*Pure & Appl. Chem.*, Vol. 51, pp.597-608.
Pergamon Press Ltd. 1979. Printed in Great Britain.

0033-4545/79/0301-0597 $02.00/0

# PRENYLTRANSFERASE

Hans C. Rilling

Department of Biochemistry, University of Utah, Salt Lake City, Utah 84132, USA

Abstract - The prenyltransferase reaction is reviewed with an emphasis on the enzyme farnesyl pyrophosphate synthetase. Substrate-binding studies have shown that this dimeric enzyme has two catalytic sites with identical substrate specificity. The product farnesyl pyrophosphate is associated with the allylic portion of the catalytic site and overlaps into the homoallylic site, thus interfering with the binding of isopentenyl pyrophosphate. The enzyme also catalyzes a pyrophosphate-stimulated solvolysis of the allylic substrate. This observation, along with studies of the reactivity of fluorine-containing substrate analogues, indicates an ionization-condensation-elimination mechanism for the prenyltransfer reaction. A model showing how the substrates are aligned in the catalytic site is presented.

## INTRODUCTION

The prenyltransferase reaction is the alkylation of an acceptor molecule by an allylic pyrophosphate with the concomitant release of inorganic pyrophosphate. There are three classes of prenyltransfer reactions which are distinguished by the nature of the acceptor molecule (Fig. 1). The first, which is the reaction

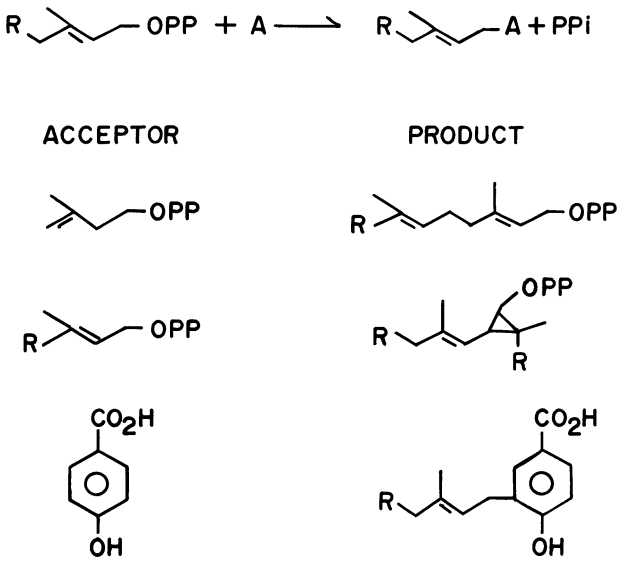



Fig. 1. The three different kinds of prenyltransfer reactions.

usually considered to be prenyltransferase (EC 2.5.1.1), is the condensation between isopentenyl pyrophosphate and an allylic pyrophosphate. The product of this reaction is the next higher homolog of the allylic substrate. Depending upon the specificity of the enzyme system, there may be only a single condensation (monoterpene synthesis) or the condensation may be repeated several thousand times (rubber biosynthesis). This reaction is the basic condensation reaction known as head-to-tail or 1'-4 and is utilized for the construction of linear polyisoprenoid chains. Also, the double-bond geometry of the products may be E or Z, again depending upon the enzyme system involved.

The second type of prenyltransfer is the head-to-head or head-to-middle ($\overline{1'\text{-}2\text{-}3}$) condensation that leads to the formation of the asymmetrical precursors of the symmetrical terpenes, squalene and phytoene. In this reaction, presqualene and prephytoene pyrophosphates are formed by the insertion of C(-1) of one allylic molecule into the C(2)-C(3) double bond of the second. Finally, a third type of prenylation is the transfer of an isoprene residue to a nonisoprenoid acceptor. The example shown is a reaction involved in the biosynthesis of coenzyme Q.

In this article, I will cover the head-to-tail prenyltransferase reaction in some detail since it has been most extensively studied. The other prenylation reactions will be included only when they serve to test the generality of what has been established for farnesyl pyrophosphate synthetase.

Early studies and stereochemistry

Prenyltransferase activity was reported initially in 1959 by Lynen, who also was the first to appreciate the use of allylic pyrophosphates in biosynthetic reactions (1). Shortly thereafter, two groups, those of Porter and Popják, achieved extensive purification from mammalian sources of farnesyl pyrophosphate synthetase, the prenyltransferase of cholesterol biosynthesis (2,3). Their work showed that a divalent cation was required in the reaction and that, most likely, one enzyme catalyzed the condensation between dimethylallyl pyrophosphate and isopentenyl pyrophosphate forming geranyl pyrophosphate which, in turn, condensed with a second mole of isopentenyl pyrophosphate, producing farnesyl pyrophosphate. Since then, prenyltransferase activities for the synthesis of geranylgeranyl pyrophosphate (4), solanesyl pyrophosphate (5), and undecaprenyl pyrophosphate (6) have been reported.

The stereochemistry of the prenyltransfer action was established by Cornforth's and Popják's investigation of the stereochemistry of squalene biosynthesis (7). In their solution of thirteen of the fourteen points of stereochemical ambiguity in squalene biosynthesis, six items dealt with the prenyltransfer reaction. They demonstrated that during the condensation reaction the carbinol carbon of the allylic substrate is inverted and that the si face of the C(3)-C(4) double bond of isopentenyl pyrophosphate is attacked. The new carbon-to-carbon bond is formed on, and the proton is lost from, the same side of isopentenyl pyrophosphate (Fig. 2). The proton lost is the pro R hydrogen. Others have determined that the stereochemistry of

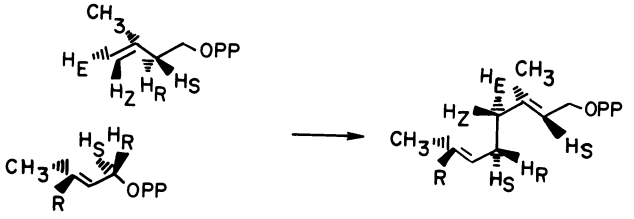



Fig. 2. The stereochemistry of prenyltransferase (7).

proton removal at C(2) seems to be dictated by the geometry of the double bond being formed. During synthesis of polyisoprenoids with E double bonds, the pro R hydrogen is removed, while the formation of Z double bonds entails the removal of the pro S hydrogen from C(2) (8-10). However, it is not known if proton loss and carbon-to-carbon bond formation are always on the same side of isopentenyl pyrophosphate, since the stereochemistry of the condensation reaction leading to Z double bonds has not been determined.

