Under similar conditions, the rhodium dichloride complex **1a** is about twice as active (per rhodium atom) toward cyclohexene as the Wilkinson complex, and the iridium dichloride complex **1b** is about a factor of three more active still. However, whereas $[RhCl(PPh_3)_3]$ catalyzes the reduction of cyclohexene fifty times faster than 1-methylcyclohexene, the corresponding factor is only about 3 for **1a** and 7 for **1b**. This probably reflects a greater steric constraint in $[RhCl(PPh_3)_3]$ than in $[Rh(C_5Me_5)Cl_2]_2$.

A measure of the steric factors involved may be obtained from the cone angle, θ.[31] For C_5Me_5–Rh it is calculated to be 185° while for Ph_3P θ = 145° and for the grouping *fac*-{$(Ph_3P)_2Cl$}Rh θ is estimated as 230°. Clearly we may therefore anticipate the C_5Me_5–M catalysts to be sterically rather less demanding than $[Rh(Ph_3P)_3Cl]$.

Arene Hydrogenation Catalysts

While the homogeneous catalysis of olefin hydrogenation has now become almost a commonplace reaction, the hydrogenation of benzene and substituted benzenes is difficult even with heterogeneous catalysts.[32] A few homogeneous catalysts have been reported to reduce benzenes to cyclohexanes[33] but they mostly appear to be rapidly deactivated or to have a limited range.

It is therefore of especial interest that Russell recently demonstrated that (pentamethylcyclopentadienyl)-rhodium complexes such as **1a** show reasonable activity for the reduction of benzene and substituted benzenes to cyclohexanes (Table I) at 50 °C and 50 atm of hydrogen.[34] Again, base was a necessary cocatalyst and 2-propanol was the solvent of choice; this indicates, as is to be expected, the importance of a heterolytic hydrogen activation step in the reaction mechanism. No detectable amounts of metal were formed under these conditions, and turnover numbers of over 400 equiv of benzene to cyclohexane per rhodium have been obtained; in general, cyclohexenes and cyclohexadienes were not detected in the products. By contrast, the iridium complex **1b** showed only 28% of the activity of **1a**; this may correlate with the greater stability of the Ir^{III}–arene by comparison with the Rh^{III}–arene complexes previously noted, and it is interesting that **1a** reduces most easily those alkylated benzenes that form the least stable complexes in 8. $[Rh(Ph_3P)_3Cl]$ only showed 3% of the activity of **1a**, and $[Co(C_5Me_5)I_2]_2$ decomposed to metal under the same conditions.

Analysis of the products formed by **1a**-catalyzed reduction of the *o*-, *m*-, and *p*-xylenes showed a high stereoselectivity for the formation of the *cis*-dimethylcyclohexanes [*cis*:*trans* = 6.2:1 (*o*-xylene), 3.8:1 (*m*-xylene), and 2:1 (*p*-xylene)]. Our preliminary results also show that the catalyst can tolerate at least some functional groups on the arene, for example, $-CO_2R$, $-COR$, $-NMe_2$, OMe.

Activity as hydrogenation catalysts certainly only represents one aspect of the chemistry of these very versatile complexes, and more applications will undoubtedly be found. The recent syntheses of alkyl-substituted tetramethylcyclopentadienes makes the formation of $[M(C_5Me_4R)X_2]_2$ complexes possible, and it should also be possible to attach (C_5Me_4R)–RhX_2 onto polymers to give supported catalysts.

The studies described here are due primarily to the dedicated efforts of my co-workers both in Sheffield and at McMaster University in Canada. I should in particular like to thank and to pay tribute to the excellent work of Dr. Colin White, Dr. J. W. Kang, Dr. K. Moseley, Dr. A. J. Oliver, Dr. H. B. Lee, Dr. D. S. Gill, Dr. S. J. Thompson, and Dr. M. J. H. Russell.

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(32) P. N. Rylander, "Catalytic Hydrogenation over Platinum Metals", Academic Press, New York, N.Y., 1967, p 309 et seq.
(33) See, for example, P. Abley, I. Jardine, and F. J. McQuillin, *J. Chem. Soc. C*, 140 (1970); P. D. Taylor and M. Orchin, *J. Org. Chem.*, 37, 3913 (1972); M. C. Rakowski, F. J. Hirsekorn, L. S. Stuhl, and E. L. Muetterties, *Inorg. Chem.*, 15, 2379 (1976), and references therein.
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The Prenyl Transfer Reaction. Enzymatic and Mechanistic Studies of the 1′–4 Coupling Reaction in the Terpene Biosynthetic Pathway

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The terpene biosynthetic pathway is used to synthesize a wide variety of small molecules which are often lipophilic and fulfill a multiplicity of functions. Important examples are sterols (structural components of eukaryotic membranes), carotenes (photoreceptors in visual and photosynthetic systems), and phosphorylated polyprenols (membrane-soluble carriers of sugars in the biosynthesis of bacterial cell wall polysaccharides and eukaryotic glycoproteins). In other systems the hydrophobic polyisoprenoid chain is used to anchor respiratory coenzymes (ubiquinone, porphyrin a) or chlorophyll to membranes. Terpenes are also involved in communication between organs (hormones) and organisms (pheromones). In addition to these few

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Hans Rilling, Professor of Biochemistry at the University of Utah, was born in Cleveland, Ohio, in 1933, and received his B.A. degree from Oberlin College. His Ph.D. thesis research at Harvard University was directed by K. Bloch, who introduced him to terpene biosynthesis. After postdoctoral work at the University of Michigan, Dr. Rilling moved to the University of Utah, where he has continued research on various aspects of terpene biosynthesis.

