tive cooperativity may be diagnostic of sequential conformational changes (Levitzki and Koshland, 1969).

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Prenyltransferase: The Mechanism of the Reaction†

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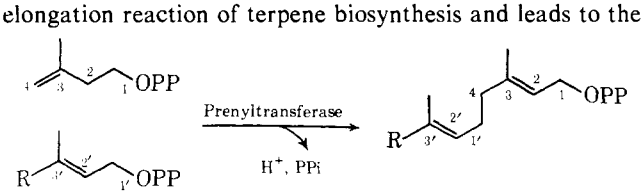
ABSTRACT: The enzyme, prenyltransferase, which normally catalyzes the addition of an allylic pyrophosphate to isopentenyl pyrophosphate, has been found to catalyze the hydrolysis of its allylic substrate. The rate of this hydrolysis is markedly stimulated by inorganic pyrophosphate. Competition experiments with 2-fluoroisopentenyl pyrophosphate and inorganic pyrophosphate demonstrated that inorganic pyrophosphate stimulated hydrolysis by binding at the isopentenyl pyrophosphate site. Hydrolysis carried out in $H_2{}^{18}O$ or with (1*S*)-[1-^{3}H]geranyl pyrophosphate show the C–O bond is broken and the C_1 carbon of geranyl pyrophosphate is inverted in the process. These results are interpreted to favor a carbonium ion mechanism for the prenyltransferase reaction.

Prenyltransferase (EC 2.5.1.1) catalyzes the condensation between C_4 of isopentenyl and C_1' of an allylic pyrophosphate, generating the five-carbon homologue of the allylic pyrophosphate. This condensation is the fundamental chain elongation reaction of terpene biosynthesis and leads to the formation of such diverse products as sterols, carotenes, dolichols, and the hydrocarbon side chains of the respiratory coenzymes.



The mechanisms which have been proposed for prenyl transfer can be grouped into two broad categories—those in which condensation is initiated by heterolytic cleavage of the carbon–oxygen bond of the allylic pyrophosphate, with or without assistance from the double bond of isopentenyl pyrophosphate, yielding cationic intermediates (Lynen et al., 1958; Rilling and Bloch, 1959; Cornforth and Popjak, 1959; Cornforth, 1968), and those in which condensation is initiated by attack of a nucleophilic group at the double bond of isopentenyl pyrophosphate with simultaneous formation of the C_1'–C_4 bond between the two substrates and rupture of the C_1'–oxygen bond (Cornforth et al., 1966; Cornforth, 1968). On the basis of the observation that C_1' is inverted during prenyl transfer, Cornforth and Popjak ar-

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gued that formation of a bond between C_1' and C_4 is concerted with cleavage of the C_1'-oxygen bond (Cornforth et al., 1966; Cornforth, 1968). They further postulated that the stereochemistry of proton elimination from C_2 of isopentenyl pyrophosphate required the participation of a nucleophilic X group. The mechanism proposed by Cornforth and Popjak is commonly accepted as the mechanism for the prenyltransferase reaction.

Recently, prenyltransferase from several sources has been purified to homogeneity (Eberhardt and Rilling, 1975; Reed and Rilling, 1975). During equilibrium dialysis experiments designed to measure the binding of substrate to the avian liver enzyme, Brent Reed in this laboratory observed that the enzyme also catalyzed the hydrolysis of the allylic substrate upon prolonged incubation. It seemed to us that examination of this novel catalytic activity of the enzyme might provide new information about the mechanism of prenyl transfer. In this paper we report the results of these experiments and present a mechanism for the reaction.

Materials and Methods

Porcine Liver Prenyltransferase. The enzyme was purified from pig liver by methods similar to those used for the preparation of avian liver enzyme and was assayed by the acid-lability method (Reed and Rilling, 1975). The protein was crystalline and homogeneous as judged by electrophoresis in natural and sodium dodecyl sulfate—containing polyacrylamide gels. The purification procedure will be published elsewhere.

Substrates. [1-^{14}C]Isopentenyl pyrophosphate purchased from Amersham/Searle was diluted to a specific activity of 10 μCi/μmol with synthetic material.