The stereochemistry of this condensation led Cornforth and Popják to propose a two-step condensation-elimination or X-group mechanism in which an unspecified X nucleophile and the C(1) of allylic substrate were added trans to the double bond of isopentenyl pyrophosphate. The X group and the departing proton were then eliminated in a trans mode (Fig. 3) (8,11,12). This mechanism was in contrast to an ionization-condensation-elimination mechanism that had been proposed earlier by Lynen (1) and Bloch (13), as well as Cornforth and Popják (14) (Fig. 4).

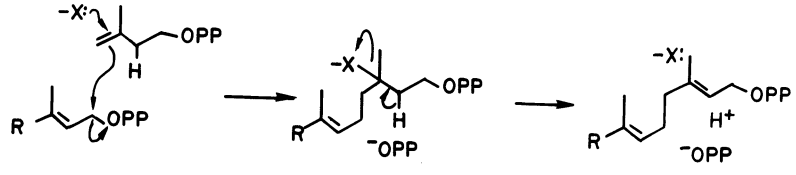



Fig. 3. The condensation-elimination or X-group mechanism for the prenyltransferase action.

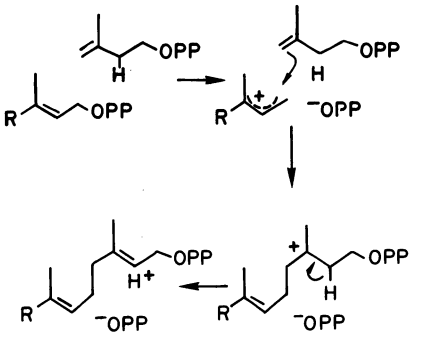



Fig. 4. The ionization-condensation-elimination mechanism for the prenyltransferase reaction.

The substrate specificity of farnesyl pyrophosphate synthetase has been studied extensively by Ogura and Popják (15-25). The pyrophosphate ester moiety is essential for activity as a substrate. However, extensive modification of the hydrocarbon portion of the allylic substrate is possible. The minimum requirements are a C(2)-C(3) double bond and a hydrogen at C(2). Ogura prepared a series of analogues by extending the hydrocarbon chain E from the allylic double bond and found two optima for substrate activity corresponding to the chain lengths of the natural substrates (20). This observation led to the postulate that the enzyme had two catalytic sites with different substrate specificities. In all, more than thirty analogues of the allylic substrate have been shown to participate in the prenyltransfer reaction. The specificity for the homoallylic substrate is more restrictive and only a few analogues have been shown to function as substrates. One particularly interesting example is homoisopentenyl pyrophosphate, which condenses with geranyl pyrophosphate to produce the Z rather than E product (23). Thus, the addition of one carbon to the homoallylic substrate produces a major change in the conformation of this substrate in the catalytic site. The rather casual substrate specificity of this enzyme has proved to be useful in experiments that will be described later.

Enzyme isolation and characterization

I will now concentrate on experiments carried out during the past several years at the University of Utah in my laboratory and in the laboratory of Professor C. D. Poulter (27). These studies have provided a more detailed knowledge of prenyltransferase and its mechanism of catalysis. Initially, homogeneous preparations of farnesyl pyrophosphate synthetase were obtained from both Saccharomyces cerevisiae (28) and Phycomyces blakesleeanus (29). Both of these proteins are dimers of about 85,000 daltons, and both catalyzed the condensation of isopentenyl pyrophosphate with either dimethylallyl pyrophosphate or geranyl pyrophosphate. Unfortunately, these enzymes are relatively unstable and thus unsuitable for more detailed studies. Homogeneous prenyltransferase was also obtained from pig liver (30). This enzyme, which is not allosteric, has the unusual property of existing in two interconvertible forms.

Pig liver prenyltransferase, when chromatographed on DEAE at pH 7.8 with a phosphate gradient, eluted as two discrete peaks of activity. The individual peaks, when rechromatographed under the same conditions, also split into two components. It was possible to demonstrate interconversion of the two forms by isoelectric focusing. Koyama et al. observed the same phenomena and presented evidence that the formation and cleavage of disulfide bonds was involved in the interconversion (31). Since we could not control the interconversion of the two forms and were never certain of which form we were assaying, we did not feel that this enzyme was a suitable subject for enzymatic and mechanistic studies. Fortunately, a stable crystalline enzyme was obtained from chicken livers (32), and this protein has been used for most of the experiments described subsequently. This prenyltransferase, like the others, has a molecular weight of about 85,000. $V_{max}$ is 2.5 units $mg^{-1}$ in the best preparations, giving a turnover number of about 100 per catalytic site per minute at 37°. The enzyme from yeast is somewhat more active, with a turnover number of about 200 at 30°. Farnesyl pyrophosphate also condenses with isopentenyl pyrophosphate at about 1% the rate of geranyl pyrophosphate. The $K_m$ values for the geranyl and isopentenyl pyrophosphates are 0.5 μM. Reversibility of the reaction was tested by incubating the enzyme with geranyl pyrophosphate, a limiting amount of isopentenyl pyrophosphate and $^{32}P$ inorganic pyrophosphate.

$$GPP + IPP \xrightarrow{Mg^{2+}} FPP + {}^{32}PPi + H^{+}$$

Geranyl pyrophosphate was isolated from the incubation mixture and found to be devoid of $^{32}P$. If the reverse reaction had proceeded at $10^{-7}$ of the rate of the forward reaction, $^{32}P$ would have been recovered with the geranyl pyrophosphate (29).

All of the prenyltransferase enzymes that have been purified to homogeneity have been found to have two subunits. The subunits have not been resolved by electrophoresis in polyacrylamide gels containing either SDS or urea at alkaline pH. End-group analysis, tryptic maps, and cyanogen bromide fragmentation patterns also provide evidence that is consistent with identical subunits (28,29,30,32). Finally, the substrate-binding studies described below also indicate catalytic sites with identical properties.

