ENZYME-CATALYZED
PHOSPHORYL TRANSFER
REATIONS

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CONTENTS

PERSPECTIVES AND SUMMARY ................................................................. 877
 Classes of enzyme involving reactions at phosphorus ............................... 878

COVALENT REACTION INTERMEDIATES, CRYPTIC
AND OTHERWISE.......................................................................................... 879
 Reactions of Phosphoric Monoesters .......................................................... 879
 Phosphates .................................................................................................. 879
 Phosphokinases ......................................................................................... 881
 Phosphomutases ....................................................................................... 886
 Reactions of Phosphoric Diesters ............................................................... 890

REACTION ENERGETICS ........................................................................... 892
 “Off” Rates ............................................................................................... 893
 Internal Thermodynamics ......................................................................... 896

THE NATURE OF THE ELEMENTARY STEP .............................................. 898
 Associative versus Dissociative Pathways ..................................................... 898
 Stereochemistry .......................................................................................... 902
 Prochiral substrate → prochiral product ..................................................... 902
 Prochiral substrate → pro-prochiral product ................................................. 903
 Pro-prochiral substrate → pro-prochiral product ......................................... 905
 Metal Ions .................................................................................................... 907
 Cation-dependent diastereoisomer preference ............................................. 908
 Substitution-inert complexes of nucleotides ............................................... 909
 Catalysts ....................................................................................................... 911

PERSPECTIVES AND SUMMARY

The enzyme-catalyzed transfer of phosphoryl groups is ubiquitous in intermediary metabolism. Such reactions are central to the energy balance of all organisms, and are also involved in cellular control mechanisms at every
level. Essentially every metabolic process that must be driven thermodynamically uphill—whether it be the maintenance of a transmembrane chemical potential, the coupling of chemical free energy to mechanical, electrical, or photochemical events, or the synthesis of a metabolite of higher free energy—involves a displacement at the phosphorus atom of a phosphoric monoester or anhydride. These reactions are coupled by the phosphokinases and ATPases to the free energy change that results from the cleavage of the phosphoric anhydride of a nucleoside triphosphate. In addition, there are many reactions that involve displacements at the phosphorus atom of phosphoric diesters and which result in the transfer of a phosphoric ester moiety rather than of an unsubstituted phosphoryl group.

Despite the existence of a rich literature on the physical-organic chemistry of reactions of phosphoric esters \(1, 2\), it is only within the last few years that the molecular details of enzyme-catalyzed reactions at phosphorus have been heavily scrutinized. The crystal structures of a number of phosphokinases are currently being pushed to resolutions higher than 2.5 Å, and these structures, coupled with the results from penetrating mechanistic studies, are providing a new understanding of enzyme catalysis in this area.

Investigation of the mechanism of enzyme-catalyzed reactions at the phosphorus locus is at three levels: the first concerns the definition of the number and kind of reaction intermediates (e.g. is there an obligatory phosphoenzyme \(3, 4\), and what is the order of substrate binding?); the second relates to the energetics of the reaction (e.g. what is the rate-limiting transition state, and how does a particular intermediate partition?); and the third faces the problem of the elementary step (e.g. is the displacement associative or dissociative, and how is each step catalyzed by the enzyme?). This review exemplifies the problems and the promise of some recent approaches made at each of these levels.

**CLASSES OF ENZYME INVOLVING REACTIONS AT PHOSPHORUS** In Figure 1 are summarized the classes of enzyme that catalyze displacements at phosphorus. The enzymes that handle phosphoric monoesters\(^1\) fall into three categories: the phosphatases, where water is the acceptor of the phosphoryl group (these include enzymes such as alkaline phosphatase that are merely hydrolytic, and enzymes such as the ATPases where the free energy available is coupled to some other metabolic function); the kinases, where a nucleoside triphosphate is the phosphoryl donor and some molecule other