[1-$^3H_2$]Geranyl pyrophosphate and geranyl pyrophosphate were prepared from the alcohols by the method of Cramer (Cramer and Weiman, 1960; Cornforth and Popjak, 1969). The pyrophosphate esters were purified by ion-exchange chromatography on Dowex 1-X8 formate. Columns (1 × 16 cm) were developed with 300 ml of a linear gradient of 0.11–0.63 M ammonium formate in methanol–water 90:10. (1*S*)-[1-^{3}H]Geraniol was prepared by the stereoselective reduction of [1-^{3}H]geraniol by yeast alcohol dehydrogenase and NADH. The resulting alcohol was pyrophosphorylated and the product isolated as described above.

All other reagents used were the purest obtainable commercially.

Hydrolysis of Geranyl Pyrophosphate. Prenyltransferase (13 μg, specific activity 580) and geranyl pyrophosphate (2 nmol, specific activity 120 μCi/μmol) were incubated at 37 °C in a mixture containing glycylglycine, pH 7.0, 10 mM; $MgCl_2$, 2 mM; mercaptoethanol, 1 mM; and varying concentrations of inorganic pyrophosphate in a volume of 50 μl. At five time intervals between 0 and 20 min, 10-μl aliquots were removed and added to 1 ml of methanol containing 0.5% ammonia and 0.2–0.3 ml of Dowex 1-X8 ion-exchange resin (formate form). After mixing, the samples were transferred to disposable Pasteur pipets which were plugged with cotton, and the eluates were collected in scintillation counting vials. The columns were washed with two 1-ml portions of methanol. A few drops of formic acid (68%) were added to the eluate followed by 20 ml of 0.4% Omnifluor (New England Nuclear) in toluene. Radioactivity was determined by liquid scintillation spectrometry. The linear portions of the velocity curve were used for calculating the rate of reaction.

Table I: Stimulation of Hydrolysis of Geranyl Pyrophosphate by Inorganic Pyrophosphate.

[Inorganic Pyrophosphate] (M)	Rate of Geraniol Formation[a] (nmol min^{-1} mg^{-1})
0	<0.2
4×10^{-6}	1.4
1.2×10^{-5}	3.7
2×10^{-5}	5.5
4×10^{-5}	6.6
2×10^{-4}	12.0
6.7×10^{-4}	12.0
2×10^{-3}	3.8
6×10^{-3}	0.5

[a] The procedures used are described in Methods.

Condensation of 2-Fluoroisopentenyl Pyrophosphate with [1-^{3}H]Geranyl Pyrophosphate. Preliminary experiments indicated that the product of this condensation was more stable to acid than the substrate geranyl pyrophosphate; so this reaction could be followed by the disappearance of [^{3}H]geranyl pyrophosphate. Incubation mixtures (55 μl) contained glycylglycine, 10 mM, pH 7.0; $MgCl_2$, 1 mM; dithiothreitol, 0.1 mM; prenyltransferase (3 μg, specific activity 900); and varying amounts of 2-fluoroisopentenyl pyrophosphate. Incubation was at 37 °C. Aliquots (10 μl) were removed from incubation mixtures and pipetted into 0.1 ml of 4:1 methanol–11 N HCl. After a 15-min incubation at 30 °C, the solution was made alkaline by the addition of 0.5 ml of 10% aqueous KOH. The products of hydrolysis were extracted into 1 ml of hexane, one-half of which was counted by liquid scintillation spectrometry. Counting efficiency was determined by internal standardization.

Results

Stimulation of Hydrolysis of Geranyl Pyrophosphate by Inorganic Pyrophosphate. The initial observations by Reed in this laboratory indicated that the hydrolysis of allylic pyrophosphate by avian liver prenyltransferase was autocatalytic. This suggested that one of the products of hydrolysis was participating in the hydrolysis reaction. Inorganic pyrophosphate seemed the most likely candidate for this role. When we incubated pig liver prenyltransferase with geranyl pyrophosphate for relatively short time intervals, negligible quantities of geraniol were produced. However, if inorganic pyrophosphate was included in the incubation mixtures, there was a pronounced conversion of geranyl pyrophosphate to hexane-soluble compounds (Table I). The lowest concentration of inorganic pyrophosphate that stimulated hydrolysis was 4×10^{-6} M, while maximal hydrolysis was attained at approximately 4×10^{-4} M. At higher concentrations of inorganic pyrophosphate, there was a decline in the rate of hydrolysis of geranyl pyrophosphate. The maximal rate was 12 nmol min^{-1} mg^{-1}, approximately 1.7% of the rate of the prenyltransferase reaction catalyzed by the enzyme.

