Heteroatom Chemistry
Volume 2, Number 1, 1991

Enzymatic and Chemical Reactivities of *sym*-Monothiopyrophosphate

Christopher J. Halkides, Eric S. Lightcap, and Perry A. Frey*

Institute for Enzyme Research, Graduate School, and Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Madison, Wisconsin 53705, U.S.A.

Received 28 June 1990.

ABSTRACT

The synthesis of sym-*monothiopyrophosphate (MTP) is described and the relative hydrolysis rate constants are reported for the dianion, trianion, and tetraanion in aqueous solution. The MTP-dianion undergoes hydrolysis at a rate 5×10^7-fold faster than the dianion of pyrophosphate (PP_i) and the trianion undergoes hydrolysis 2.7×10^7-fold faster than PP_i-trianion. The entropy of activation for the hydrolysis of MTP-trianion is 0.2 cal/deg/mol, and the enthalpy of activation is 19.7 kcal/mol. The hydrolysis appears to involve a dissociative transition state. The hydrolysis of the tetramethyl ester of MTP, in contrast, appears to involve an associative transition state. The Mg complex of MTP is a reasonable substrate for PP_i-dependent phosphofructokinase and inorganic pyrophosphatase, despite its larger size relative to PP_i. Therefore, MTP is tolerated within the active sites and in the transition states of enzymes that catalyze phosphoryl transfer from PP_i to acceptor molecules. These observations are consistent with the transition states for these enzymes being dissociative in nature, although they do not prove that they are dissociative. MTP is, on the other hand, not a substrate for acetyl-CoA synthase, an enzyme that utilizes the Mg complex of PP_i as a specific acceptor for the adenylyl group, an alkylphosphoryl group. Inasmuch as nonenzymatic reactions of this type involve associative transition states, it may be that the larger size of MTP relative to PP_i undermines the catalytic process in the active site of acetyl-CoA synthase, which may catalyze adenylyl transfer through an associative transition state.*

INTRODUCTION

Enzymatic phosphoryl group and phosphoryl ester group transfer reactions are among the most important reactions in biochemistry. Phosphoryl group transfer reactions are crucial to the production and utilization of ATP as a source of chemical free energy in metabolism and as the principal source of energy for mechanical work in muscular contraction and transport processes. Phosphoryl transfer reactions are also important as a major means by which metabolic intermediates acquire electrostatic charges that make them impermeable to cellular membranes and allow them to be compartmentalized within cells. Phosphoryl ester group transfer reactions are essential steps in the biosynthesis of many molecules in living cells, including coenzymes, phospholipids, second messengers, proteins, and nucleic acids.

Despite their importance, the mechanisms of action of phosphotransferases and nucleotidyltransferases are not very well understood in chemical terms. The stereochemistry of enzymatic substitution at P is well developed [1]; however, little is known of the nature of the transition states [2]. Although the transition states for enzymatic reactions are expected to be similar to those of comparable nonenzymatic reactions, this cannot be assumed to be the case, since special microenvironments may exist in enzymic active sites that cannot be duplicated in solutions. Moreover, enzymes accelerate reactions by such large factors, 10^8–10^{14} or more, that the mechanisms may not

*To whom correspondence should be addressed.

© 1991 VCH Publishers, Inc.

1042-7163/91/$3.50 + .25

involve the same transition states as nonenzymatic reactions. The situation is further complicated for enzymatic substitution at P by the fact that the chemical mechanism of phosphoryl transfer in solution continues to be controversial.