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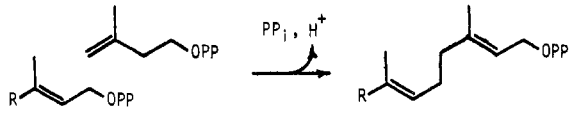
examples, there is a host of compounds produced by the terpene biosynthetic pathway whose biological functions are unknown.[1]

It is likely that terpene biosynthesis is ubiquitous, since at least portions of the pathway are found in all prokaryotic and eukaryotic organisms that have been surveyed. Some organisms, such as fungi, contain many segments of the pathway, including the synthesis of dolichol, ubiquinones, sterols, and carotenes,[2] while others, such as insects, rely on diet as a source of one or more classes of terpenes—in this case sterols—but have maintained the ability to synthesize dolichols, ubiquinones,[3] and juvenile hormones.[4] In contrast, some bacteria may be restricted to undecaprenol synthesis.

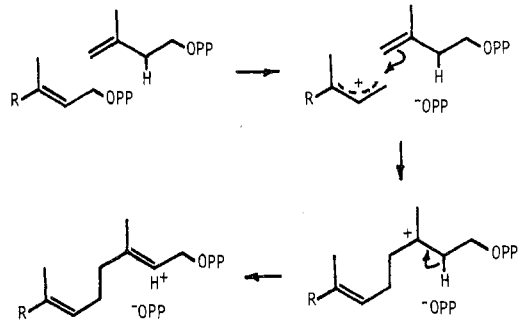
Biosynthetically, terpenes are compounds composed of 2-methylbutyl (isoprene) units, which may either be used to construct the entire carbon skeleton of a molecule or may be attached to a nonterpene fragment. The biological process for attaching a terpene fragment to another moiety is a prenyl transfer reaction—a bisubstrate reaction in which one of the partners is an allylic, terpene pyrophosphate and the other can be one of a variety of terpene or nonterpene compounds. The premier example of a prenyl transfer reaction is the 1′–4 (head-to-tail) condensation[6] between 3-methyl-3-butenyl pyrophosphate (isopentenyl pyrophosphate) and



an allylic pyrophosphate with the concomitant release of inorganic pyrophosphate from the allylic molecule. This reaction was discovered by Lynen, who was first to realize the importance of allylic pyrophosphates in biosynthetic reactions.[7] Another important prenyl transfer reaction is the 1′–2–3 coupling (head-to-middle) found in the sterol and carotenoid pathways when two molecules of farnesyl or geranylgeranyl pyrophosphate are joined to yield the cyclopropylcarbinyl intermediates presqualene or prephytoene pyrophosphate, respectively.[8] Although this reaction is not usually classified as a prenyl transfer, it may have mechanistic features in common with the head-to-tail condensation.[9] A third category involves the condensation of the organic portion of an allylic pyrophosphate with an acceptor that is not isoprenoid. Examples of products thus formed are respiratory quinones, cytochrome a_3, ubiquinone, chlorophyll, and some alkaloids. Thus many variations in the products of the prenyl transfer reaction are possible, including the length of the polyprenyl chain (molecular weight), the stereochemistry of the double bonds, and the structure of the prenyl acceptor. Although this diversity implies that a family of prenyl transferases with a wide range of selectivities toward substrates must exist, only a few of these enzymes have been purified to homogeneity, and none have been fully characterized.

Scheme I

Ionization–Condensation–Elimination



Most attention has been focused on the farnesyl pyrophosphate synthetase system (EC 2.5.1.1) because of its role in sterologenesis, and we will restrict our discussion to this enzyme. Early work on isolation and characterization of the synthetase came from the laboratories of Porter[10] and Popjak.[11] The former group obtained a highly purified preparation from pig liver and suggested that one enzyme catalyzed the condensation of isopentenyl pyrophosphate with either dimethylallyl pyrophosphate or geranyl pyrophosphate, a point later proved with homogeneous proteins. It was also shown that either Mg^{2+} or Mn^{2+} was required to support enzymatic activity. Popjak reported a kinetic analysis of the enzyme and concluded from patterns of product inhibition that the reaction was ordered sequential.[11]

The substrate specificity of prenyl transferase (farnesyl pyrophosphate synthetase) has been extensively studied in the laboratories of Ogura and Popjak.[12] They demonstrate that the pyrophosphate moieties of both substrates are essential. The specificity for the organic part of the homoallylic substrate was thought to be relatively high; however several analogues have recently been shown to be reactive.[12l] Simply extending the carbon chain by a single atom (4-methylpent-4-en-1-yl pyrophosphate) leads to a major perturbation

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(4) R. C. Jennings, K. J. Judy, and D. A. Schooley, *J. Chem. Soc., Chem. Commun.*, 21 (1975).

(5) Bactoprenol is the primary product of mevalonate metabolism in some bacteria (K. J. L. Thorne and E. Kodicek, *Biochem. J.*, **99**, 123 (1966), and it is quite possible that anaerobically grown bacteria would elaborate only this terpene.

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(7) F. Lynen, H. Eggerer, U. Henning, and I. Kessel, *Angew. Chem.*, **70**, 738 (1958).

(8) (a) W. W. Epstein and H. C. Rilling, *J. Biol. Chem.*, **245**, 4597 (1970); (b) G. Popjak, J. Edmond, and S. Wong, *J. Am. Chem. Soc.*, **95**, 2713 (1973); (c) L. J. Altman, L. Ash, R. C. Kowerski, W. W. Epstein, B. R. Larsen, H. C. Rilling, F. Muscio, and D. E. Gregonis, *J. Am. Chem. Soc.*, **94**, 3257 (1972); (d) A. A. Qureshi, F. J. Barnes, E. J. Semmler, and J. W. Porter, *J. Biol. Chem.*, **248**, 2755 (1973).