Identification of the Products of Hydrolysis. An incubation mixture containing prenyltransferase, geranyl pyrophosphate, and inorganic pyrophosphate was stopped by the addition of methanol when the reaction was about half completed. Thin-layer chromatography of the hexane-soluble products showed that nearly all of the radioactivity migrated with geraniol. A small, but significant, portion (1%) ran

Table II: Normalized Ion Intensities from the Mass Spectra of the Geranyl Benzoates.

Ion	Theoretical ^{16}O	Sample Generated in Water Containing ^{16}O	^{18}O Run 1	^{18}O Run 2
189	100	100.0	100.0	100.0
190	13.7	14.9	22.3	23.5
191	1.27	0.9	21.1	27.5
192	0.09	0	3.4	9.8

nearly to the front and was assumed to represent hydrocarbon products. In other experiments hydrocarbon products were separated from the alcohol by chromatography on small columns of grade II alumina. Small (0.15–0.6% of products) quantities of hydrocarbons were observed. These amounts were significantly greater than found in appropriate control experiments. In the initial experiment, the radioactive compounds remaining in the aqueous alcohol phase were extracted into 1-butanol and chromatographed on a thin-layer system that would separate geranyl pyrophosphate from the monophosphate and the free alcohol (Sofer and Rilling, 1969). After development, analysis of the distribution of radioactivity on the thin-layer plate revealed 8% geranyl monophosphate and 92% geranyl pyrophosphate. The presence of the monophosphate is a consequence of about 5% contamination of this substance in the starting material. Inorganic pyrophosphate was identified as a product of the reaction in an indirect manner. A hydrolysis mixture from which 0.1 μmol of geraniol was recovered was analyzed for orthophosphate (Richards and Boyer, 1965). Less than 0.01 μmol of orthophosphate was detected. Thus, less than 5% of the pyrophosphate moiety of geranyl pyrophosphate had been converted to orthophosphate. It is apparent then that the products of the prenyltransferase-catalyzed hydrolysis of geranyl pyrophosphate are almost exclusively geraniol and pyrophosphate.

One way in which inorganic pyrophosphate could facilitate the hydrolysis of geranyl pyrophosphate would be by participating in the reaction as an analogue of isopentenyl pyrophosphate. A kinetic analysis of this could not be achieved because of the large (100-fold) difference in rates of prenyl transfer and hydrolysis. Also, other experiments had shown that inorganic pyrophosphate acted as a competitive inhibitor at the geranyl pyrophosphate binding site and as a noncompetitive inhibitor at the isopentenyl pyrophosphate binding site (Holloway and Popjak, 1967). We had previously prepared 2-fluoroisopentenyl pyrophosphate and found that it inhibited prenyltransferase (Muscio et al., unpublished results). Further experiments showed that this analogue would participate in the prenyltransferase reaction. The product, whose structure has not been completely elucidated, was relatively stable to acid. Treatment of the product with alkaline phosphatase liberated an alcohol that migrated with farnesol on both normal-phase and reverse-phase thin-layer chromatography, indicating that it resulted from the condensation of the two substrates. Curve A in Figure 1 shows the condensation of 2-fluoroisopentenyl pyrophosphate with geranyl pyrophosphate as a function of time. Maximum velocity is about 5% of prenyl transfer to the natural substrate and other experiments showed an apparent K_m of 0.3 mM for the fluoro analogue.

When 2-fluoroisopentenyl pyrophosphate was included in the incubation mixtures with prenyltransferase, geranyl pyrophosphate, and inorganic pyrophosphate, there was negligible hydrolysis of the allylic substrate (curve C in Figure 1). Curve B in the figure shows the hydrolysis of geranyl pyrophosphate under the same conditions represented in curve C, except that the fluoro analogue was omitted. Since condensation with 2-fluoroisopentenyl pyrophosphate depletes geranyl pyrophosphate, it was necessary to establish that residual geranyl pyrophosphate was available for hydrolysis. The geranyl pyrophosphate concentration was estimated by measuring residual acid-labile material during the course of the reaction. The data represented by curve A in the figure indicates that geranyl pyrophosphate was present for the first 10 min of the reaction. This experiment clearly illustrates that inorganic pyrophosphate and the fluorine-containing analogue compete for the same site on the enzyme.