The starting point in any investigation of the mechanism of an enzymatic reaction is the nonenzymatic counterpart. The reason for this is twofold. First, there are nonenzymatic analogs for most enzymatic reactions, and the available information for well-investigated enzymes suggests that the transition states may be similar to those of the comparable nonenzymatic processes. Second, it would seem in theory that the evolution of an enzyme over many millions of years would require, as the starting point, a nonenzymatic reaction, the mechanism of which could be optimized through evolution by incremental improvements in enzyme substrate binding interactions to minimize the activation energy for reaching the transition state. In this view of incremental evolution, the preexisting transition states are the only ones that would have been accessible to early enzymes. Other transition states would necessarily have been higher in energy, and it is not obvious how a higher energy transition state could be gradually carved from one of lower energy through an evolutionary process that simultaneously minimizes the activation energy. Despite these expectations for enzymes in general, enzymatic phosphoryl transfer may be a special case, since it is difficult to envision catalysis of phosphoryl transfer through the chemical interactions that are known to be available at enzyme active sites [2].

In considering the mechanisms of enzymatic phosphoryl and alkylphosphoryl group transfer we begin with the nonenzymatic transition states. Early research on the mechanism of phosphoryl group transfer reactions indicated that monomeric metaphosphate monoanion (PO_3^-) is an intermediate in the hydrolysis of alkylphosphate monoanions, of acyl phosphate monoanions and of corresponding dianions with good leaving groups [2, 3]. Evidence supporting this hypothesis included the observation of low entropies of activation, little or no solvent kinetic isotope effects in ^2H_2O, and relative insensitivity to the basicity of the leaving group in a series of related esters. Recent stereochemical experiments showed that PO_3^- cannot be a discrete intermediate in water, although it might be in other solvents [4, 5]. Recent physical organic kinetic analyses of phosphoryl transfer reactions in water show that the transition state contains both the leaving group and the phosphoryl acceptor, but that there is little bonding between the phosphoryl group and either the leaving group or the acceptor [6–8]. Alkylphosphoryl group transfers seem to proceed through associative transition states, which are characterized by a high degree of bonding between phosphorus and both the entering acceptor and the leaving group. This is supported by several lines of evidence, including the fact that discrete pentavalent adducts are formed as intermediates in favorable cases [9]. The entropies of activation for alkylphosphoryl transfer reactions are negative and large, the rates are sensitive to the basicities of the leaving groups, and solvolysis of phosphodiesters in ^2H_2O proceed with significant solvent kinetic isotope effects. Dissociative and associative transition states for phosphoryl and alkylphosphoryl group transfer are illustrated in Figure 1 in trigonal pyramidal geometry, with little bonding to entering and leaving groups in the dissociative case and substantial bonding in the associative case.

sym-Monothiopyrophosphate (MTP) is a structural and chemical analog of pyrophosphate (PP_i), a substrate for many enzymes. The chemical and structural differences between MTP and PP_i can be used to interpret the behavior of enzymes toward MTP as a substrate in place of PP_i (Figure 2). The differences include the fact that MTP is larger than PP_i, and the P—S bonds in MTP are both longer and weaker than the P—O bonds in PP_i. The weakness of the P—S bonds in MTP, and its moderately larger size relative to PP_i, make it an attractive substrate analog for examining the nature of enzymatic transition states. The active sites of enzymes are sterically circumscribed by the need to provide specific binding groups to hold substrates in correct orientations for reactions and to immobilize the substrates and eliminate unproductive degrees of freedom. Therefore, a large substrate analog is normally not tolerated by an enzyme, although smaller analogs may react at slow rates.

MTP is an interesting case of a larger analog of PP_i that might be a valuable probe for examining the transition states of enzymatic reactions. Consider an enzyme that catalyzes phosphoryl transfer

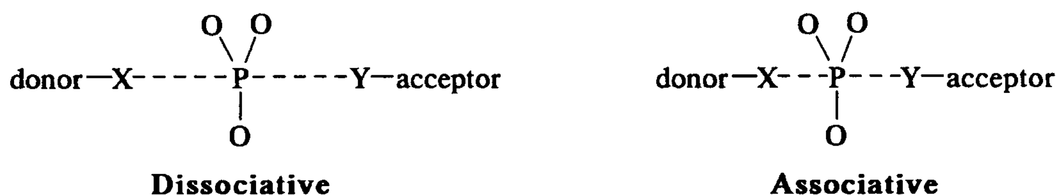


FIGURE 1 Dissociative and Associative Transition States for Phosphoryl and Alkylphosphoryl Group Transfer.