Substrate-binding studies

Substrate-binding studies were initiated to solve the problem of the specificity of the catalytic sites in a two-subunit enzyme that catalyzes the condensation of both a $C_5$ and $C_{10}$ allylic substrate with isopentenyl pyrophosphate. Since a divalent cation, $Mg^{2+}$ or $Mn^{2+}$, is required for enzymatic activity, the initial studies were in the presence of $Mg^{2+}$. Reed demonstrated that the enzyme bound two moles of either of the three allylic substrates (Fig. 5) (33). Mutual competition was also demonstrated between the allylic substrates with a maximum of two allylic pyrophosphates bound per mole of enzyme (Table I). Substrate-

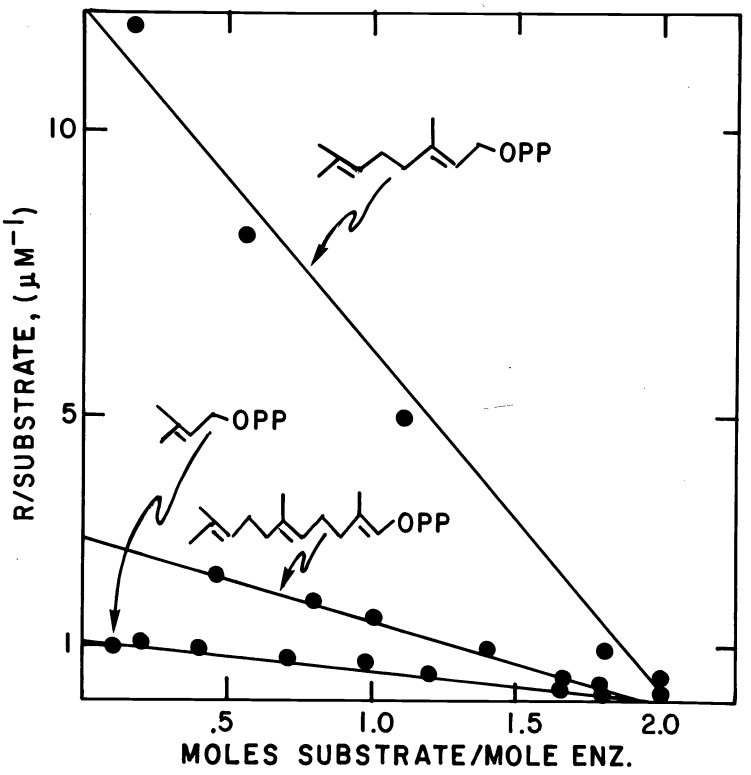



Fig. 5. Scatchard plots of the binding of the three allylic pyrophosphate substrates to prenyltransferase in the presence of $Mg^{2+}$. R is the number of moles of substrate bound per mole enzyme. Data from (33).

TABLE I. Competition between dimethylallyl and geranyl pyrophosphate for binding to prenyltransferase in the presence of $Mg^{2+}$.

| Concentration of geranyl pyrophosphate (μM) | Moles ligand bound per mole enzyme | | |
|---|---|---|---|
| | Dimethylallyl pyrophosphate | Geranyl pyrophosphate | Total |
| 0 | 1.8 | 0 | 1.8 |
| 4 | 1.3 | .5 | 1.8 |
| 10 | .9 | 1.1 | 2.0 |
| 20 | .5 | 1.7 | 2.2 |
| 30 | .4 | 1.9 | 2.3 |

The concentration of dimethylallyl pyrophosphate (20 μM) and enzyme (5.5 μM) were fixed. Data from (33).

binding experiments with the homoallylic substrate gave surprising results in that four moles of this substrate were bound per mole of protein. Thus, it seemed this substrate could occupy both the allylic and homoallylic regions of the catalytic site. This is not unexpected since isopentenyl pyrophosphate and dimethylallyl pyrophosphate are structurally quite similar. However, there is no indication for the converse; i.e.,

for dimethylallyl pyrophosphate binding in the isopentenyl pyrophosphate region of the catalytic site. This observation is consistent with the more rigid substrate specificity found for the homoallylic site. In addition, there is kinetic evidence for the interaction of isopentenyl pyrophosphate at the allylic site since isopentenyl pyrophosphate inhibits prenyltransferase at relatively low concentrations (1 μM) (32,34). By including citronellyl pyrophosphate, a nonreactive analogue of geranyl pyrophosphate, it was possible to reduce the binding of isopentenyl pyrophosphate to two moles per mole of enzyme (33). These studies indicated very clearly that the enzyme contained two catalytic sites with indistinguishable affinities for both of the allylic substrates and for isopentenyl pyrophosphate. Subsequently, additional experiments were undertaken to evaluate the role of the metal ion in the prenyltransfer reaction. When divalent cations were omitted, the allylic substrates still were bound efficiently with only a slight increase in the dissociation constant (35). However, isopentenyl pyrophosphate was bound to the extent of two moles per mole enzyme rather than four (Fig. 6). Thus, the presence of metal ion is required for the interaction of isopentenyl pyrophosphate with the allylic site. In the absence of a metal ion, it was possible to demonstrate the

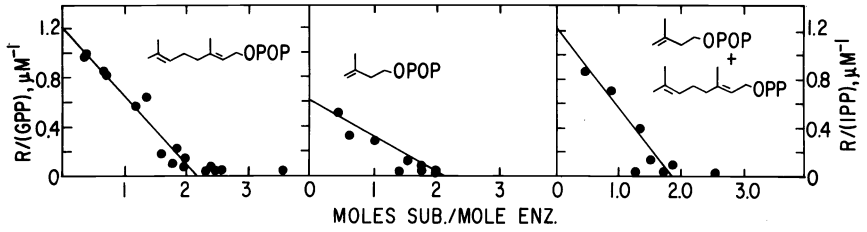



Fig. 6. Scatchard plots of the binding of geranyl pyrophosphate and isopentenyl pyrophosphate to prenyltransferase in the absence of a divalent cation. R is the number of moles of substrate bound per mole enzyme. Data from (35).

simultaneous binding of both geranyl and isopentenyl pyrophosphate without significant catalysis. The affinity of isopentenyl pyrophosphate for the enzyme was only slightly enhanced by the presence of the second substrate. This experiment also clearly demonstrated that isopentenyl pyrophosphate was bound to the homoallylic site when metal ion was excluded (35).

In the absence of metal ion, and consequently catalytic activity, it was possible to demonstrate competition between isopentenyl pyrophosphate and farnesyl pyrophosphate. We had previously shown that the product, farnesyl pyrophosphate, competed with the other allylic substrates for the enzyme. Now, in the absence of metal, farnesyl pyrophosphate was also competitive with isopentenyl pyrophosphate for binding to the enzyme. However, in this situation, as the concentration of isopentenyl pyrophosphate was increased, an increasing amount was bound to the enzyme without displacing farnesyl pyrophosphate (Table 2); and

TABLE 2. Competition for binding to prenyltransferase between isopentenyl pyrophosphate and farnesyl pyrophosphate in the absence of $Mg^{2+}$.

| Concentration of isopentenyl pyrophosphate (μM) | Moles ligand bound per mole enzyme | | |
|---|---|---|---|
| | Isopentenyl pyrophosphate | Farnesyl pyrophosphate | Total |
| 0 | 0 | 2.1 | 2.1 |
| 15 | .2 | 1.9 | 2.1 |
| 25 | .2 | 1.9 | 2.1 |
| 75 | .75 | 1.9 | 2.65 |
| 125 | 1.5 | 1.7 | 3.2 |

The concentration of farnesyl pyrophosphate (20 μM) and enzyme (4.8 μM) were fixed. Data from (35).

finally, at 125 μM isopentenyl pyrophosphate, two moles were bound along with two moles of farnesyl pyrophosphate. The absence of metal ion was not responsible for competition between these two pyrophosphates since the relatively nonreactive 2-fluorofarnesyl pyrophosphate and isopentenyl pyrophosphate were mutually competitive in the presence of $Mg^{2+}$ (35).