\(^{1}\)For simplicity we classify displacements at the \(\gamma\)-phosphoryl group of ATP with the reactions of phosphoric monoesters because an unsubstituted phosphoryl group is transferred in all these cases. Analogously, we classify displacements at the \(\alpha\) or \(\beta\) phosphorus of ATP with reactions of phosphoric diesters.
Figure 1 Classes of enzyme that catalyze reactions at phosphorus. a, the starred atom may be an oxygen of a hydroxyl, carboxyl, or phosphoryl group, or the nitrogen of a guanidino group; b, the phosphoryl group is transferred (formally intramolecularly) between two hydroxyl groups or between a hydroxyl and a carboxyl group; c, phospholipase C and D; d, Adenosine; e, includes such ligases as aminoacyl tRNA synthetases; f, includes such ligases as glutamine synthetase and acetyl-CoA carboxylase.

than water is the acceptor; and the mutases, for which the acceptor is another functional group on the donor molecule. The enzymes that handle phosphoric diesters are either hydrolytic (e.g. the nucleases) or nucleotidyl transfer catalysts. As will become clear below, this functional categorization is mirrored mechanistically.

COVALENT REACTION INTERMEDIATES, CRYPTIC AND OTHERWISE

Reactions of Phosphoric Monoesters

PHOSPHATASES The most thoroughly characterized phosphatase is undoubtedly the alkaline phosphatase from Escherichia coli (5), the mechanistic pathway for which involves the obligatory formation of a phosphoenzyme intermediate. The evidence for this intermediate comes from the following: (a) $k_{cat}$ is independent of the alcohol moiety for a variety of phosphoric monoesters; (b) the enzyme catalyzes transphosphorylation between alcohols, the kinetic behavior being consistent with a phosphoenzyme intermediate; (c) a phosphoenzyme can be isolated by incubating the enzyme with $P_i$ or substrate at low pH (or, in lower yield, by quenching from the steady state at higher pH values); (d) the enzyme is phosphorylated at a unique serine residue; (e) despite early failures, a "burst" of product alcohol can be observed with phosphate-free enzyme at low temperatures; and (f) the enzyme catalyzes the exchange of $^{18}O$ between $H_2O$ and $P_i$. In practice, the elucidation of the mechanistic pathway (and the meaning of some of the above observations) is complicated by the fact that $P_i$ dissociation becomes rate-limiting at high pH (6). The rate constants for successive steps (phosphorylation, dephosphorylation, and $P_i$
dissociation) only differ by about an order of magnitude, so that rather small changes in rate constants can affect the contribution of a particular step to the observed catalytic rate (see also 7, 8). Sterechemically, alkaline phosphatase proceeds with overall retention at phosphorus in transphosphorylation (9). This behavior is not surprising, since alcoholyisis of the phosphoenzyme intermediate is nearly (not exactly—different alcohols were used) the microscopic reverse of the formation of the phosphoenzyme, and the stereochemical course of the phosphorylation should be repeated in the dephosphorylation, giving retention overall.

Acid phosphatases (e.g. from potato, wheat germ, or human prostate) also appear to involve phosphoenzyme intermediates, the evidence in this case deriving from: (a) the observation of burst kinetics (10, 11); (b) common ratios of transphosphorylated products from a number of different phosphate donors, which imply a common intermediate (12); (c) enzyme-catalyzed 18O exchange between H2O and P1 (13); and (d) the trapping of a phosphoenzyme in which a histidine residue is phosphorylated (10).

The third group of phosphatasies comprises the ATPases, which are responsible for the coupling of ATP hydrolysis to other metabolic processes such as in oxidative phosphorylation (the mitochondrial enzyme), photosphorylation (the chloroplast enzyme), muscle action (the myosin ATPase and its fragments), and the maintenance of ion gradients (the Na,K-ATPase, the Ca-ATPase of sarcoplasmic reticulum). The isotopic exchange reaction between H2O and P1 has been exploited for many of these systems, largely by Boyer’s group (14–18). They have investigated (a) the rate of appearance of solvent-derived 18O in P1 either when P1 is the substrate or when it is the product of ATP hydrolysis, (b) the corresponding rates of 18O appearance in ATP, and (c) the rate of phosphorylation either of the enzyme [e.g. for sarcoplasmic reticulum ATPase (18)] or of the product [e.g. for inorganic pyrophosphatase (19)]. In terms of the present discussion, they have concluded that most if not all of the observed oxygen exchanges between P1 and H2O are accounted for by the reversible formation of covalent phosphorylated intermediates between P1 and either the enzyme or a cosubstrate (20). With both the sarcoplasmic reticulum (21) and Na,K-ATPase (22), the enzyme is transiently phosphorylated on an aspartic acid residue.