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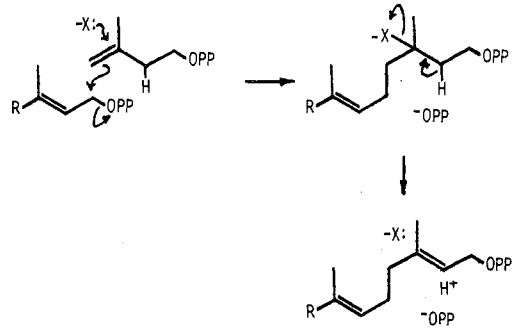
(12) (a) G. Popjak, P. W. Holloway, and J. M. Baron, *Biochem. J.*, **111**, 325 (1969); (b) G. Popjak, J. L. Rabinowitz, and J. M. Baron, *ibid.*, **113**, 861 (1969); (c) K. Ogura, T. Nishino, T. Koyama, and S. Seto, *J. Am. Chem. Soc.*, **92**, 6036 (1970); (d) T. Nishino, K. Ogura, and S. Seto, *J. Am. Chem. Soc.*, **93**, 794 (1971); (e) T. Nishino, K. Ogura, and S. Seto, *Biochim. Biophys. Acta*, **235**, 322 (1971); (f) T. Nishino, K. Ogura, and S. Seto, *ibid.*, **302**, 33 (1973); (g) C. D. Poulter, D. M. Satterwhite, and H. C. Rilling, *J. Am. Chem. Soc.*, **98**, 3376 (1976); (h) T. Koyama, K. Ogura, and S. Seto, *J. Am. Chem. Soc.*, **99**, 1999 (1977); (i) K. Ogura, A. Saito, and S. Seto, *J. Am. Chem. Soc.*, **96**, 4037 (1974); (j) C. D. Poulter, J. C. Argyle, O. J. Muscio, and H. C. Rilling, unpublished results; (k) C. D. Poulter, J. C. Argyle, and E. A. Mash, *J. Am. Chem. Soc.*, **99**, 957 (1977); (l) A. Saito, Ph.D. Dissertation, Tohoku University, Sendai, Japan, 1976.

Scheme II

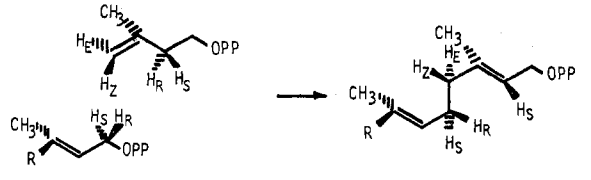
Condensation-Elimination



in the alignment of the homoallylic substrate in the active site, producing a *Z* rather than *E* product.[12i] Requirements for the allylic substrates are less demanding, although the enzyme requires a trisubstituted C(2)–C(3) double bond with a hydrogen at C(2).[13] As the hydrocarbon tail of the substrate is extended from the *E* location at C(2) by a linear chain, two optima are found corresponding to the chain lengths of the two natural substrates.[12f] Thus far, over 30 allylic analogues have been reported to function as substrates.

Two mechanisms have been proposed for the 1′–4 condensation reaction. An ionization–condensation–elimination mechanism appeared in the late 1950s when the role of the prenyl transfer reaction in sterol biosynthesis was first determined.[7,12k,15] The central features of this mechanism, as shown in Scheme I, include ionization of the allylic pyrophosphate, a cationic alkylation of the C(3)–C(4) double bond of isopentenyl pyrophosphate, and elimination of a proton from C(2) of the condensed cation, to generate the new double bond between C(2) and C(3).

The second mechanism (Scheme II) grew from the stereochemical work of Popjak and Cornforth on squalene biosynthesis.[15c,16] In a series of careful investigations, they discovered that prenyl transfer occurs with (1) inversion of configuration at C(1) of the allylic pyrophosphate, (2) attachment of C(1) to C(4) of isopentenyl pyrophosphate on the *si* face of the C(3)–C(4) double bond, and (3) loss of H_R from C(2) upon formation of the new C(2)–C(3) double bond. Later it was



determined that the stereochemistry of proton removal from C(2) is related to the stereochemistry of the newly formed double bond. Prenyl transferases which generate *E* double bonds remove H_R and those which generate *Z* double bonds remove H_S.[15c,17] A new mechanism, which we refer to as a condensation–elimination mechanism or X-group mechanism, was thought to offer a better explanation of the stereochemical data. Popjak and Cornforth[15c,16] envisioned participation by an unknown nucleophile (the X group) in an intermolecular variation of an S_N2' displacement,[18] where displacement of the pyrophosphate moiety in the allylic substrate results in the formal trans addition of C(1) and X to the C(3)–C(4) double bond of isopentenyl pyrophosphate. In the second step a trans elimination of H from C(2) and X from C(3) generates the new double bond. It can readily be seen from models that loss of H_R produces an *E* double bond and loss of H_S, a *Z* double bond. Both mechanisms shown in Scheme I are logical and have ample precedent in chemical systems.

When we began our work with farnesyl pyrophosphate synthetase, the enzyme had not been purified to homogeneity nor, in our opinion, had any definitive work appeared which would permit one to distinguish between the two mechanisms proposed for the prenyl transfer reaction. In this Account we describe recent progress in our collaborative effort to determine the structure of farnesyl pyrophosphate synthetase and the mechanism of the reaction catalyzed by this enzyme.

Isolation of the Enzyme and Binding Studies

Homogeneous preparations of a cytoplasmic enzyme, farnesyl pyrophosphate synthetase, have been obtained from several sources. Purification steps include fractionation with ammonium sulfate, chromatography, first on DEAE-cellulose and then on hydroxyapatite, and crystallization. The enzyme from yeast was unstable, so most studies on the properties of the protein and mechanism of the reaction have been with the enzymes from liver.