One can envision two ways that the product of prenyltransferase could compete with both of the substrates (Fig. 7). One model shows the pyrophosphate moiety of farnesyl bound to the isopentenyl pyrophosphate site and the hydrocarbon portion filling both the allylic and homoallylic sites. The second model shows farnesyl pyrophosphate binding in the allylic site but so crowded in the hydrocarbon portion that the tail of the molecule is forced back into the homoallylic site. The substrate binding shown in Table 2 indicates that the second model is quite likely. Other experiments have provided additional evidence for this. Inorganic pyrophosphate was used as a substrate analogue in the first of these experiments. In the presence of $Mg^{2+}$, four moles of inorganic pyrophosphate are bound per mole enzyme, with strong positive cooperativity. We

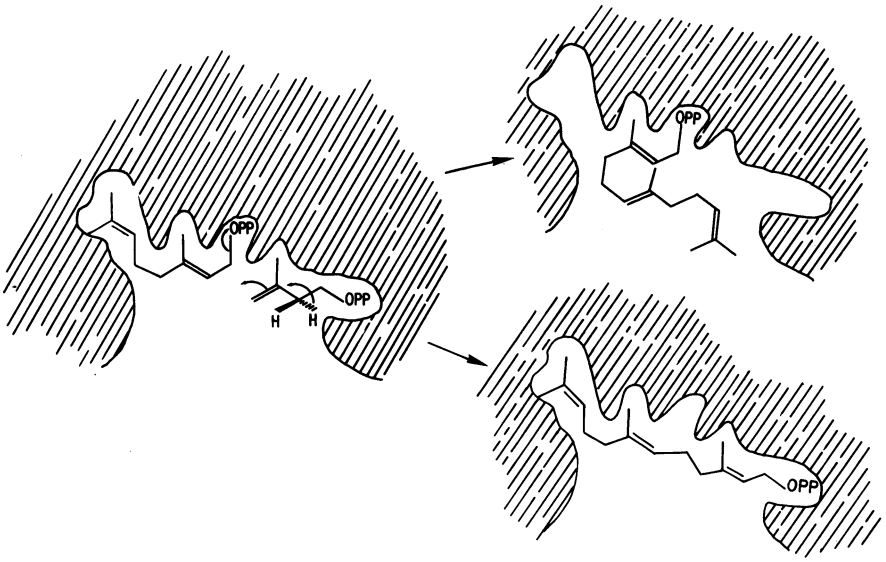



Fig. 7. Two models showing how the product, farnesyl pyrophosphate, could be bound to the catalytic site of prenyltransferase.

reasoned that at low concentrations of inorganic pyrophosphate one site on the enzyme would be preferentially occupied. Then the binding of pyrophosphate was measured in the presence of increasing concentrations of farnesyl pyrophosphate, isopentenyl pyrophosphate, and citronellyl pyrophosphate, an analogue of geranyl pyrophosphate. Both farnesyl and citronellyl pyrophosphate suppressed binding of pyrophosphate at all concentrations tested while, at low concentrations, isopentenyl pyrophosphate actually enhanced the binding of inorganic pyrophosphate (36). Since farnesyl pyrophosphate depresses the binding of inorganic pyrophosphate like the geranyl pyrophosphate analogue rather than enhancing its binding as isopentenyl pyrophosphate does, it is most likely that the pyrophosphate portion of farnesyl pyrophosphate is residing in the allylic site on the enzyme.

In experiments to be discussed below, we demonstrated that prenyltransferase can solvolyze its allylic substrate, a process which is facilitated by inorganic pyrophosphate acting in the homoallylic site. The maximum velocity for solvolysis of the three substrates is 1.8, 12 and .17 μmole $min^{-1}mg^{-1}$ for the $C_5$, $C_{10}$, and $C_{15}$ pyrophosphates, respectively. However, the $K_m$ value for all allylic substrates is quite similar, being $36 \pm 3$ μM and the $K_m$ for inorganic pyrophosphates is about 20 μM for all three solvolytic reactions (36). Thus, the $K_m$ values indicate that the three allylic pyrophosphates are interacting at the same site on the enzyme and that farnesyl pyrophosphate does not compete with inorganic pyrophosphate in the solvolytic reaction. The reduced solvolytic rate for farnesyl pyrophosphate probably reflects an inefficient alignment of this substrate in the catalytic site.

Since both substrates, geranyl and isopentenyl pyrophosphate, can be bound to the enzyme in the absence of metal ion, it is clear that $Mg^{2+}$ or $Mn^{2+}$ is required for catalysis and not binding of substrates. Nonetheless, the metal ion must be interacting with the enzyme substrate complex in order to facilitate catalysis. Since $Mn^{2+}$ supports catalysis by prenyltransferase, we were able to use $^{54}Mn^{2+}$ for binding studies. In the absence of substrate, the protein did not bind significant amounts of this ion (35). However, in the presence of either substrate, four moles of metal were bound per enzyme or two per catalytic site. For binding in the presence of geranyl pyrophosphate, the Scatchard plots were linear. In the presence of isopentenyl pyrophosphate, the plots were curved, and positive cooperativity was indicated. Binding of $^{54}Mn^{2+}$ was also examined in the presence of analogues of both substrates, 2-fluorogeranyl pyrophosphate and 2-fluoroisopentenyl pyrophosphate. These analogues are substrates for prenyltransferase with $K_m$ values approaching the natural substrates but reaction velocities sufficiently damped so that significant condensation does not occur during a binding assay. Here again, in the presence of both substrates, four metal ions were bound per mole enzyme. Thus, with either or both substrates, two metal ions are associated with each catalytic site (35). The substrate binding experiments are summarized in Table 3.