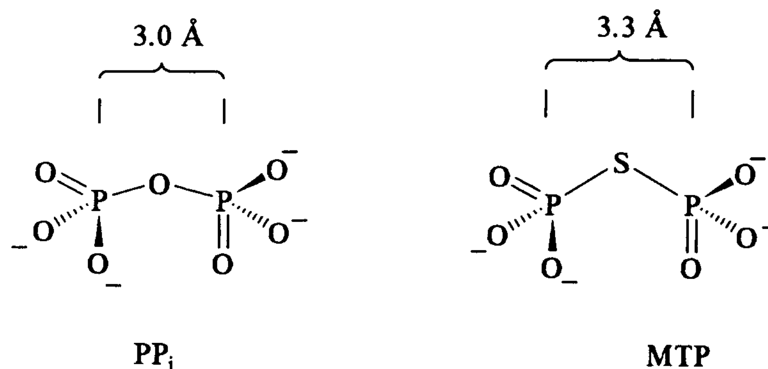


FIGURE 2 Chemical and Structural Differences between MTP and PP$_i$.

using PP$_i$ as the specific substrate. One might expect MTP to be accepted in a dissociative transition state, since it is elongated and might accommodate the steric requirements of MTP, whereas it is difficult to imagine how MTP could fit into a more compact associative transition state that is stabilized by enzymatic binding interactions. Moreover, the weak P—S bonds can be expected to be compatible with a dissociative transition state, which is characterized by a high degree of bond cleavage and little bond formation. Thus, when one compares ground states with transition states, the relative strengths of bonds in ground states of related molecules become important determinants of relative rates among substrates, when the transition states involve a high degree of bond cleavage and the molecules react by the same mechanism.

CHEMICAL PROPERTIES OF MTP

The synthesis and investigation of organophosphorus compounds with sulfur bridging two phosphorus atoms was developed by Professor Jan Michalski and his associates [10]. The synthetic method is of special interest because of the means by which this labile system is generated in high yields, by using electrophilic S and nucleophilic P, in contrast to the use of electrophilic P and nucleophilic O in the synthesis of the P—O—P linkage. It is clear that this is the only practical way to synthesize the P—S—P grouping. The preparation of MTP was first reported by Loewus and Eckstein [11], who demethylated tetramethyl monothiopyrophosphate (Me_4MTP), which had been prepared as described by Michalski and coworkers [10]. The demethylation employed initial silylation with trimethylsilyl iodide (TMSI), followed by desilylation with propanol and diisopropylethylamine. Loewus and Eckstein described MTP as a highly labile material that could be generated but was difficult to obtain in a pure and stable form. The hydrolysis rate constants reported by Loewus and Eckstein suggested, however, that the tetraanion of MTP could be prepared in pure form and stored indefinitely at low temperatures.

We reproduced the results of Loewus and Eckstein and observed that TMSI presented some problems. Therefore, we substituted trimethylsilyl triflate and tetrabutylammonium iodide for TMSI, and all subsequent demethylation reactions proceeded without complication. The Li_4MTP was routinely purified in the presence of 10 mM LiOH and stored in frozen solutions at −77°C for longer than six months with no detectable decomposition.

The rate constants for the hydrolysis of three ionic forms of MTP have been measured and can be compared with corresponding data on the dianion and trianion of PP$_i$, which have been published [12, 13]. MTP is far more reactive in hydrolysis than PP$_i$, 2.7 × 10^7-fold for the trianion and 5 × 10^7-fold for the dianion. Little information is available on the hydrolysis of PP$_i$-tetraanion; however, estimates based on extrapolated data indicate that MTP-tetraanion is several millionfold more reactive than PP$_i$-tetraanion.