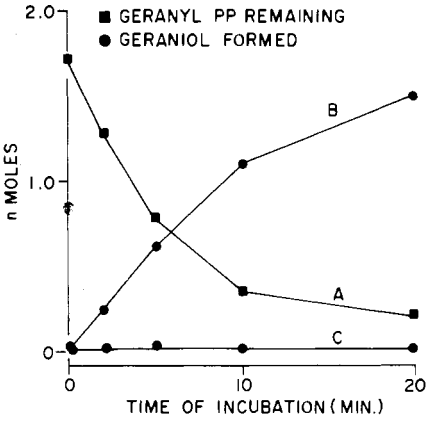


FIGURE 1: Inhibition of pyrophosphate stimulated hydrolysis of geranyl pyrophosphate by 2-fluoroisopentenyl pyrophosphate. Incubation conditions are described in Methods. At the indicated times, samples were withdrawn and analyzed for residual acid-labile material (■) or for the formation of geraniol (●). The incubation represented by curve A contained 2-fluoroisopentenyl pyrosphosphate, 0.81 mM. The incubation represented by curve B contained 0.18 mM inorganic pyrophosphate. Incubation represented by curve C contained both.

Origin of the Oxygen and Inversion of C_1' in Geraniol. During the normal condensation with isopentenyl pyrophosphate, the C–O bond of the allylic substrate is cleaved. Consequently, it is important to establish the origin of the oxygen of geraniol produced by hydrolysis. The enzyme-catalyzed hydrolysis of geranyl pyrophosphate was carried out at 37 °C for 1 h in water containing 19.9 atom % excess of ^{18}O, geranyl pyrophosphate, 0.44 mM; $MgCl_2$, 4 mM; inorganic pyrophosphate, 0.8 mM; glycylglycine, pH 7.6, 20 mM; and prenyltransferase, 0.31 mg, specific activity 625 in a volume of 0.5 ml. An appropriate control experiment was run in ^{16}O water. The geraniol produced was extracted into pentane and converted to the benzoate ester. After preliminary purification by chromatography on grade II alumina, the esters were analyzed on an LKB 9000-S gas chromatograph–mass spectrometer in the laboratory of Dr. James McCloskey of this university. A 3-ft 3% SE-30 column was used for the separation. The ions representing the loss of an isoprene unit (M-69) from the molecular ion (*m/e* 258) were chosen for analysis because they contained both oxygen atoms, were relatively intense, and required little background correction. The results (Table II) showed the geraniol generated in the presence of $H_2{}^{18}O$ incorporated 96 ± 16% of the theoretical maximum ^{18}O incorporation.

Another feature of the normal catalytic process of prenyltransferase is the inversion of C_1' in the allylic substrate. Since the experiment with ^{18}O water clearly demonstrated that the C–O bond of geranyl pyrophosphate was cleaved,

we felt that it was important to know if the carbinol carbon had inverted during hydrolysis. (1*S*)-[1-^{3}H]- and [1-$^3H_2$]-geranyl pyrophosphate (10 nmol) were incubated for 45 min with prenyltransferase (13 μg) under identical conditions (inorganic pyrophosphate, 0.3 mM; $MgCl_2$, 1 mM; phosphate buffer, 10 mM, pH 7.0). The radioactive geraniol produced by hydrolysis was extracted into pentane and 1 μg of carrier geraniol added. The solvent was removed carefully under a stream of nitrogen and a mixture of liver alcohol dehydrogenase, 1 mg in experiment 1, 2 mg in experiment 2; NAD, 4 μmol, and ethanol, 100 μmol, was then added in a volume of 0.6 ml containing 50 mM phosphate buffer, pH 7.8.[1] Incubation was at 37° for 30 min. The ethanol was in 15 000-fold M excess over geraniol and as such acted as a sink for "hydride" hydrogen. As anticipated, half of the radioactivity of the geraniol produced from [1-$^3H_2$]-geranyl pyrophosphate was lost during the incubation with alcohol dehydrogenase, while the alcohol produced from the asymetrically labeled substrate retained about 3% of the radioactivity (Table III). When these values are corrected for loss during work-up, the results indicate that, at most, 3.3% of the asymmetrically labeled substrate retained its configuration during hydrolysis. Thus, inversion was nearly quantitative.

Table III: Stereochemistry of the Hydrolysis of Geranyl Pyrophosphate by Prenyltransferase.[a]

	Experiment 1		Experiment 2	
	[1-$^3H_2$]	(1*S*)-[1-^{3}H]	[1-$^3H_2$]	(1*S*)-[1-^{3}H]
Recovered from hydrolysis	5.97×10^5	1.67×10^4	1.76×10^5	1.84×10^4
Recovered from ADH incubation	2.34×10^5	4.56×10^2	8.36×10^4	5.64×10^2
% recovered	39.4	2.73	47.5	3.06
Corrected for loss in control		3.4		3.3

[a] Experimental details are given in Methods.