Mechanistic studies

Prenyltransferase has been subjected to kinetic analysis several times. Halloway and Popják utilized the pattern of product inhibition and concluded that the mechanism was ordered sequential (3). Quite recently, Laskovics in Poulter's laboratory carried out an analysis of dead-end inhibition patterns utilizing fluorine-containing substrate analogues as inhibitors (34). Their results have demonstrated clearly that the sequence of addition of substrates to the enzyme is random rather than ordered. This observation is consistent with the

TABLE 3. Ligand binding, dissociation constants, and saturation values.

| Measured ligand | Fixed ligand | Saturation value | $K_{diss}$ (μM) |
|---|---|---|---|
| DMAPP | Mg | 2 | 2.4 |
| GPP | Mg | 2 | 0.28 |
| FPP | Mg | 2 | 0.73 |
| IPP | Mg | 3.8 | 2.5 |
| GPP | Mn | 1.9 | 0.23 |
| IPP | Mn | 3.6 | 0.8 |
| GPP | None | 2.2 | 1.8 |
| IPP | None | 2.0 | 4.3 |
| IPP | GPP | 1.9 | 1.5 |
| Mn | GPP | 4.3 | 9.3 |
| Mn | FPP | 4.2 | 9.2 |
| Mn | IPP | 4.0 | 3 |
| Mn | 2-F-IPP + 2-F-GPP | 4.3 | 6.3 |

substrate-binding experiment which showed no evidence for sequential binding of the substrates (33,35). During the kinetic studies at Utah, a clever, independent proof of the random mechanism was developed. In these experiments the enzyme was preincubated at 0° with $Mg^{2+}$ and one radioactive substrate. Then a large excess of the same substrate, nonradioactive, and the second substrate were added. The reaction was then stopped at various times and the extent of incorporation of both substrates into product was determined. If the substrate initially bound to the enzyme was in rapid equilibrium with the solvent, the relative incorporation of this substrate into product would be the simple average of the specific activity of the initial substrate and the diluting substrate. If, however, the residence time for the substrate on the enzyme approached the turnover time for the enzyme, then the original substrate would be preferentially incorporated into product on the first catalytic turnover of the enzyme. The results indicate that a very significant amount of geranyl pyrophosphate is trapped in the catalytic site. Similar experiments indicate that isopentenyl pyrophosphate can also be trapped. For each substrate, a little more than 50% of the catalytic sites remains occupied by the initial substrate and is not diluted out by the nonradioactive substrate that is introduced with the second substrate (34). These experiments provide definitive and direct evidence that the order of addition of substrates is random.

A property of the enzyme discovered during initial substrate-binding experiments was its ability to hydrolyze the allylic substrates (37). The reaction was autocatalytic, which suggested that one of the products was facilitating the hydrolysis. It was known from inhibition studies that the allylic alcohols did not interact with the enzyme while inorganic pyrophosphate did (38). Therefore, it seemed most likely that inorganic pyrophosphate was participating in the reaction. It was also reasonable to assume that, since the allylic substrate was being modified, the inorganic pyrophosphate must be acting in the homoallylic site. This was proved by demonstrating that inclusion of 2-fluoroisopentenyl pyrophosphate, a relatively nonreactive analogue of isopentenyl pyrophosphate, completely blocked the pyrophosphate-stimulated solvolysis (39). In the normal course of the prenyltransfer reaction, the C-O bond of the allylic substrate is cleaved and the carbinol carbon is inverted. The following experiments tested to see if this happened during the hydrolysis of the allylic substrate. Geranyl pyrophosphate, inorganic pyrophosphate, and $Mg^{2+}$ were incubated with the enzyme in $H_2{}^{18}O$. The geraniol produced by hydrolysis was analyzed by mass spectrometry and found to have incorporated $^{18}O$ to the extent anticipated for quantitative cleavage of the carbon-oxygen bond and replacement of the alcohol by oxygen from water. Thus, it was apparent that the enzyme was not acting as a phosphatase which cleaves oxygen phosphorus bonds, and we concluded that the observed hydrolysis was an aberration of the normal catalytic reaction. 1-S-$^3$H Geranyl pyrophosphate was prepared from 1-S-$^3$H geraniol, which was obtained by enzymatic reduction of 1-$^3$H geraniol. Enzyme-catalyzed solvolysis of this stereochemically labeled material produced 1-$^3$H-R-geraniol, as judged by enzymatic oxidation utilizing alcohol dehydrogenase. Thus, the pyrophosphate-stimulated solvolysis of the allylic substrate has all of the attributes of the normal prenyltransfer reaction except that water rather than isopentenyl pyrophosphate is the acceptor molecule. Fig. 8 depicts the catalytic site of prenyltransferase with homoallylic substrate or with pyrophosphate as an indication of how the solvolytic reaction might occur. The fact that the enzyme, in conjunction with inorganic pyrophosphate, can cleave the carbon-oxygen bond of the allylic substrate unassisted by the double bond of the homoallylic substrate provides strong evidence that ionization is the initiating event in the normal condensation reaction (39).

Convincing proof for the ionization-condensation-elimination reaction has come from studies utilizing fluorine-containing substrate analogues (40,41,42). Fluorine by its electron withdrawing effect when substituted about the allylic double bond should destabilize the formation of a carbonium ion. Analogues so substituted should have a substantially suppressed rate of ionization. Also, because of its size, fluorine should be able to replace hydrogen without disturbing the size or conformation of the substrate molecule. Poulter and his coworkers have prepared several fluorine-containing analogues, E and Z trifluorodimethyl

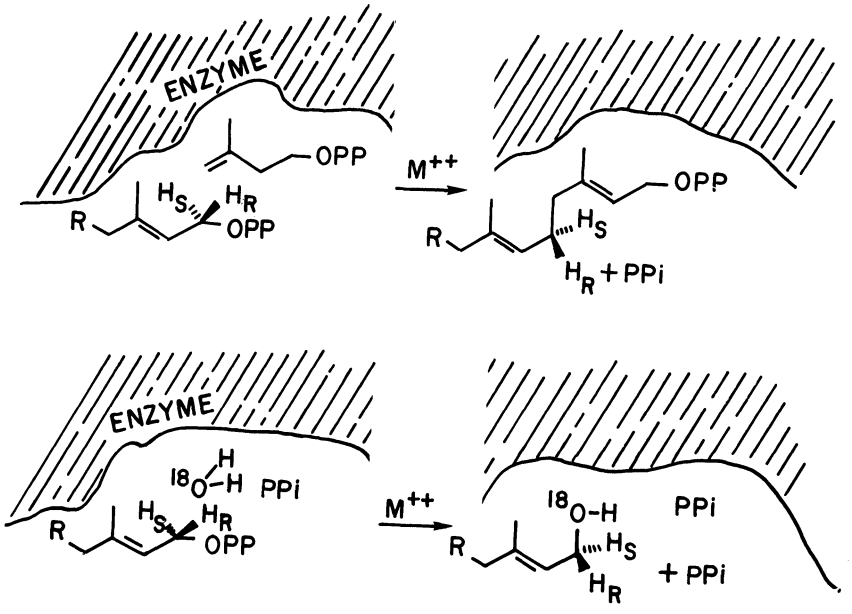
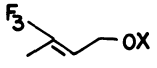
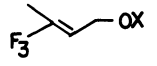
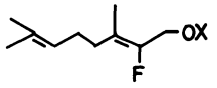