The proteins from yeast,[19a] avian liver,[19b] and porcine liver[19c] have molecular weights of about 85 000 and are comprised of two subunits which cannot be resolved by electrophoresis in sodium dodecyl sulfate containing gels. The avian enzyme has a single N-terminal amino acid and the number of tryptic peptides anticipated for identical subunits was obtained.[19b]

Since the enzyme catalyzed the condensation of isopentenyl pyrophosphate with either dimethylallyl pyrophosphate or geranyl pyrophosphate, the possibility that individual subunits have different substrate specificities was investigated. A study of the number and specificity of the substrate binding sites was undertaken with the avian enzyme to resolve this problem. Initially Mg^{2+} containing buffer was used since a divalent cation is required for enzymatic activity.[20] Under these conditions a maximum of 2 mol of either of the two allylic substrates or the product was bound per mol of enzyme, giving a ratio of 1:1 substrate:enzyme subunit. The binding constants for dimethylallyl,

(13) This may be primarily a kinetic effect rather than a binding phenomenon since replacing the methyl group at C(3) by a hydrogen will reduce the reactivity of the allylic pyrophosphate toward ionization by a substantial amount.[14]

(14) K. K. Astin and M. C. Whiting, *J. Chem. Soc., Perkin Trans. 2*, 1157 (1976).

(15) (a) H. C. Rilling and K. Bloch, *J. Biol. Chem.*, **234**, 1424 (1959); (b) J. W. Cornforth and G. Popjak, *Tetrahedron Lett.*, **No. 29**, 311 (1959); (c) J. W. Cornforth, *Angew. Chem., Int. Ed. Engl.*, **7**, 903 (1968).

(16) (a) J. W. Cornforth, R. H. Cornforth, G. Popjak, and L. Yengoyan, *J. Biol. Chem.*, **241**, 3970 (1966); (b) G. Popjak and J. W. Cornforth, *Biochem. J.*, **101**, 553 (1966).

(17) (a) B. L. Archer, D. Barnard, E. G. Cockbain, J. W. Cornforth, R. H. Cornforth, and G. Popjak, *Proc. R. Soc. London, Ser. B*, **163**, 5A (1966); (b) D. P. Gough and F. W. Hemming, *Biochem. J.*, **118**, 163 (1970).

(18) Cornforth[15c] discussed a range of possibilities for the X group mechanism, including a situation where X is not covalently attached to C(3) but controls stereochemistry via ion pairing.

(19) (a) N. L. Eberhardt and H. C. Rilling, *J. Biol. Chem.*, **250**, 863 (1975); (b) B. C. Reed and H. C. Rilling, *Biochemistry*, **14**, 50 (1975); (c) L.-S. Yeh and H. C. Rilling, *Arch. Biochem. Biophys.*, **183**, 718 (1977).

(20) B. C. Reed and H. C. Rilling, *Biochemistry*, **15**, 3739 (1976).

geranyl, and farnesyl pyrophosphates were 1.8, 0.17, and 0.73 μM, respectively. The tight binding of the product, farnesyl pyrophosphate, was surprising since this compound is a poor substrate for condensation. When the enzyme was presented with two allylic substrates simultaneously, their binding was mutually competitive with a maximum of two substrate molecules bound per enzyme dimer. These experiments demonstrate that both of the allylic binding sites on the enzyme have the same substrate specificity, thus indicating the enzyme contains two identical catalytic sites, one per monomer. Since the enzyme is not allosteric, we assume the substrates are binding only at the catalytic site.

A total of 4 mol of isopentenyl pyrophosphate were bound per mol of enzyme in the presence of Mg^{2+}. The inclusion of citronellyl pyrophosphate, an analogue of geranyl pyrophosphate reduced this to two. Thus, isopentenyl pyrophosphate, which is a close structural analogue of dimethylallyl pyrophosphate, binds in the allylic site as well as the homoallylic site, and binding at the allylic site is competitive with an analogue of the allylic substrate.[20]

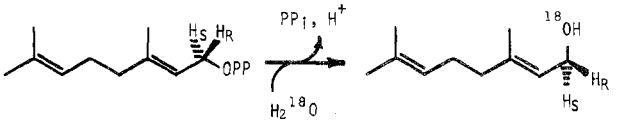
Subsequent experiments demonstrated that a divalent cation was not required for substrate binding, although either Mn^{2+} or Mg^{2+} increased the affinity of the enzyme for its substrates several fold.[21] This experiment demonstrates that these ions are required for catalysis rather than substrate binding. In fact, both allylic and homoallylic substrates can be bound simultaneously to the enzyme in the absence of metal ions with a mutual enhancement of binding and without condensation.