For those enzymes that catalyze the transfer of a phosphoryl group to water, therefore, the favored path seems to involve a phosphoenzyme intermediate. There seems to be little preference amongst the available enzyme nucleophiles capable of acting as acceptors, since as we have seen above, phosphoryl group transfer may be to a hydroxyl, imidazolyl, or carboxylate group. [From the point of view simply of what enzymic nucleophiles can be phosphorylated, we should include here phosphothioredoxin, which contains a thiolphosphate link (23, 24), poliovirus RNA-linked protein,
which is bound to the 5' end of poliovirion RNA by a phosphodiester link involving a tyrosine residue (25; see also 26, 27), and the adenylylated intermediate in the reaction catalyzed by DNA ligase, in which a lysine residue is involved in a phosphoramidate link (28).

PHOSPHOKINASES The second major group of phosphoryl transfer enzymes, the kinases, catalyze the transfer of the γ-phosphoryl group of a nucleoside triphosphate to an acceptor molecule other than water (29). There are so many phosphokinases that, rather than provide an inadequate catalogue of results on all kinases, it is preferable to discuss one, yeast hexokinase, the mechanism of which has been investigated with most thoroughness. At this time it seems likely that many of the features displayed by hexokinase will prove to apply to phosphokinases generally.

Steady-state kinetic studies (30, 31) on hexokinase show intersecting double-reciprocal plots, which indicate a sequential rather than a ping-pong pathway. That is, phosphoryl transfer occurs within ternary complexes of the enzyme and its substrates. It should be emphasized, however, that such classical experiments may be misleading; apparently parallel double-reciprocal plots may in fact be just slightly converging (this error was made for mammalian hexokinase, which was originally believed to show a parallel initial velocity pattern), and there are also circumstances in which false intersections can be observed [e.g. if one substrate is contaminated with its coproduct, or if the intermediate in a ping-pong pathway is chemically labile (32)]. The study of kinase reactions is further complicated by such problems as what to do about the essential divalent metal ion (should one simply keep the total metal ion concentration constant, or should one buffer the metal ion to maintain a constant level of free cation?), and how to treat the questions of multiple binding sites (e.g. 33), substrate inhibition or activation, and salt effects (e.g. 34). These elements of ill-behavior often result in nonlinear double-reciprocal plots (e.g. 35), the interpretation of which (intersecting or parallel) becomes more a matter of whim than judgment. In the light of the above, inhibition studies using products, product analogues, and substrate analogues are essential before reliable deductions can be made from steady-state kinetics (32).

While it is clear that hexokinase follows a sequential pathway, the degree of order in substrate addition is less unambiguous. The consensus now is that the pathway is a largely ordered one with glucose leading, but the alternative order of ATP binding before glucose can become dominant under conditions of high nucleotide concentration (36, 37). This explains the observation of effectively random substrate addition at certain substrate concentrations. The "mainly ordered" sequence (Figure 2) is supported by the equilibrium flux measurements of Britton & Clarke (38), who found that the ratio of the two fluxes: glucose 6-phosphate = ATP and glucose 6-
phosphate ⇌ glucose, is unaffected by the glucose concentration, but rises as the ATP concentration increases. This result is expected for an ordered pathway with glucose leading. However, it had earlier been shown that each of the equilibrium exchange reactions: glucose 6-phosphate ⇌ glucose and ADP ⇌ ATP, rises to a plateau level when the concentration of the other substrate pair is raised (39). If the only possible way of loading the enzyme were by the binding of glucose followed by ATP, one would have predicted a suppression of the glucose 6-phosphate ⇌ glucose exchange reaction by very high ATP + ADP levels. The fact that such suppression was not observed suggests that the alternate order of binding may become preferred at high nucleotide levels (40). The skeletal scheme of Figure 2 has been fleshed out considerably by recent work from Rose's group involving the use of the isotope trapping method (41, 42). These experiments are described below in the section on reaction energetics.