Fig. 8. A schematic representation of the catalytic site of prenyltransfer catalyzing prenyltransfer to isopentenyl pyrophosphate or water.

alcohol (40), and 2-fluorogeraniol (41,42). The methane sulfonate esters of these analogues were prepared and their rates of solvolysis compared to the methane sulfonate of dimethylallyl alcohol. The trifluoro-substituted alcohols had their rates of solvolysis suppressed by a factor of nearly $10^7$. Similarly, 2-fluoro-geranyl methane sulfonate, when solvolyzed, decomposed at a rate $4 \times 10^{-3}$ that of geranyl methane sulfonate. In contrast, substitutions of fluorine for hydrogen slightly accelerate SN-2 displacement in allylic systems. The pyrophosphate esters of these allylic alcohols were then prepared. All of them served as competitive inhibitors of prenyltransferase. The 2-fluorogeranyl pyrophosphate had a $K_i$ value that approached the $K_m$ for geranyl pyrophosphate, while the trifluoro analogues were relatively poor inhibitors. All three of these analogues were tested as substrates for prenyltransferase and found to be utilized by the enzyme. However, the rate of condensation with geranyl pyrophosphate was greatly reduced compared to that of the normal substrate. The degree of suppression was almost identical to that observed in the solvolysis of these analogues (Table 4). Thus, the substitution of fluorine in an allylic system has identical

TABLE 4. Rate suppression of fluorine-containing substrate analogues compared to rates obtained with unsubstituted allylic methane sulfonates or pyrophosphate esters.

| | RATE RETARDATION | | |
|---|---|---|---|
| | SOLVOLYSIS | PRENYLTRANSFER | $K_i$ (μM) |
|  | $10^{-7}$ | $3 \times 10^{-7}$ | 23 |
|  | $10^{-7}$ | $3 \times 10^{-7}$ | 29 |
|  | $4.4 \times 10^{-3}$ | $8.4 \times 10^{-4}$ | 1.1 |

SOLVOLYSIS X = $-\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}-CH_3$ TRANSFER X = $-PP$

Data from (40,41,42).

effects on both the condensation reaction of prenyltransferase and on the chemical ionization of these analogues (40,41,42). This is convincing proof for an ionization-condensation-elimination mechanism for prenyltransferase.

This mechanism does not eliminate the question of participation by an X group in the reaction. However, experiments with 2-fluoroisopentenyl pyrophosphate produced evidence against the participation of an X group. 2-Fluoroisopentenyl pyrophosphate functions as a substrate as well as an inhibitor ($K_i = 0.8$ μM) and 2-fluorofarnesyl pyrophosphate is synthesized at several percent of the rate obtained with the natural substrate. About half of the fluorine-containing analogue is consumed during the reaction, which is consistent with one of the enantiomers functioning as a substrate. During prolonged incubation (several days) with 2-fluoroisopentenyl pyrophosphate and geranyl pyrophosphate, the enzyme fully retained its activity (24). If an X mechanism were functioning, one would have anticipated that the enzyme would have been derivatized by the (R) 2-fluoroisopentenyl pyrophosphate which would have formed a stable complex with the enzyme with subsequent loss of catalytic activity. If the X group were the pyrophosphate of isopentenyl pyrophosphate, we would have recovered a fifteen-carbon fluorine-containing diol rather than 2-fluorofarnesol on enzymatic hydrolysis of the product.

Our substrate-binding studies had demonstrated that metal ions were not required for binding of substrates; so they must be essential for catalysis. In fact, it was possible to bind both substrates to the enzyme without metal ions and observe no catalysis. Thus, it is possible that the role of the metal ions is to assist in ionization of the allylic substrate. Also there was an indication that cations destabilized allylic pyrophosphate (43). Consequently, we made a more precise analysis of metal ion promoted solvolysis of allylic pyrophosphate. The solvolysis was at 55° C at constant ionic strength and neutral pH. As is shown in Table 5, there

TABLE 5. The effect of $Mg^{2+}$ and $Mn^{2+}$ on the solvolysis of geranyl pyrophosphate.

| $M^{2+}$ concentration M | Rate x $10^4$ $sec^{-1}$ in the presence of $Mn^{2+}$ | $Mg^{2+}$ |
|---|---|---|
| 0 | 0.28 | 0.28 |
| 0.1 | 2 | 0.8 |
| 0.2 | 4 | 1.0 |
| 1 | 15 | 3.5 |
| 2 | 30 | 5.5 |

A constant ionic strength of 4.8 was maintained. Data from (44).

was a marked increase in the solvolysis of geranyl pyrophosphate at high concentrations of both $Mg^{2+}$ and $Mn^{2+}$ (44). An analysis of the products showed the production of linalool and geraniol in a ratio of 5:1, thus confirming solvolytic cleavage of the carbon-oxygen bond. Thus, it is clear that a salt or complex between $M^{2+}$ and geranyl pyrophosphate labilizes the pyrophosphate ester bond. We had previously determined the dissociation constant for geranyl pyrophosphate and its monomagnesium or manganous salt (35). This constant indicates that geranyl pyrophosphate is essentially quantitatively converted to its monometal salt at mM $Mg^{2+}$ or $Mn^{2+}$. This concentration of metal ion does not enhance the solvolysis of geranyl pyrophosphate. Thus, it must be the dimetal salt that is solvolyzing. The requirement for a second cation to accelerate the solvolysis of geranyl pyrophosphate is of special interest since we know that two metal ions are bound per catalytic site of prenyltransferase when substrate is present. Since these ions are required for catalysis, we have postulated that the ions are bound with the allylic substrate, thus neutralizing the charge on the pyrophosphate ion and facilitating the ionization of this substrate (27,44).