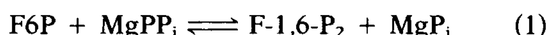
The difference in hydrolysis rates for MTP compared with PP$_i$ cannot be explained simply by the fact that thiophosphate is a better leaving group than phosphate, since the pK_a values for the relevant species differ by less than two log units [15]. The greater reactivity of MTP more likely results from the weakness of the P—S bonds and a dissociative transition state for hydrolysis, which features a high degree of bond cleavage. Moreover, the entropy of activation for hydrolysis of the trianion is 0.2 cal/deg/mol and the enthalpy of activation is 19.7 kcal/mol, values that reflect a dissociative transition state. Loewus and Eckstein reported an entropy of activation of −9.6 cal/deg/mol and enthalpy of 21.1 kcal/mol at pH 10 [11], where MTP exists almost exclusively as the tetraanion.

The hydrolysis rate constants for the tetramethyl ester, Me_4MTP, in aqueous solution at pH 7 and 25°C is 0.020 s^{-1}, which is only 48-fold faster than the reported value for the tetramethyl ester of PP$_i$ [14]. The entropy of activation for the hydrolysis of Me_4MTP is relatively large, −22.7 cal/deg/mol, and the enthalpy of activation is 13 kcal/mol. The hydrolysis of Me_4MTP appears to involve an associative transition state, in which bond cleavage to

the leaving group is not very advanced and there is substantial bonding to the entering acceptor. This is typical of phosphotriesters and phosphodiesters (3).

MTP AS AN ENZYMATIC PHOSPHORYL DONOR

The pyrophosphate-dependent phosphofructokinase (PP_i-PFK) from *Pseudomonas freudenreichii* catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate by the Mg complex of pyrophosphate (Equation 1).



$$\mathrm{F6P + MgPP_i \rightleftharpoons F\text{-}1,6\text{-}P_2 + MgP_i} \quad (1)$$

The conventional phosphofructokinases (PFK) in most cells catalyze the chemically analogous reaction of Equation 2, in which the phosphoryl group donor is the Mg complex of ATP.

$$\mathrm{F6P + MgATP \rightleftharpoons F\text{-}1,6\text{-}P_2 + MgADP} \quad (2)$$

The PP_i-PFK is a convenient phosphotransferase for use in studies of MTP because it is PP_i-specific, it is commercially available, and the reaction is chemically typical of many ATP-dependent phosphoryl transfers catalyzed by enzymes such as hexokinase, glycerokinase, and other phosphofructokinases. Moreover, the kinetics for PP_i-PFK have been thoroughly examined by Cook and coworkers [16, 17], who have found that the kinetic mechanism involves random equilibrium binding of substrates and products, with rate limiting conversion of ternary complexes to products. The kinetic mechanism for the forward reaction is illustrated in Figure 3, where the constants K_{F6P} and K_{PP} are the dissociation constants for fructose-6-phosphate and pyrophosphate, respectively. Because the binding of substrates and products are equilibrium processes, the random equilibrium binding mechanism makes PP_i-PFK particularly suitable for evaluating MTP as a substrate in comparison with PP_i, so that the diffusion of products from the enzyme does not limit the reaction rate. That is, the rate limiting step is the conversion of the ternary complex E·F6P·PP_i to products; the process that determines the overall reaction rate is very likely the phosphoryl group transfer step itself.

The maximum velocity for PP_i-PFK with MTP is 1/40th that with PP_i as phosphoryl donor substrate. By ordinary standards of comparison, the relative values of the maximal velocity for MTP and PP_i appear to indicate that MTP is a poor substrate for PP_i-PFK. However, it is in reality a rather good substrate when one considers the catalytic efficiency of this enzyme. A reasonable estimate of the factor by which this enzyme increases the rate of reaction 1 is 7×10^{11}, which should be within an order of magnitude of the true value. On this scale, the factor of 40 between the maximum rates for MTP and PP_i reflects a minor difference, and MTP may be regarded as a reasonable substrate. From the standpoint of K_m-values, MTP is also a reasonable but less tightly bound substrate. And it appears that the binding of fructose-6-phosphate by the enzyme-MTP binary complex is slightly retarded, possibly because of a slight degree of steric crowding owing to the greater size of MTP relative to PP_i. In sum, however, we must conclude that MTP is tolerated in the active site of PP_i-PFK and in the transition state for phosphoryl transfer catalyzed by this enzyme. The greater size of MTP relative to the specific substrate does not seem to exclude it from reacting as a phosphoryl donor.