The above discussion brings us to the point where the nature of the interconversion of the two ternary complexes, e·glucose·ATP ⇌ e·glucose 6-phosphate·ADP, has to be faced: is the phosphoryl group transferred directly between the bound substrates, or is it transferred via a phosphoenzyme complex, e·P·glucose·ADP? Three results have been cited in support of the latter (falsely, as will be seen). First, in common with many phosphokinases, hexokinase shows an intrinsic hydrolytic activity toward ATP (43), and while this reaction is 40000 times slower than that of glucose phosphorylation, unsubstantiated analogy with the phosphatases has led to suggestions of the intermediacy of a phosphoenzyme for the ATPase reaction. However, the striking promotion of the ATPase activity of hexokinase by glucose analogues such as lyxose (44) indicates that the ATPase reaction may only be a trivial diversion from the normal phosphorylation of glucose within the ternary complex. In the absence of glucose the active site will not, after all, be empty, and solvent water could act as a poor surrogate for the 6-hydroxyl group of glucose. Secondly, hexokinase catalyzes the slow isotopic exchange of ATP and ADP in the absence of glucose (43, 45). Such half-reactions (Equation 1):

enzyme + ATP ⇌ phosphoenzyme + ADP
phosphoenzyme + acceptor ⇌ phosphoacceptor + enzyme

1.
have been cited widely (4) as evidence for phosphoenzyme intermediates in phosphokinase reactions, and although in the case of hexokinase the exchange occurs at a rate about 10000 times slower than the normal reaction and is not accelerated by lyxose (46), “substrate synergism” (47) could always be invoked. [Substrate synergism describes the need to bind the cosubstrate in order to achieve the maximal rate of the half-reaction (47).] Unfortunately, the phosphokinase field is peppered with erroneous conclusions that arise from the presence of substrate or enzyme contaminants, and for many such studies, efforts have not been reported to ensure the absence of cosubstrate or cosubstrate analogues (the presence of which naturally allows ATP-ADP exchange by reversal of the overall reaction) or to ensure the absence of contaminating enzymes (such as adenylate kinase) that would catalyze the observed exchange (48, 49). Thirdly, hexokinase is phosphorylated on incubation with ATP and D-xylose (50). While this phosphoenzyme is catalytically inactive, the phosphorylation can be reversed with ADP and D-xylose (50).

The results reported above have led to suggestions that the phosphoryl group transfer occurs via the enzyme (an “active” phosphoenzyme that in the presence of D-xylose rearranges to an inactive form). The alternative is that the slow reactions referred to are merely side-reactions irrelevant to the normal pathway. Certainly the transient phosphorylation of a substrate surrogate [whether it be water (43, 44), an amino acid side chain (50), or even a peptide link (51)] could give rise to these observations. Nearly every phosphokinase that has been scrutinized has its complement of slow partial reactions; the problem is to decide whether they are misleading artefacts or useful clues pointing to a phosphoenzyme.