Since divalent cations are not required for substrate binding, we wanted to determine the conditions necessary for interaction between enzyme, metal, and substrates. In the absence of substrate, $^{54}Mn^{2+}$ was not bound significantly to the enzyme. However, in the presence of either an allylic or the homoallylic substrate, two metal ions were bound per catalytic site. When geranyl pyrophosphate was the fixed ligand, linear Scatchard plots were obtained for the binding of $^{54}Mn^{2+}$, indicating that both metal ions were bound with similar affinity. In related experiments with the homoallylic substrate as the fixed ligand, Scatchard plots of the data were curved, indicating different affinities for the Mn^{2+} ions, with positive cooperativity. The binding of metal was also measured in the simultaneous presence of analogues of both substrates, using relatively nonreactive fluorine analogues of geranyl and isopentenyl pyrophosphate (see below). Here again 2 mol of $^{54}Mn^{2+}$ were bound per catalytic site, and the data were suggestive of positive cooperativity. Since isopentenyl pyrophosphate binds at the allylic site as well as at the homoallylic site in the presence of a divalent cation, it is not possible to establish at which site the homoallylic substrate is facilitating Mn^{2+} binding. However, since two metals are bound in the presence of either or both substrates, it seems likely that the metal ions are coordinated between the pyrophosphate residues of the substrates.[21]

Mechanistic Studies

As stated earlier, stereochemical studies were not sufficient to permit a distinction between ionization–condensation–elimination and condensation–elimination mechanisms. Several more definitive approaches were available, including use of allylic substrates whose reactivities are predictably different for the two different mechanisms or a substrate analogue which could serve as an affinity label for the proposed X group. We have carried out experiments based on both ideas; however, an early clue about the nature of the reaction came from the observation that the allylic substrates were hydrolyzed upon prolonged incubation in the absence of isopentenyl pyrophosphate.[22] The hydrolysis was autocatalytic, suggesting stimulation by a product. Subsequently, the reaction was shown to be absolutely dependent upon the presence of inorganic pyrophosphate with a maximum rate of 2% of the normal prenyl transfer reaction and a K_m for pyrophosphate of 33 mM.[23] Thin layer chromatography of the products of the enzyme–pyrophosphate-mediated hydrolysis of [^{3}H]geranyl pyrophosphate showed geraniol to be the major component, while a small, but significant, portion (ca. 1%) was a hydrocarbon. 2-Fluoroisopentenyl pyrophosphate, a competitive inhibitor against isopentenyl pyrophosphate, completely inhibited the hydrolysis of geranyl pyrophosphate (see below). This strongly suggested that inorganic pyrophosphate was interacting with the enzyme at the isopentenyl pyrophosphate binding site and, in so doing, assisting in the hydrolysis of the allylic substrate. Water would be excluded from the isopentenyl pyrophosphate site by the normal substrate or the fluorine-containing analogue, and hydrolysis would not be significant when the enzyme performs its normal catalytic function.

This hypothesis was substantiated as follows. During the reaction normally catalyzed by prenyl transferase, the C–O bond of the allylic substrate is cleaved and the carbinol carbon is inverted. If the pyrophosphate-stimulated solvolysis of the allylic substrate represents an abortion of the normal catalytic sequence, one would anticipate that the oxygen of the geraniol thus formed would be derived from water. When geranyl pyrophosphate was hydrolyzed in $H_2{}^{18}O$, ^{18}O was recovered in the alcohol in the theoretical amount anticipated for rupture of the C–O bond. Similarly solvolysis of (1*S*)-[1-^{3}H]geranyl pyrophosphate gave the (1*R*)-[1-^{3}H] alcohol as the product. Thus, solvolysis of the allylic substrate stimulated by inorganic pyrophosphate has all of the attributes of the normal prenyl transferase reaction with pyrophosphate as an analogue of isopentenyl pyrophosphate and water as the prenyl acceptor. This would result from water occupying the



portion of the isopentenyl site where the hydrocarbon moiety of the normal substrate usually resides. Although these studies provide evidence for an ionization mechanism, there is still the possibility that water, normally a weak nucleophile, is activated toward displacement of pyrophosphate from the allylic substrate by functionality in the active site.

(21) H. L. King, Jr., and H. C. Rilling, *Biochemistry*, 16, 3815 (1977).

(22) B. C. Reed, Ph.D. Thesis, University of Utah, 1976.

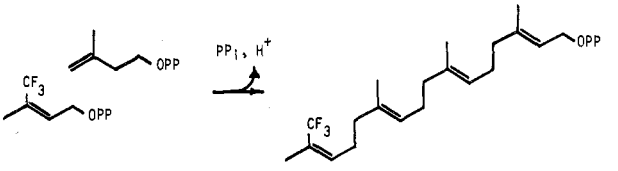
(23) C. D. Poulter and H. C. Rilling, *Biochemistry*, 15, 1079 (1976).

With evidence beginning to accumulate against a displacement–elimination mechanism, we decided to seek verification for an ionization–condensation–elimination sequence. Our approach involved selectively replacing hydrogens around the C(2)–C(3) double bond of the allylic substrate with fluorine. The powerful electron-withdrawing effect of fluorine should retard the rate of an ionization step which depletes the electron density of the allylic moiety but should not drastically alter the rate of a nucleophilic displacement at C(1). If one makes the reasonable assumption that cleavage of the carbon–oxygen bond in the allylic substrate is rate limiting, destabilization of a positively charged allylic moiety by fluorine should be reflected in a reduction of the rate of the enzyme-catalyzed reaction. In using this approach it is essential to satisfy several criteria. The enzyme must recognize the analogue as an allylic substrate, as demonstrated by competitive inhibition with the normal allylic substrate; the analogue should participate in the prenyl transfer reaction to give the expected product; and the rate of the prenyl transfer reaction between isopentenyl pyrophosphate and the analogue must be consistent with the chemical properties of the analogue.

The first set of allylic analogues to be studied were the five-carbon compounds (*E*)- and (*Z*)-trifluoromethyl-2-buten-1-yl pyrophosphate.[12g] A trifluoromethyl moiety was selected because it is not much larger than the methyl group it replaces,[24] and its σ^+ constant (0.612)[25] indicates that it should drastically reduce the rate of ionization of an allylic pyrophosphate. These predictions were verified by solvolysis of methanesulfonate derivatives of dimethylallyl alcohol and both trifluoromethyl alcohols, where substitution of methyl by trifluoromethyl depressed the rate by almost 10^7.[12g] In contrast, the trifluoromethyl moiety slightly accelerates the rate of S_N2 displacement of chloride by iodide in the (*E*)-2-butenyl system.[26] Thus, replacing a methyl group by trifluoromethyl in an allylic compound slightly accelerates a direct displacement and severely depresses ionization, with a total difference of about 10^8!