Inorganic pyrophosphatase catalyzes the hydrolysis of the Mg^{2+} complex of PP_i according to Equation 3.

$$\mathrm{MgPP_i + H_2O \longrightarrow MgP_i + P_i} \quad (3)$$

This enzyme also accepts the Mg^{2+} complex of MTP as a very good substrate. In fact, the maximal velocity (V_m) for the pyrophosphatase-catalyzed hydrolysis of MTP is 3 times that for PP_i, which shows that MTP is very well accommodated in the active site and the transition state for the reaction. The efficiency of the enzyme in hydrolyzing MTP is slightly less under nonsaturating conditions, as indicated by the fact that the value of V_m/K_m for MTP is one third the value for PP_i.

Both PP_i-PFK and inorganic pyrophosphatase are PP_i-specific enzymes that catalyze phosphoryl transfer, and both accommodate MTP in place of PP_i in their binding sites and in their transition states. Given the intrinsic tendency of MTP to undergo phosphoryl transfer reactions through a

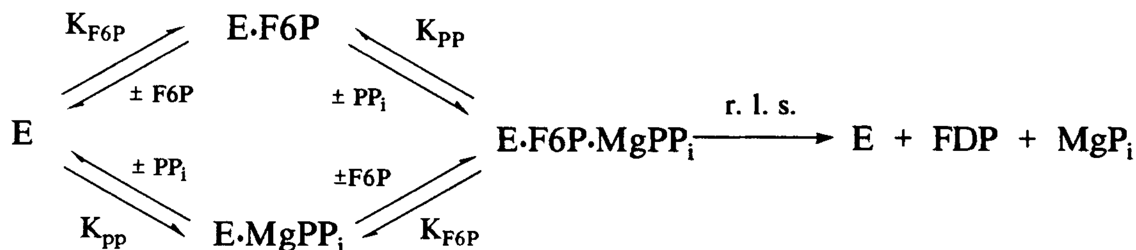


FIGURE 3 The Kinetic Mechanism for the Pyrophosphate-Dependent Phosphofructokinase Reaction. The substrate binding steps are equilibrium processes, and the breakdown of the ternary enzyme substrate complex to products is the rate limiting step.

dissociative transitions state, it is highly probable that this reactivity is realized in its reactions as an enzymic substrate.

MTP AS AN ENZYMATIC NUCLEOTIDYL GROUP ACCEPTOR

Many important enzymes catalyze biosynthetic reactions in which ATP or another nucleoside triphosphate is cleaved to PP_i and a nucleotide derivative. These enzymes include most nucleotidyltransferases such as DNA polymerase, RNA polymerase, DNA ligase, and many coenzyme synthesizing enzymes. These reactions all involve the cleavage of the phosphoanhydride bond between P_α and P_β of the nucleoside triphosphate, a process that would proceed through an associative transition state in a nonenzymatic reaction. Some of these reactions are readily reversible; that is, the enzyme also catalyzes the reverse cleavage of a nucleotide derivative to a nucleoside triphosphate. Acetyl-CoA synthase, for example, catalyzes the reversible formation of acetyl-CoA from acetate, CoA, and ATP by a two-step mechanism. The two steps of the reverse reaction are shown in Figure 4. In the first step AMP cleaves acetyl-CoA to form CoA and acetyl adenylate, which is bracketed in Figure 2 to emphasize its transient nature as an enzyme-bound intermediate. Without undergoing dissociation from the enzyme, acetyl adenylate is cleaved by PP_i to acetate and ATP, which are finally released from the enzyme. This second step is an example of an alkylphosphoryl group transfer, in which the group donor is a carboxylic-phosphoric anhydride. In a nonenzymatic reaction, this process would be expected to involve an associative transition state.

