^{2+}, 1 mM Mn^{2+} or 3 mM Co^{2+}. Half optimum activities were observed at 0.9 and 30 mM for Mg^{2+}, 0.5 and 5 mM for Mn^{2+}, and 1.2 and 10.5 mM for Co^{2+}. Ni^{2+} was a very poor cofactor and Ca^{2+} was ineffective. The *E*/*Z* ratio of C_{15}-products formed from GPP + IPP changed in the presence of different metals. With Mg^{2+} and Mn^{2+} this ratio was 4. With Co^{2+}, the ratio increased to 8 and no *Z*-products were formed in the presence of Ni^{2+}. This differential effect of metals could be interpreted in terms of the existence of two or more stereospecific prenyltransferases which could differ in their metal specificity. However, it is also possible to visualize a single enzyme generating carbocations whose geometry could be defined by the nature of the bound metal ion. Ni^{2+} would allow only a conformation leading to *E*-products, whereas the other metals could be less effective in freezing the intermediate carbocation.

The heating of purified prenyltransferase at 60° for 10 min at pH 7.4 completely inactivated the enzyme (Fig. 2). IPP, GPP, Mg^{2+} or DTT could not protect it from inactivation, but if the enzyme was heated in the presence of 10 mM Mg^{2+} plus 22 μM GPP it retained almost its full activity (Fig. 2). The products formed by the enzyme were analysed, and only the *E*-activity was retained after heating in the presence of GPP–Mg (Fig. 1). Something similar happened when the enzyme was aged at 4° for 1 month in the presence or absence of GPP–Mg (Fig. 1).

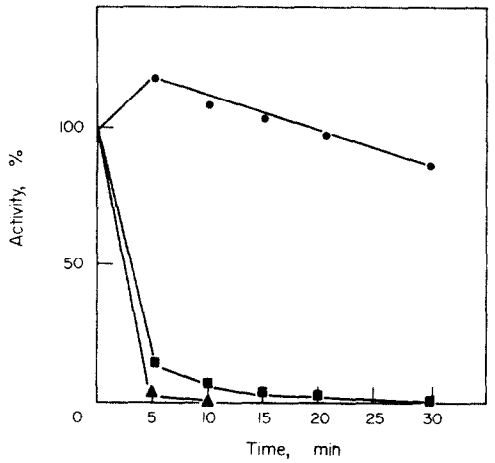


Fig. 2. Protection of prenyltransferase from heat inactivation by Mg–GPP. Heat inactivation was carried out for variable time intervals at 60° in 100 mM Pi buffer (pH 7.4). Protein concentration was 40 μg/ml. The reaction was stopped by cooling the tubes to 0° and prenyltransferase was assayed as described in the Experimental. ▲—▲, No addition or addition of 22 μM GPP or of 1 mM DTT; ■—■, addition of 10 mM $MgCl_2$; ●—●, addition of 22 μM GPP + 10 mM $MgCl_2$.

Inhibitors and inactivators

These effects were studied with GPP + IPP as substrates. Table 2 shows that NPP and the non-allylic analogue citronellyl pyrophosphate were the most effective inhibitors of *E*-prenylsynthetase from *Citrus sinensis*. The presence of an esterified pyrophosphoryl group contributes to binding more than the structure of the organic moiety, since allylic phosphomonoesters are less effective inhibitors than the C_2–C_3-saturated C_{10}-pyrophosphate.

Incubation of purified *E*-prenylsynthetase with 40 μM PCMB produced an inactivation with a half life of 15 min. The process could not be reversed by the addition of 1 mM DTT. In contrast with this, DTNB produced a comparable rate of inactivation at a concentration of 4 μM. This curve exhibited a second slower phase, even in the presence of 120 μM reagent, which shows that the shape of the curve was not due to limiting concentrations of the inhibitor (Fig. 3B). An 80% reactivation was achieved by incubating the enzyme with 1 mM DTT. This agrees with the requirement of intact SH groups reported for other prenyltransferases [4, 26]. As shown in Fig. 3A, the Mg^{2+} complex of GPP protected the enzyme from DTNB inactivation whereas this rate was increased by free GPP. Mg^{2+} alone had no effect. Since free GPP and the Mg–GPP complex have opposite effects on the rate of inactivation, it may be assumed that they may cause different conformational changes when binding the enzyme. The association constant of Mg–GPP is of the order $7 \times 10^3\ M^{-1}$ [33] and this allows us to estimate that under our experimental conditions more than 95% of the substrate was in the GPP–Mg form. Furthermore, Mg^{2+} increases the binding of GPP to avian liver prenyltransferase [34] by a factor of 12. These facts, the opposite effects of free and bound GPP on the inactivation rate by DTNB, and the protection from heat inactivation only by Mg–GPP, are consistent with the view that Mg–GPP is the true substrate of prenyltransferases.