When the (*E*)- and (*Z*)-trifluoromethyl analogues were incubated with isopentenyl pyrophosphate and porcine farnesyl pyrophosphate synthetase, the rate of condensation was 3×10^{-7} that of the normal catalytic rate. Although the similar rate depressions for solvolytic and enzymatic reactions were encouraging, the analogues did not satisfy the other criteria we had determined to be important. Inhibition constants for the *E* isomer (K_i = 51 μM against geranyl pyrophosphate and K_i = 23 μM against dimethylallyl pyrophosphate) and the *Z* isomer (K_i = 62 μM against geranyl pyrophosphate and K_i = 29 μM against dimethylallyl pyrophosphate) were considerably larger than the Michaelis constants for the normal substrates, indicating a substantially reduced affinity. Also, Lineweaver–Burk plots gave mixed-linear patterns. This observation was surprising since substrate binding studies with the avian enzyme indicated that dimethylallyl pyrophosphate only binds to the allylic site while the kinetic data suggest interactions of the fluoro analogues with both sites.[12g]

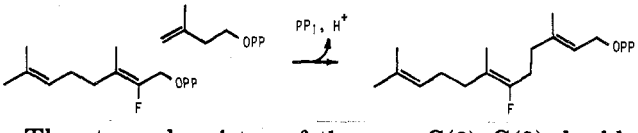
Incubation of (*E*)- and (*Z*)-trifluoromethyldimethylallyl pyrophosphate with [^{14}C]isopentenyl pyrophosphate and the porcine enzyme each gave a product which, after hydrolysis with alkaline phosphatase, eluted slightly before geranylgeraniol on a molecular sizing column.[27] Since geranylgeranyl pyrophosphate is not a substrate for the enzyme and the catalytic rate of the C_{15} to C_{20} conversion is 10^{-2} that of the normal rate, C_{20} materials, presumably the C_{15} trifluoromethyl derivatives of geranylgeranyl pyrophosphate, should result from the condensation of the C_5 fluoro analogues with isopentenyl pyrophosphate.



Unfortunately, we were not able to produce enough C_{20} material for mass spectral analysis.

In order to rectify these problems we switched to 2-fluorogeranyl pyrophosphate as an analogue. The single fluorine at C(2) should retard ionization of the C–O bond, but not to the degree of a trifluoromethyl group, and the ten-carbon analogue should not bind to the site for isopentenyl pyrophosphate.

These predictions were confirmed in a series of experiments with the 2-fluoro analogue.[12k] Substitution of the hydrogen at C(2) by fluorine retarded the solvolysis of geranyl methanesulfonate by 4.4×10^{-3} and accelerated by a factor of two the displacement of chlorine from geranyl chloride with cyanide,[28] while the rate of prenyl transfer with the pyrophosphate derivative was depressed by 8.4×10^{-4}. The fluoro analogue was a competitive inhibitor (K_i = 2.4 μM) for geranyl pyrophosphate, and the Michaelis constant for 2-fluorogeranyl pyrophosphate (K_m = 1.1 μM) was close to that of the normal substrate (K_m = 0.8 μM). The sole product of the reaction was identified as 6-fluorofarnesyl pyrophosphate.



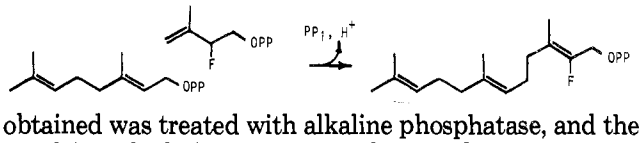
The stereochemistry of the new C(2)–C(3) double bond was determined enzymatically by incubating the C_{10} fluoro analogue with (*R*)- and (*S*)-2-[^{3}H]isopentenyl pyrophosphate and porcine enzyme. The *R* stereoisomer gave a product with quantitative retention of label, while tritium was absent in the other.[29] The observations are consistent with the generation of a new *E* double bond. Thus, the 2-fluorogeranyl pyrophosphate satisfies the three criteria we originally outlined and demonstrated that the prenyl transfer reaction proceeds via an ionization–condensation–elimination mechanism.

The indication that cations are required for catalysis, taken with an earlier observation that divalent cations enhanced the hydrolysis of allylic pyrophosphates,[30]

(24) (a) W. F. Edgell, G. B. Miller, and J. W. Amy, *J. Am. Chem. Soc.*, 79, 2391 (1957); (b) J. L. DeCorn, G. Elefante, A. M. Liquori, and A. Damiani, *Nature (London)*, 216, 910 (1967).
(25) Y. Okamoto, T. Inukai, and H. C. Brown, *J. Am. Chem. Soc.*, 80, 4969 (1958).
(26) E. T. McBee, O. R. Pierce, and D. D. Smith, *J. Am. Chem. Soc.*, 76, 3725 (1954).
(27) C. D. Poulter and D. M. Satterwhite, *Biochemistry*, 16, 5470 (1977).
(28) C. D. Poulter and E. A. Mash, unpublished results.
(29) C. D. Poulter, unpublished results.