The observation on metal binding along with previous stereochemical studies (7,8,11,12) on prenyltransferase can be used to predict the orientation of the substrates with respect to each other during condensation. The stereochemical studies had shown that the carbon-to-carbon bond is formed between the si face of the double bond in isopentenyl pyrophosphate and the back side of the carbinol carbon of the allylic substrate. This requires the orientation of the plane of the double bond of isopentenyl pyrophosphate to be perpendicular to a line passing through the C-O bond of the other substrate. On the basis of these considerations, a model was proposed for the orientation of the substrates (3,45). However, since only one point of interaction between the substrates was fixed, a 360° rotation of these molecules with respect to each other was possible in the model. Two metal ions are bound at each catalytic site whenever product or either substrate (or both) is present (35). To account for these observations, any model must show two metal ions interacting with both substrates. This could be accomplished by the metal ions acting as bridges between the pyrophosphate moieties of the substrates. The result of this interaction is to fix the orientation of the substrates with respect to each other as is shown in Fig. 9. In addition, models show that the 2-pro-R hydrogen of isopentenyl pyrophosphate is surrounded by oxygens of the allylic pyrophosphate. Thus, the pyrophosphate when ionized from the allylic substrate provides the base necessary to remove this proton (27,35,39,45).

Following condensation, the newly synthesized geranyl pyrophosphate drops back into the allylic site and condenses with another isopentenyl pyrophosphate. The farnesyl pyrophosphate thus formed again slides

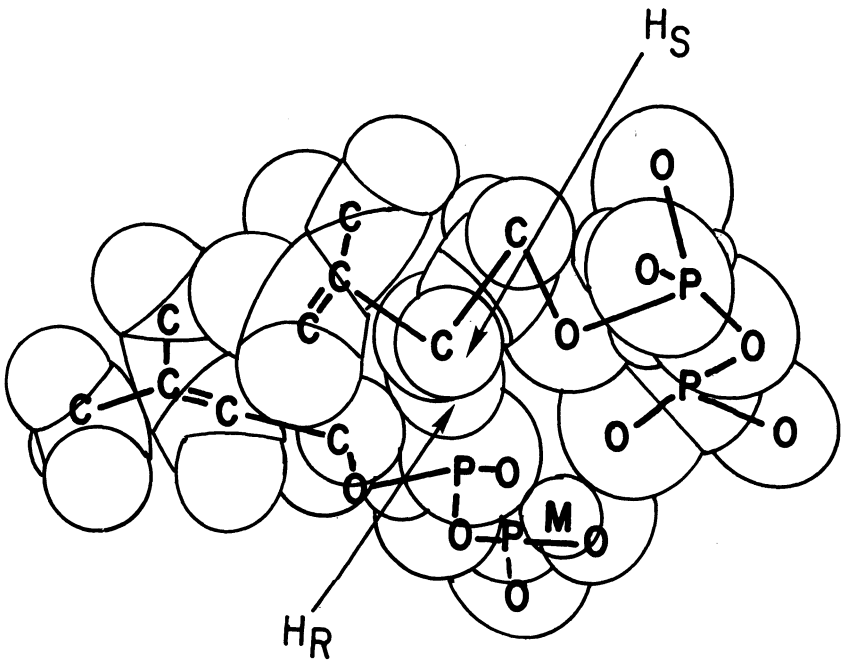



Fig. 9. Molecular models showing the orientation of dimethylallyl pyrophosphate and isopentenyl pyrophosphate in the catalytic site of prenyltransferase. M is a $Mg^{2+}$ ion and $H_S$ and $H_R$ are the pro S and pro R hydrogens on C(2) of isopentenyl pyrophosphate.

back into the allylic site with its hydrocarbon tail projecting into the homoallylic site, hindering the entry of another mole of isopentenyl pyrophosphate as is shown in Fig. 7.

Affinity labeling

In order to gain a better insight as to how the enzyme directs these condensations, we have initiated studies to determine the structure of the peptides at the catalytic site. The wide variety of substrate analogues that function as substrates or as inhibitors clearly indicated that prenyltransferase will tolerate substantial variation in the structure of the organic portion of the substrate. Photoreactive analogues of the substrates have been prepared for labeling the catalytic site. One analogue, o-azidophenethyl pyrophosphate, has been prepared and shown to be an effective inhibitor of the enzyme with $K_i$ values of 9 and 18 μM against isopentenyl and geranyl pyrophosphates, respectively (46). Binding studies also demonstrated that the analogue was competitive with both substrates. The enzyme and analogue were photolyzed together in a series of five consecutive treatments. The enzyme lost activity progressively at each photolysis step until only 6% of its catalytic activity remained (Table 6). The appropriate control indicated that nearly all of this

TABLE 6. Photoinactivation of prenyltransferase by o-azidophenethyl pyrophosphate

| Number of irradiations | Percent of initial activity | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 |
| + Affinity label* | 100 | 69 | 35 | 20 | 10 | 6 |
| - Affinity label | 100 | 104 | 98 | 98 | 101 | 90 |

*Prenyltransferase was irradiated at 254 nm in the presence of o-azidophenethyl pyrophosphate (.6 μM) at 0°. After each period of irradiation, an aliquot of the enzyme was assayed for activity. The photoaffinity label was then replenished and the irradiation repeated.

loss of activity could be attributed to photoderivatization of the enzyme by the analogue. Isoelectric focusing of the treated enzyme showed a nearly complete loss of native prenyltransferase and the appearance of a new, rather diffuse, band of protein. We have attempted to steer the analogue into one portion or the other of the catalytic site by including an excess of either geranyl pyrophosphate or isopentenyl pyrophosphate in the photolysis mixtures. However, in these experiments the enzyme lost its ability to bind to both substrates in parallel with the loss in catalytic activity (46). Thus, single derivatization of the catalytic site may completely destroy its binding capacity for either substrate.

Other prenyltransferases

Farnesyl pyrophosphate synthetase clearly functions by an ionization-condensation-elimination mechanism, and we have postulated a role for metal ions in this reaction. It will be interesting to see if the other types of prenyltransfer reactions proceed by the same mechanism. Two other enzymes that catalyze prenyltransfer reaction have been purified to homogeneity and thus are suitable subjects for investigation. Dimethylallyl pyrophosphate:tryptophan dimethylallyl transferase has been purified by Lee, Floss and Heinstein (47) and by a different procedure by L. Chayet at Utah (48) from Claviceps sp. Also, pure dimethylallyl pyrophosphate : aspulvinone dimethylallyl transferase has been obtained in Ogura's laboratory (49). Neither of these enzymes requires a divalent cation for catalytic activity. Although one of these enzymes was reported to be stimulated by $Ca^{2+}$ (47), we have been unable to find stimulation by divalent cations (48). Obviously there is not a general requirement for metal ion in prenyltransfer reactions or these enzymes are metaloproteins.