One important approach to the detection of cryptic phosphoenzymes (that is, those that cannot be isolated, or that are ill-behaved eitherchemically or kinetically) was devised by Midelfort & Rose (52). These authors recognized that even if a kinase reaction involved the formation of a phosphoenzyme from ATP, the ADP might not freely dissociate, and the expected ATP-ADP exchange reaction would not then be observed. The knowledge that even enzyme-bound protons can be “sticky,” in the sense that exchange of a substrate molecule with those in solution can occur more rapidly than the exchange of a proton from the enzyme’s active site (53, 54), suggests that something the size of ADP could easily be sticky. Accordingly, on the reasonable basis that even tightly-bound ADP could still suffer rotation of its β-phosphoryl group, Midelfort & Rose (52) developed the “β,γ-bridge to nonbridge” positional isotope exchange experiment illustrated in Figure 3. Using this ingenious method, the transient formation of a phosphorylated intermediate in the glutamine synthetase reaction was established. When, however, the ATP positional isotope exchange test is
applied to hexokinase (using highly purified enzyme in the complete absence of glucose), it fails, even in the presence of lyxose (I. A. Rose, private communication). [The absence of positional isotope exchange leaves one uncertain as to whether there is no phosphoenzyme intermediate or whether the \(\beta\)-phosphoryl group of ADP cannot rotate during the lifetime of the intermediate. This ambiguity exists for starch phosphorylase, for example, where positional exchange of the C-O-P bridge and nonbridge phosphoryl oxygens of glucose 1-phosphate is seen in the presence of maltotriose as primer, but not during starch elongation (55). In this case, the rate of formation of the putative intermediate may be slower than the rate of its collapse. An analogous case is glucose 1,6-bisphosphate synthetase (56), which proceeds via a phosphoenzyme intermediate (57), but does not catalyze the nonbridge to bridge randomization of a carbonyl \(^{18}\)O-label in the substrate 1,3-bisphosphoglycerate (57).]

The clearest evidence on the nature of the phosphoryl group transfer in the interconversion of the ternary complexes \(e\cdot\text{glucose}\cdot\text{ATP}\) and \(e\cdot\text{glucose} 6\text{-phosphate}\cdot\text{ADP}\) comes from stereochemical studies. The transfer of the phosphoryl group from ATP to glucose has been shown to proceed with inversion of configuration at phosphorus (58, 59) (Table 1), which strongly suggests that transfer occurs directly between the substrate molecules within the ternary complex without the covalent intervention of the enzyme.

To what extent is hexokinase archetypal? Other kinases for which an extensive literature exists, such as pyruvate kinase (68, 69), certainly appear to follow the pattern of a sequential reaction where the phosphoryl group is transferred directly between substrates with "in-line" geometry (58, 59). The scene is not, however, always so tidy. Of all the phosphokinases (except nucleoside diphosphokinase, which appears to be a special case: see below), acetate kinase is the enzyme for which the most persuasive arguments have

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**Figure 3** The positional isotope exchange method of Midelfort & Rose (52).
Table 1  Stereochemical course of displacements at phosphoric monoesters

<table>
<thead>
<tr>
<th>Method</th>
<th>16O−18O−S</th>
<th>16O−17O−18O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
<td>Result</td>
<td>Ref.</td>
</tr>
<tr>
<td>Glycerol kinase</td>
<td>Inversion</td>
<td>58−60</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>Inversion</td>
<td>58, 59</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>Inversion</td>
<td>58, 59</td>
</tr>
<tr>
<td>Adenylate kinase</td>
<td>Inversion</td>
<td>61−63</td>
</tr>
<tr>
<td>Adenosine kinase</td>
<td>Inversion</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>2 inversions or</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 retentions</td>
<td></td>
</tr>
<tr>
<td>Nucleoside diphosphate</td>
<td>Overall</td>
<td>65</td>
</tr>
<tr>
<td>kinase</td>
<td>retention</td>
<td></td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
<td>Inversion</td>
<td>b</td>
</tr>
</tbody>
</table>

*a* P. A. Frey, private communication.