The PP_i-dependent cleavage of acetyl-CoA catalyzed by acetyl-CoA synthase is quite straightforwardly observed by measuring either the rate of ATP formation or the rate of CoA formation from acetyl-CoA, AMP, and PP_i. However, acetyl-CoA synthase will not accept MTP as a substrate in place of PP_i under any conditions that we have explored. Even very large amounts of enzyme that are sufficient to generate ATP in NMR tubes for direct observation do not catalyze any detectable reaction with MTP. This is remarkable in view of the fact that the purely chemical reactivities of PP_i and MTP as group acceptors are expected to be similar.

The simplest rationale for the failure of MTP to substitute for PP_i in the acetyl-CoA synthase reaction is that it simply does not fit properly into the active site. The site is presumably tailored to the dimensions of PP_i and cannot accommodate the added bulk of MTP in such a way as to allow it to react as an acceptor of AMP from acetyl adenylate. The failure of MTP with acetyl-CoA synthase contrasts sharply with its ability to fit into the active sites of PP_i-specific phosphoryl transferring enzymes and to act as a phosphoryl group donor to natural acceptor substrates.

CONCLUSIONS AND PROSPECTS

Enzymatic catalysis requires very specific binding interactions between substrates and active sites, especially in close proximity to the reacting functional groups. Any slight perturbations in these interactions can be expected to cause severe retardation in rates. This alone can account for the fact that MTP is a poor analog for PP_i in the acetyl-CoA synthase reaction. To understand how MTP can be a reasonably good substrate for PP_i-PFK, one must explain how it is able to react as well or nearly as

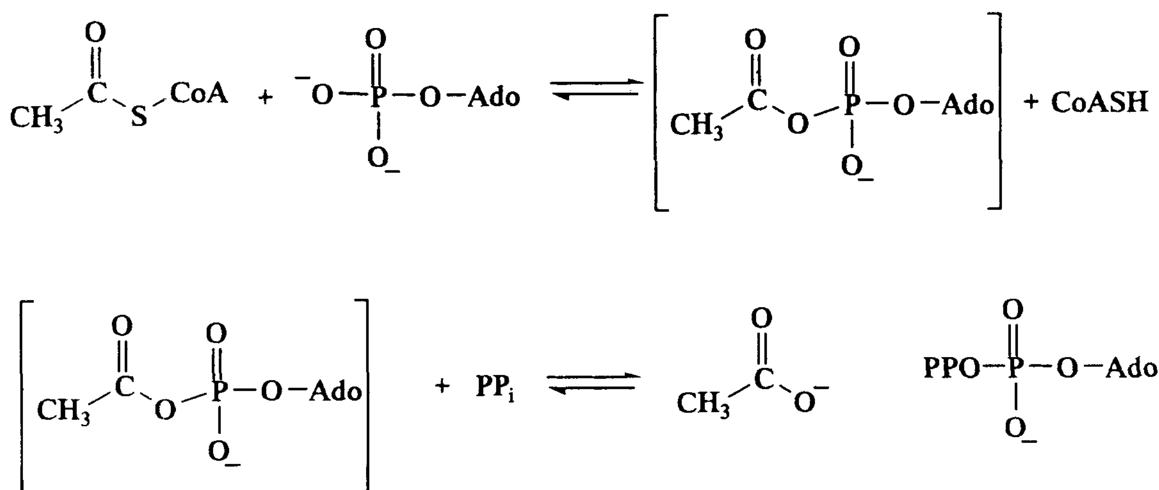


FIGURE 4 The Two Step Chemical Mechanism of the Reaction Catalyzed by Acetyl-CoA Synthase. Both steps of the reaction occur with substrates bound to the active site of the enzyme. The bracketed intermediate, acetyl adenylate, never dissociates from the active site, but it reacts either with CoA to form acetyl-CoA or with PP_i to form ATP, depending on the relative concentrations of PP_i and CoA.