suggested a mechanism for triggering the enzymatic reaction by depleting negative charge in the pyrophosphate moiety via coordination to metal, thereby enhancing its reactivity as a leaving group. In a more careful study, we examined the effects of Mg^{2+} and Mn^{2+} on the solvolysis of geranyl pyrophosphate.[31] This substrate was solvolyzed at 55 °C at constant ionic strength in buffered aqueous solution. At concentrations of less than 0.05 M, neither Mn^{2+} nor Mg^{2+} had an effect on the rate of solvolysis of the allylic compound. However, at higher concentrations there was a 30-fold acceleration which leveled off at about 1.5 M metal ion. The maximum rate of solvolysis with Mn^{2+} was 3×10^{-3} s^{-1} and was 6-fold greater than found with Mg^{2+}. The enhanced solvolysis as a function of cation concentration strongly suggests that the solvolysis resulted from the formation of a complex between geranyl pyrophosphate and a divalent cation. From the dissociation constants of Mn^{2+}–geranyl pyrophosphate and Mg^{2+}–geranyl pyrophosphate, it can be shown that the substrate exists as the monometal salt when the cation concentration is 1 mM, a concentration that does not affect the rate of solvolysis. Consequently, the formation and decomposition of a $(metal)_2$–geranyl pyrophosphate complex must be responsible for the enhanced solvolysis. This requirement for a second cation is particularly interesting since prenyl transferase binds two metals per catalytic site in the presence of substrate.[21]

The question of participation by an X group in the condensation is not precluded in an ionization–condensation–elimination mechanism.[15c] We decided to probe for the involvement of an X group in the reaction by using 2-fluoroisopentenyl pyrophosphate as a potential affinity label.[12j] The fluorine, by thwarting elimination after attachment of the X group, would irreversibly attach the allylic substrate and the fluoro analogue to the enzyme.[32] Initial experiments demonstrated that this was not the case. Addition of 2-fluoroisopentenyl pyrophosphate along with isopentenyl pyrophosphate and geranyl pyrophosphate slowed down the rate of normal reaction, but after dialysis to remove small molecules, full activity was restored to the enzyme. Also 2-fluoroisopentenyl pyrophosphate is an excellent competitive inhibitor for the normal substrate with a $K_i = 0.8$ μM, and the fluoro analogue participates in the prenyl transfer reaction with a rate $^1/_{25}$th that of isopentenyl pyrophosphate. The product thus



obtained was treated with alkaline phosphatase, and the resulting alcohol was converted to the benzoate. The ester was identified as 2-fluorofarnesyl benzoate by mass spectrometry, by NMR, and finally by synthesis. The stereochemistries of the C(2)–C(3) *E* and *Z* isomers were established by comparing chemical shifts of the methyl groups at C(3) and ^1H–^{19}F long-range coupling to the methyl protons and with chemical shift studies using paramagnetic shift reagent.[12k] Thus, prenyl transferase catalyzes the condensation between 2-fluoroisopentenyl pyrophosphate and geranyl pyrophosphate to give a product identical with the normal product, except the hydrogen at C(2) has been replaced by fluorine.

During incubations with the fluoro analogue and geranyl pyrophosphate, the rate of condensation dropped as the reaction proceeded, and upon prolonged incubations slightly less than 50% of the homoallylic substrate was consumed. Based on the normal stereochemistry for proton removal at C(2) of isopentenyl pyrophosphate, it is likely, but still not proved, that the enzyme is selective for (*S*)-2-fluoroisopentenyl pyrophosphate. Since only (*R*)-2-fluoroisopentenyl pyrophosphate should serve as an affinity label for the elusive X group, our data do not rigorously exclude its participation in the reaction. However, it is instructive to note that the enzyme retains most of its activity after incubations of up to 4 days in the presence of racemic 2-fluoroisopentenyl pyrophosphate and geranyl pyrophosphate.

Mechanism of the Reaction

Sufficient data now exist to justify an analysis of the 1′–4 coupling reaction catalyzed by farnesyl pyrophosphate synthetase in the context of an ionization–condensation–elimination mechanism. The kinetic results of Popjak suggest an ordered sequential pattern.[11,33] Thus both substrates are present in the active site prior to initiation of the condensation. Binding studies indicate that the enzyme has two catalytic sites, with identical specificities for binding the allylic substrates.[20]

Proper alignment of the two substrates is an obvious and important requirement for the enzyme. Although the precise topology of the enzyme–substrate complex is not known, some reasonable assumptions can be made. The stereochemical data of Popjak and Cornforth define the orientation of C(1) of the allylic substrate with respect to the C(3)–C(4) double bond of isopentenyl pyrophosphate. Since C(1) in the allylic substrate is joined to C(4) of the neighboring double bond, it is reasonable to assume that the two atoms are adjacent in the enzyme–substrate complex. Also, formation of an *E* double bond between C(2) and C(3) in isopentenyl pyrophosphate requires that the dihedral angle between the C(1)–C(2) and C(3)–C(4) bonds be greater than 90°, and ideally the dihedral angle should approach 180°. The metal binding experiments suggest that the metal ions are coordinated between the pyrophosphate residues of the two substrates.[21] This coordination serves to stack the pyrophosphates and orient the substrates as shown in the figure. Although nothing is known about the amino acids in the active site, polar and nonpolar regions must be present to accommodate the amphiphilic substrates. Also, in this model, the allylic site accepts both C_5 and C_{10} allylic substrates.

(30) C. George-Nascimento, R. Pont-Lezica and O. Cori, *Biochem. Biophys. Res. Commun.*, **45**, 119 (1971).
(31) D. M. Brems and H. C. Rilling, *J. Am. Chem. Soc.*, **99**, 8351 (1977).
(32) This assumes that the X group is covalently attached to the enzyme.[15c]
(33) The enzymatic reaction was shown to be irreversible by failure to incorporate [^{32}P]pyrophosphate into geranyl pyrophosphate. The allylic substrate, [^{32}P]pyrophosphate, and the enzyzme were incubated together or with either isopentenyl pyrophosphate or 2-fluoroisopentenyl pyrophosphate. Geranyl pyrophosphate then was recovered and purified by thin layer chromatography. If the reverse reaction had progressed at the same rate as the forward reaction, 5×10^8 cpm of ^{32}P would have been associated with geranyl pyrophosphate. However, insignificant amounts of radioactivity were found.