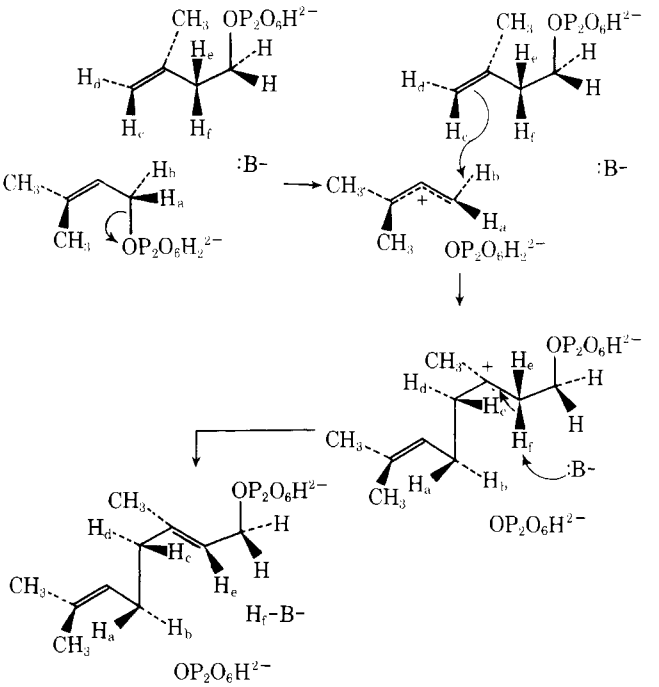
Discussion

In the absence of isopentenyl pyrophosphate, prenyltransferase catalyzes the hydrolysis of geranyl pyrophosphate with a maximal rate (12 nmol min^{-1} mg^{-1}) that is approximately 1.7% of the rate for the normal condensation reaction. Since an oxygen from water is incorporated into geraniol at C_1' with inversion of configuration, it is obvious that the C_1'-oxygen bond is cleaved during the hydrolysis. In contrast, phosphatases hydrolyze their substrates by cleaving the oxygen–phosphorus bond (Stein and Koshland, 1952). Solvolysis of geranyl derivatives in aqueous solvents normally yields geraniol and linalool (Winstein et al., 1972). However, in the enzyme-catalyzed hydrolysis of geranyl pyrophosphate, geraniol is the only detectable product. Attack by water at C_3' leading to linalool could be prevented by factors such as steric shielding of C_3' by hydrophobic interactions between the enzyme and the organic moiety, polarization of the allylic cation by the pyrophosphate counterion (Bernardi et al., 1975), or orientation of the water molecule by the hydrophilic pyrosphosphate groups. In addition, the small amount of hydrocarbon which accompanies the formation of geraniol is readily accounted for by elimination of a proton from an allylic cation.

The observation that 2-fluoroisopentenyl pyrophosphate, a competitive inhibitor for isopentenyl pyrophosphate, inhibits hydrolysis while simultaneously condensing with geranyl pyrophosphate (Figure 1) establishes that the same catalytic site is involved in prenyl transfer and hydrolysis. Inorganic pyrophosphate is known to inhibit prenyl transfer by binding to the allylic pyrophosphate and isopentenyl pyrophosphate sites (Holloway and Popjak, 1967). Binding at the allylic site may account for the decreased rates of hydrolysis at high concentrations of inorganic pyrophosphate (Table I). At low concentrations of pyrophosphate the rate of hydrolysis increases with increasing concentration of pyrophosphate, and it is reasonable to assume that the allylic site is occupied by its normal substrate and the site for isopentenyl pyrophosphate by inorganic pyrophosphate. Stimulation of the hydrolysis reaction by inorganic pyrophosphate could result from binding of water by prenyltransferase in the presence of inorganic pyrophosphate since the absence of an organic moiety at the isopentenyl pyrophosphate site leaves a hole for the water molecule adjacent to the hydrophilic anion. Water would be excluded from the isopentenyl pyrophosphate site by the normal substrate, and hydrolysis should not be significant when the enzyme performs its normal catalytic function.