Table 2. Effect of various inhibitors on prenyltransferase

Compound	$(I)_{0.5}/K_m$*
Citronellyl pyrophosphate	11
Neryl pyrophosphate	25
Geranyl monophosphate	50
Octyl pyrophosphate	60
Neryl monophosphate	70
Inorganic pyrophosphate	250

*$I_{0.5}$ is the concentration of inhibitor required to inhibit the enzyme reaction by 50% at saturating substrate concentrations. It is expressed as a ratio referred to the apparent K_m for GPP of 0.73 μM. This ratio is equivalent to K_i/K_m in a non-competitive inhibition and to $\frac{K_i}{K_m}\left(1 + \frac{S}{K_m}\right)$ in a competitive inhibition.

The results presented show that prenyltransferases from orange flavedo form C_{10}- and C_{15}-prenyl pyrophosphates, but do not catalyse any further chain lengthening. The products may have *E*- or *Z*-conformation around a 2–3 double bond. This is a stereochemical biosynthesis and not an isomerization. Partial dissociation of the activities catalysing the formation of the two diastereomeric products suggests that *E*- and *Z*-prenylsynthetases may be different enzymes. Metal specificity points in the same direction, but this may not be ascertained until complete separation of these two enzymes is achieved.

The presence of mono- and sesqui-terpenoids of *E*- and *Z*-conformation in orange essential oils could be the result of the activity of these product stereospecific prenyltransferases followed by the hydrolysis of the isomeric pyrophosphates catalysed by prenyl phosphatases [21].

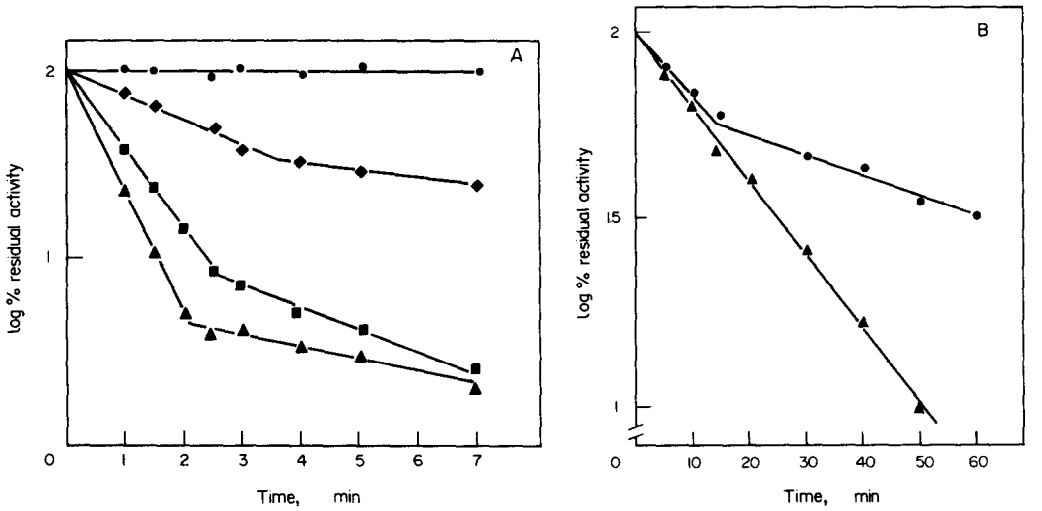


Fig. 3. Inactivation of prenyltransferase by DTNB or PCMB. The enzyme (0.6 mg/ml protein) was incubated at 0° for different time intervals in 100 mM Pi buffer (pH 7.4) in the presence or absence of additions as indicated. The reaction was stopped by diluting an aliquot in the assay medium, and activity was measured as described in the Experimental. DTNB or PCMB at the concentrations resulting from dilution have no inhibitory effect on enzyme activity. (A) ●—●, No addition; ■—■, addition of 0.8 mM DTNB or of 0.8 mM DTNB + 10 mM $MgCl_2$; ▲—▲, addition of 0.8 mM DTNB + 22 μM GPP; ♦—♦, addition of 0.8 mM DTNB + 22 μM GPP + 10 mM $MgCl_2$. (B) ●—●, 4 μM DTNB; ▲—▲, 40 μM PCMB.

EXPERIMENTAL

Substrates. GPP, NPP, DMAPP and citronellyl pyrophosphate were prepared by phosphorylation [35] of the corresponding alcohols. The pyrophosphates were separated by ion exchange chromatography [36].