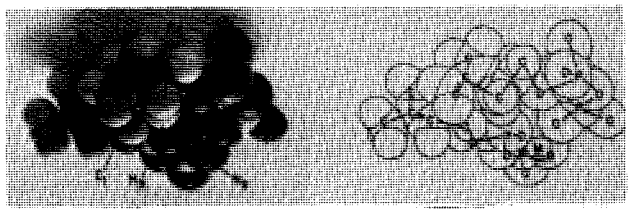


Figure 1. A proposed alignment for isopentenyl pyrophosphate, dimethylallyl pyrophosphate, and magnesium prior to ionization.

Since the enzyme binds both pyrophosphates in the absence of the divalent cations and only binds Mg^{2+} and Mn^{2+} when a pyrophosphate moiety is present, it is reasonable to assume that the metal is assisting the catalytic step. Pyrophosphates are dianions at physiological pH and are relatively stable in that form. However, removal of negative charge from the pyrophosphate enhances its reactivity as a leaving group, and it is likely that metal ion catalysis involves the formation of a complex which maximally depletes the negative charge at P(1), thereby activating the allylic substrate for ionization of the carbon–oxygen bond.

Ionization produces an allylic cation which condenses with isopentenyl pyrophosphate. This ionization could be assisted by the π electrons in the double bond of isopentenyl pyrophosphate, in which case bonding between the two substrates would be synchronous with ionization, or ionization and condensation could be two discrete steps. We have no data which permit us to distinguish between these two possibilities. The parallel between rate retardations observed when hydrogen is replaced by fluorine for solvolysis where π participation is not involved and the prenyl transfer reaction suggest that similar amounts of developing positive charge are delocalized into the allylic double bond in both instances.[12g,k] Covalent bonding between the substrates upon ionization is not needed to preserve the stereochemistry at C(1) in the allylic substrate since the allylic cations are resonance-stabilized species with three trigonal carbons [C(1), C(2), and C(3)] and directly bonded atoms in a common plane. The energy barrier for rotation about the C(1)–C(2) bond is not precisely known, but a barrier of more than 28 kcal/mol is anticipated.[34] Once the substrates are aligned as shown in the figure,[36] the carbon–carbon bond will be formed with inversion at C(1) unless the entire allylic cation flips in the active site prior to condensation. A movement of that magnitude for a highly reactive species is not likely. It should be mentioned that the pyrophosphate-dependent hydrolysis of the allylic pyrophosphate indicates the enzyme can ionize the allylic substrate without anchimeric assistance by isopentenyl pyrophosphate.[23]

The condensation reaction is completed by elimination of a proton from C(2) to form a double bond between C(2) and C(3). If the pyrophosphate moieties are stacked prior to ionization, the pyrophosphate group just released from the allylic substrate is in a position to assist in removing the proton,[37] and the stereochemistry of the elimination step is a consequence of the alignment of the substrates in the active site. Since Cornforth and Popjak 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). For orientations which permit formation of an *E* double bond, only the *pro-R* proton is available to the pyrophosphate. If the substrates are arranged as shown in the figure, the region of space surrounding the proton to be eliminated is so congested that intervention of another base is unlikely. By a similar argument, the *pro-S* proton must be lost from C(2) when a *Z* double bond is formed. Examples of *Z* transferases are known, and the proton is eliminated from the *pro-S* position.[17]

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 one could still envision participation by an X group in noncovalent electrostatic stabilization, we see no justification for its continued inclusion in mechanistic schemes at this time. The ionization–condensation–elimination mechanism is fully consistent with the chemical properties of the substrates and substrate analogues, the stereochemistry of prenyl transfer, and the observation that the enzyme catalyzed hydrolysis of its allylic substrates.

Overview

Recent work with farnesyl pyrophosphate synthetase has answered basic questions about size, subunit structure, and binding properties. The results of our studies with fluoro analogues clearly point toward an ionization–condensation–elimination mechanism for the reaction. In the future we anticipate that the structure of the enzyme will be refined, especially with regard to the composition of functional groups in the active site and the topology of the enzyme–substrate complex. Several questions about the mechanism, such as the timing of the ionization–condensation–elimination sequence, remain to be answered. Finally, the generality of the mechanism found for farnesyl pyrophosphate synthetase has not been established for enzymes which catalyze prenyl transfer to acceptors other than isopentenyl pyrophosphate.

It is a pleasure for us to acknowledge the contributions of our co-workers, J. C. Argyle, D. M. Satterwhite, E. A. Mash, O. J. Muscio, N. L. Eberhardt, B. C. Reed, L.-S. Yeh, H. L. King, and D. M. Brems, to the work described in this account. Financial support was generously provided by the National Institutes of Health, GM 21328 (CDP) and AM 13140 (HCR), the Alfred P. Sloan Foundation (CDP), and a Research Career Development Award from the National Institutes of Health, HL 00084 (CDP).

(34) The barrier for the parent cation is estimated to be 27.9 kcal/mol,[35] and substitution of alkyl groups at C(3) should increase the barrier to rotation about the C(1)–C(2) bond.

(35) N. L. Allinger and J. H. Siefert, *J. Am. Chem. Soc.*, 97, 752 (1975).

(36) This orientation is only one of several which fits stereochemical and binding data.

(37) G. Popjak, *Harvey* Lect., 65, 127 (1971).