The unexpected hydrolytic activity provides additional information concerning the catalytic properties of prenyltransferase and can be used to further define the mechanism of the reaction. We wish to propose a mechanism involving cationic intermediates (Scheme I) that is consistent with the available data for prenyl transfer and differs from the commonly accepted X-group mechanism (Cornforth et al., 1966; Cornforth, 1968). It is reasonable to assume that the allylic substrate is activated in a similar manner during both hydrolysis and prenyl transfer. Allylic pyrophosphates

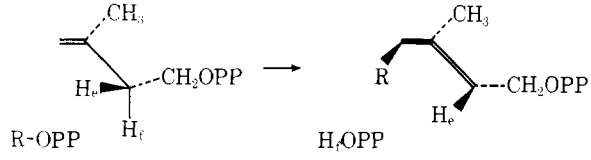
Scheme I: Ionization–Condensation–Elimination.



[1] Alcohol dehydrogenase from yeast (used to prepare the substrate) and from liver (used in this experiment) has the same stereochemistry for alcohol oxidation (Lemieux and Howard, 1963; Donninger and Ryback, 1964).

are easily activated when negative charge is removed from the pyrophosphate moiety as evidenced by a rate ratio of at least 10^3 for hydrolysis of the mono- and dianions of dimethylallyl pyrophosphate (Tidd, 1971). The enzyme could trigger prenyl transfer or hydrolysis by removing negative charge from the pyrophosphate moiety of the allylic substrate. From data currently av ole, we cannot determine at what point in the condensat the bond between C_1' of geranyl pyrophosphate and C_4 of isopentenyl pyrophosphate is formed. Ionization could initially give an allylic cation which subsequently condenses with isopentenyl pyrophosphate, or ionization could be assisted by the π-electrons in the double bond of isopentenyl pyrophosphate, implying bonding between the two substrates is synchronous with ionization. Covalent bonding between C_4 and C_1' is not necessary to preserve the stereochemistry of the latter center. The geranyl cation is a resonance stabilized species with the three trigonal carbon atoms, (C_1', C_2', and C_3') all in the same plane. The energy barrier for a 90° rotation from the plane of a terminal carbon depends on the substitution pattern of an allylic cation, but for rotation about the C_1'-C_2' bond of the geranyl system, a barrier of more than 28 kcal/mol is anticipated (Allinger and Seifert, 1975). Once the substrates are aligned as shown in Scheme I, C_1' will be completely inverted during prenyl transfer unless C_1', C_2', and C_3' flip over together after ionization and before condensation. A movement of that magnitude is not anticipated. It should be emphasized that ionization without anchimeric assistance by the double bond in isopentenyl pyrophosphate is possible since hydrolysis occurs in the absence of the homoallylic substrate.

The condensation reaction is completed by elimination of a proton (H_f) from C_2 to form a double bond between C_2 and C_3. The pyrophosphate group just released from the allylic substrate would be in position to assist in removing the proton, and we view the stereochemistry of the elimination step as an expected consequence of the alignment of the substrates in the active site prior to reaction. When isopentenyl pyrophosphate is bound in a conformation where the dihedral angle between the C_1-C_2 and C_3-C_4 bonds is greater than 90°, an *E*-double bond will be formed between C_2 and C_3. Since Cornforth and Popjak (Cornforth et al., 1966) have shown that the allylic group is attached to the *si* face of the C_3-C_4 double bond, the pyrophosphate fragment just released from the allylic substrate must be located on the *si* face of the trigonal center at C_3. In this orientation, only H_f is available to the pyrophosphate when the *E* isomer is formed. By similar argument, H_e must be lost from



C_2 when a *Z*-double bond is formed. Examples of *Z* transferases are known and H_e is eliminated (Overton and Roberts, 1974, and references therein).

In summary, we suggest that prenyl transfer takes place by an ionization-condensation-elimination mechanism. The exact timing of condensation with regard to ionization is unknown, but the presence of the C_3-C_4 double bond of isopentenyl pyrophosphate is not required for the enzyme to trigger ionization of the allylic pyrophosphate. Although our data do not rigorously exclude the displacement-elimination mechanism proposed by Cornforth and Popjak, our proposed mechanism involving carbonium ion intermediates is fully consistent with the chemical properties of the substrates, compatible with the stereochemistry of prenyl transfer and explains why the enzyme catalyzes hydrolysis of its allylic substrates in the absence of isopentenyl pyrophosphate. Experiments in progress in our laboratories with other substrate analogoues should provide adequate evidence to distinguish between these mechanisms.

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