Enzyme preparation. The Me_2CO powder from orange flavedo was prepared as described before [21] and homogenized for 3 min at 0° in an Omnimixer with 15 vol. 0.1 M K-Pi buffer (pH 7.4) containing 10% glycerol and 0.1 mM DTT, per g of powder. The homogenate obtained was strained through cheesecloth and centrifuged for 20 min at 25 000 *g* and 0°. EDTA was added to the extract up to 45 mM final concn, and proteins were precipitated between 45 and 65% saturation of $(NH_4)_2SO_4$. (The temp. must not exceed 4° during this procedure [37]). The ppt. obtained was dissolved in a vol. of 0.1 M K-Pi buffer (pH 7.4) containing 40 mM EDTA, 0.1 mM DTT and 10% glycerol equivalent to 1/25 of the vol. of the extract. The $(NH_4)_2SO_4$ fraction was applied to a 1.2 × 100 cm Sephadex G-100 column equilibrated with 10 mM K-Pi buffer (pH 7.4) containing 2 mM EDTA and 0.1 mM DTT. The sample was allowed to enter *ca* 1 cm into the gel before adding 10 mM K-Pi buffer (pH 7.4) containing 40 mM EDTA, 0.1 mM DTT and 2.37 M $(NH_4)_2SO_4$. The enzyme emerged with all the proteins in the void vol. of the column and was practically free of the pigments present in the extract. The fraction containing prenyltransferase activity was concd through a Diaflo membrane PM 30. It was then applied to a 1.6 × 40 cm column of DEAE-cellulose previously equilibrated in 10 mM K-Pi buffer (pH 7.4) containing 2 mM EDTA and 0.1 mM DTT. Prenyltransferase activity was eluted with a linear gradient of K-Pi (pH 7.4) between 10 and 150 mM containing 2 mM EDTA and 0.1 mM DTT. The fraction obtained was freeze-dried and stored at −20°. Proteins were determined by turbidimetry [38] in the extract, the $(NH_4)_2SO_4$ fraction and the Sephadex G-100 fractions. Lowry's method was used after ion exchange chromatography, when the enzyme fraction was almost free of pigments [39].

Enzyme assay. The assay was based on the measurement of radioactivity incorporated into acid-labile allylic pyrophosphates [4] when [1-^{14}C]IPP plus GPP or DMAPP were used as substrates. Unless otherwise stated, the enzyme was incubated at 37° in glass-stoppered conical tubes in 0.5 ml of the following medium: 100 mM K-Pi buffer (pH 7.4), 8 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM GPP or DMAPP and 0.05 mM [1-^{14}C]IPP (2.04 × 10^{12} dpm/mmol) and 0.03 mg of protein. The early fractions were assayed in the presence of 10 mM ATP, which inhibited phosphatases and increased the yield of allylic pyrophosphates. After 15 min the reaction was stopped by cooling the tubes to 0° and the aq. phase was extracted with 1.5 ml of petrol (40–60°). The petrol extract [40] was divided into two or more portions, one for the measurement of radioactivity by conventional scintillation spectrometry and the other portions for product analysis. The aq. phase remaining after extraction with petrol was assayed for acid-labile allylic compounds [15]. Radioactivity in this 'allylic' or acid-labile fraction represents mainly prenyltransferase activity. IPP isomerase activity in the enzyme fractions was controlled in the absence of the allylic substrate [4, 22] and the data corrected for this radioactivity. Experiments with boiled enzyme were always included as controls. One unit of prenyltransferase was defined as the amount of enzyme which generated 1 mol of the phosphorylated allylic product per sec at 37°. Results are expressed in nkat [41]. Other portions of the aq. phase were assayed for prenyl phosphates [15] and the unrearranged prenols thus obtained by enzymic hydrolysis of their pyrophosphates were further analysed after the addition of 0.5–1 mg of authentic carriers.

Analysis of products. Reverse-phase TLC of the petrol fractions [42] was performed with both C_{10}- and C_{15}-carrier alcohols. I_2 vapour was used to visualize the spots corresponding to the standard alcohols, and then the plates were scraped into vials containing 5 ml of scintillation fluid and counted [16]. TLC of diastereomeric farnesols was performed on 0.25 mm Si gel plates coated with a soln of 2% ethylene glycol adipate in Me_2CO. The plates were developed in 5% EtOAc in petrol. Spots corresponding to the standard farnesols were visualized with I_2 vapour. In this system, the R_f values obtained were 0.6 for (2*Z*,6*E*)-farnesol and 0.51 for (2*E*,6*E*)-farnesol. The plates were scraped into scintillation vials and counted as above.

GLC was performed on stainless steel columns of 0.635 o.d. × 300 cm packed with Chromosorb W-HMDS, 60–80 mesh. Liquid phases: 2% ethylene glycol adipate or 2% SE-30; gas flow: 40 ml He/min; temp.: 250° for the thermal conductivity detector, 200° for the injector and 165° isothermal for the column oven. Effluents from GLC were introduced directly into a heated proportional radioactivity counter or collected in scintillation fluid at −70° and counted. Silylated compounds were also prepared from the concentrated petrol phases containing adequate carriers and the trimethylsilyl derivatives were analysed by GC/RC [15].

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