We have initiated testing the fluorine-containing substrates with the tryptophan transferase. The $K_m$ values for this enzyme are 125 μM and 250 μM for dimethylallyl pyrophosphate and tryptophan, respectively. The $K_{0.5}$ values for trifluorodimethylallyl, Z trifluorodimethylallyl, and 2-fluorodimethylallyl pyrophosphates were found to be 308 μM, 1.6 mM, and 0.9 mM. The E trifluoro and the 2-fluoro analogues have been tested as substrates in this enzymatic reaction. We have not been able to detect the formation of product with the E-trifluoro compound. However, our techniques have been sensitive only to $10^{-3}$ that of the rate with the natural substrate. This analogue participated in farnesyl pyrophosphate synthetase at about $10^{-8}$ that of the natural substrate; so it is possible that we are not detecting a slow reaction with the tryptophan system. In contrast, 2-fluorodimethylallyl pyrophosphate was readily accepted as a substrate at about 3% the rate of the natural substrate. In comparison, the 2-fluorogeranyl pyrophosphate was converted to 2-fluorofarnesyl pyrophosphate at a thousand-fold reduced rate compared to the natural substrates (48). Although these results are suggestive of an ionization-condensation-elimination mechanism, it is clearly too soon to propose a general mechanism for all the prenyltransfer reactions.

Acknowledgment - The contributions of my colleagues N. L. Eberhardt, B. C. Reed, L. S. Yeh, H. L. King, D. N. Brems, L. Chayet, and A. Saito are gratefully acknowledged. Also the participation of Professor C. D. Poulter and his cohorts has been important to much of the research reported in this article. Work in the author's laboratory was supported by a grant from the National Institutes of Health (AM 13140).

REFERENCES

1. F. Lynen, H. Eggerer, U. Henning, and I. Kessel, Angew. Chem. 70, 738 (1958).
2. J. K. Dorsey, J. A. Dorsey, and J. W. Porter, J. Biol. Chem. 241, 5453 (1966).
3. P. W. Holloway and G. Popják, Biochem. J. 104, 57 (1967).
4. A. A. Kandutsch, H. Paulus, E. Levin, and K. Bloch, J. Biol. Chem. 239, 2507 (1964).
5. H. Sagami, K. Ogura, and S. Seto, Biochemistry 16, 4616 (1977).
6. C. M. Allen, Jr., M. V. Keenan, and J. Sack, Arch. Biochem. Biophys. 175, 236 (1976).
7. G. Popják and J. W. Cornforth, Biochem. J. 101, 553 (1966).
8. J. W. Cornforth, Angew. Chem., Int. Ed. Engl. 7, 903 (1968).
9. B. L. Archer, D. Barnard, E. G. Cockbain, J. W. Cornforth, R. H. Cornforth, and G. Popják, Proc. R. Soc. London, Ser. B 163, 5A (1966).
10. D. P. Gough and F. W. Hemming, Biochem. J. 118, 163 (1970).
11. J. W. Cornforth, G. Popják, and L. Yengoyan, J. Biol. Chem. 241, 3970 (1966).
12. G. Popják and J. W. Cornforth, Biochem. J. 101, 553 (1966).
13. H. C. Rilling and K. Bloch, J. Biol. Chem. 234, 1424 (1959).
14. J. W. Cornforth and G. Popják, Tetrahedron Lett. 29 (1959).
15. G. Popják, P. W. Holloway, and J. M. Baron, Biochem. J. 111, 325 (1969).
16. G. Popják, J. L. Rabinowitz, and J. M. Baron, Biochem. J. 113, 861 (1969).
17. K. Ogura, T. Nishino, T. Koyama, and S. Seto, J. Am. Chem. Soc. 92, 6036 (1970).
18. T. Nishino, K. Ogura, and S. Seto, J. Am. Chem. Soc. 93, 794 (1971).
19. T. Nishino, K. Ogura, and S. Seto, Biochim. Biophys. Acta 235, 322 (1971).
20. T. Nishino, K. Ogura, and S. Seto, Biochim. Biophys. Acta 302, 33 (1973).
21. C. D. Poulter, D. M. Satterwhite, and H. C. Rilling, J. Am. Chem. Soc. 98, 3376 (1976).
22. T. Koyama, K. Ogura, and S. Seto, J. Am. Chem. Soc. 99, 1999 (1977).
23. K. Ogura, A. Saito, and S. Seto, J. Am. Chem. Soc. 96, 4037 (1974).
24. C. D. Poulter, J. C. Argyle, O. J. Muscio, and H. C. Rilling, unpublished results.
25. C. D. Poulter, J. C. Argyle, and E. A. Mash, J. Am. Chem. Soc. 99, 957 (1977).
26. A. Saito, Ph.D. Dissertation, Tohuku University, Sendai, Japan, 1976.
27. C. D. Poulter and H. C. Rilling, Accounts of Chemical Research. In press.
28. N. L. Eberhardt and H. C. Rilling, J. Biol. Chem. 250, 863 (1975).
29. H. C. Rilling, unpublished observations.

30. L. S. Yeh and H. C. Rilling, Arch. Biochem. Biophys. 183, 718 (1977).
31. T. Koyama, Y. Saito, K. Ogura, and S. Seto, J. Biochem. 82, 1585 (1977).
32. B. C. Reed and H. C. Rilling, Biochemistry 14, 50 (1975).
33. B. C. Reed and H. C. Rilling, Biochemistry 15, 3739 (1976).
34. M. Laskovics and C. D. Poulter, unpublished observation.
35. H. L. King, Jr., and H. C. Rilling, Biochemistry 16, 3815 (1977).
36. A. Saito and H. C. Rilling, unpublished observation.
37. B. C. Reed, Ph.D. Dissertation, University of Utah, 1976.
38. G. Popják, P. W. Hollaway, R. P. M. Bond, and M. Roberts, Biochem. J. 111, 333 (1969).
39. C. D. Poulter and H. C. Rilling, Biochemistry 15, 1079 (1976).
40. C. D. Poulter and D. M. Satterwhite, Biochemistry 16, 5470 (1977).
41. C. D. Poulter, J. C. Argyle, and E. A. Mash, J. Am. Chem. Soc. 99, 957 (1977).
42. C. D. Poulter, J. C. Argyle, and E. A. Mash, J. Biol. Chem. In press.
43. C. George-Nascimento, R. Pont-Lezica, and O. Cori, Biochem. Biophys. Res. Commun. 45, 199 (1971).
44. D. N. Brems and H. C. Rilling, J. Am. Chem. Soc. 99, 8351 (1977).
45. G. Popják, Harvey Lect. 65, 127 (1971).
46. D. N. Brems and H. C. Rilling, unpublished observation.
47. S.-L. Lee, H. G. Floss, and P. Heinstein, Arch. Biochem. Biophys. 177, 84 (1976).
48. L. Chayet and H. C. Rilling, unpublished observations.
49. I. Takahashi, N. Ojima, K. Ogura, and S. Seto, Biochemistry 17, 2696 (1978).