well as PP_i. A satisfactory explanation must also account for the fact that MTP does not react faster than PP_i, as it does in nonenzymatic hydrolysis. One view is that PP_i-PFK does not, in fact, catalyze phosphoryl transfer from MTP very well, but that a major factor in the observed rate is the intrinsic chemical reactivity of MTP. An estimate of the enzymatic rate acceleration factor for MTP acting as the phosphoryl donor of PP_i-PFK can be made by comparing the enzymatic turnover number (k_{cat}) with the appropriate hydrolysis rate constant. We estimate this factor at about 2×10^4, which is about 10^7 smaller than the comparable factor of 10^{11} to 10^{12} for PP_i. It is clear, from this, that PP_i-PFK is not a particularly effective catalyst of phosphoryl transfer from MTP.

The measured rate for MTP is, nevertheless, quite high and approaches that for PP_i within a factor of 40 in maximum velocity (Table 2). The reason for this is the high intrinsic chemical reactivity of MTP, which largely compensates for the reduced catalysis by the enzyme. In this view, the enzyme catalyzes phosphoryl transfer from PP_i through the effects of specific binding interactions in the ground and transition states. Much of this catalysis, on the order of 10^7 in rate enhancement, is ineffective with MTP owing to its larger size and altered shape. Most of the lost rate enhancement is compensated by the intrinsic reactivity of MTP as a phosphoryl donor, which amounts to a factor of over 10^7 relative to PP_i, as estimated from the relative hydrolysis rate constants in Table 1. The intrinsic reactivity of MTP can be expressed in this way only through a dissociative transition state, because it does not exhibit exceptional reactivity through associative transition states.

ACKNOWLEDGMENT

The work described in this paper was supported by Grant No. GM 30480 from the National Institute of General Medical Sciences of the USPHS.

REFERENCES

[1] P. A. Frey, *Advances in Enzymology and Related Topics in Molecular Biology*, *62*, 1989, 119.
[2] J. R. Knowles, *Ann. Rev. Biochem.*, *49*, 1980, 877.
[3] S. J. Benkovic, K. J. Schray, *The Enzymes*, *8*, 1973, 201.
[4] S. L. Buchwald, J. M. Friedman, J. R. Knowles, *J. Am. Chem. Soc.*, *106*, 4911.
[5] J. M. Friedman, S. Freeman, J. R. Knowles, *J. Am. Chem. Soc.*, *110*, 1988, 1268.
[6] M. T. Skoog, W. P. Jencks, *J. Am. Chem. Soc.*, *106*, 1984, 7597.
[7] N. Bourne, A. Williams, *J. Am. Chem. Soc.*, *106*, 1984, 7591.
[8] D. Herschlag, W. P. Jencks, *J. Am. Chem. Soc.*, *108*, 1986, 7938.
[9] F. H. Westheimer, in P. De Mayo (ed), *Rearrangements in Ground and Excited States*, Vol. 2, Academic Press, New York, p. 229 (1980).
[10] J. Michalski, B. Mlotkowska, A. Skowronska, *J. Chem. Soc., Perkin Trans. I*, 1974, 319.
[11] D. I. Loewus, F. Eckstein, *J. Am. Chem. Soc.*, *105*, 1983, 3287.
[12] D. O. Campbell, M. L. Kilpatrick, *J. Am. Chem. Soc.*, *76*, 1954, 893.
[13] J. R. Van Wazer, E. J. Griffith, J. F. McCollough, *J. Am. Chem. Soc.*, *77*, 1955, 287.
[14] A. D. F. Toy, *J. Am. Chem. Soc.*, *70*, 1948, 3882.
[15] P. A. Frey, *Science*, *228*, 1985, 541.
[16] B. L. Bertagnolli, P. F. Cook, *Biochemistry*, *23*, 1984, 4101.
[17] Y.-K. Cho, P. F. Cook, *J. Biol. Chem.*, *263*, 1988, 5135.