*b* M. R. Webb and D. R. Trentham, private communication.

been presented to support a pathway via a phosphoenzyme intermediate. Thus Spector and his group (70, 71) reported that acetate kinase catalyzes both of the expected partial reactions (ATP-ADP exchange in the absence of added acetate, and acetyl phosphate-acetate exchange in the absence of added nucleotide), and they isolated a phosphorylated enzyme from incubations of partially purified enzyme with either [γ-32P]ATP or acetyl [32P]phosphate. This phosphoenzyme appeared to be chemically competent, in the sense that the phosphoryl group could be transferred to either ADP or acetate (71). It was not, however, kinetically competent, since although the ATP-ADP exchange was seven times faster than enzyme turnover, the acetyl phosphate-acetate exchange was only 0.015 of the overall reaction rate (70). This kinetic inadequacy could, of course, be ascribed to substrate synergism (47) (see above), but these data disagree with Rose's earlier findings (72) that the partial exchange reactions of acetate kinase were not detectable, and with a subsequent report (73) that whereas acetate does not affect the observed ATP-ADP exchange, acetyl phosphate-acetate exchange is undetectable in the absence of added nucleotide. The literature on the steady-state kinetic behavior of acetate kinase is also in conflict, a first report of ping-pong kinetics (74) being followed by observations (based on both substrate and inhibitor studies) only consistent with a sequential pathway (75). Moreover, the early isolation of a phosphoenzyme (71) was followed by a report that the yield of this species was very variable (76), the reasons for the variation remaining obscure. [For a different system, phosphoglycerate kinase, a similar variability was found to derive from tiny amounts of substrate contaminant (48), and the “phosphoenzyme” turned out not to be a phosphoenzyme at all!] On the other hand, the possibility of a phosphoenzyme for acetate kinase is supported by the findings that the “nucleoside diphosphate kinase activity of acetate kinase”
(77) (paralleling the ATP-ADP exchange reaction) follows ping-pong kinetics, and that reaction of the putative phosphoenzyme with hydroxylamine causes a concomitant loss of acetate kinase activity (77). The reader may be forgiven a feeling of exasperation at this point, but the mechanism of phosphoryl transfer catalyzed by this enzyme now appears to have been resolved by stereochemical analysis. Since acetate kinase, like hexokinase, transfers the phosphoryl group with inversion of the configuration at phosphorus (66), the simplest view is that the phosphorylation of acetate by ATP occurs directly, within the ternary complex. The controversial partial reactions and the fickle protein phosphorylation must either derive from protein contaminants or from side-reactions catalyzed by acetate kinase itself.

Despite the persuasive evidence of Table 1, there is evidently no mechanistic imperative that requires cosubstrate phosphorylation to proceed directly. For example, the first steps of succinyl-CoA synthetase involve the production of enzyme-bound succinyl phosphate from ATP and succinate, and this reaction does appear to involve a phosphoenzyme intermediate (78, 79). Moreover, there is one simple kinase—nucleoside diphosphate kinase—that behaves well in all experiments. This enzyme shows clean ping-pong kinetics, a chemically and kinetically competent phosphoenzyme can be isolated (80), and the phosphoryl group is transferred as expected with overall retention of configuration (64) (Table 1). The lesson seems to be that if a kinase follows a pathway involving only binary complexes and a phosphoenzyme then the available methodology should readily diagnose the mechanism. In contrast, kinases that proceed via ternary complexes evidently require more careful study if one is to avoid being misled.

PHOSPHOMUTASES In the reactions catalyzed by the phosphomutases, the phosphoryl group is transferred to a different site in the same substrate, although the reaction is usually not truly intramolecular. The donor-acceptor functionalities are either a pair of hydroxyl groups [as in the case of phosphoglucomutase (glucose 6-phosphate = glucose 1-phosphate)], or a hydroxyl group and a carboxyl group [e.g. bisphosphoglycerate mutase (2,3-bisphosphoglycerate = 1,3-bisphosphoglycerate)]. There are two mechanistic classes of the first group of mutases (hydroxyl-to-hydroxyl). The first class, exemplified by phosphoglucomutase and by phosphoglycerate mutase from animal sources, shows a requirement for the bisphospho-substrate as a cofactor, and these enzymes catalyze the intermolecular transfer of phosphoryl groups amongst the two monophosphorylated substrates and the bisphosphorylated cofactor (81). The cofactor is evidently required to maintain the enzyme in its active phosphorylated form, this phosphoryl group being transferred to either of the two monophosphorylated substrates or, much more slowly, to water (81). For rabbit muscle phosphoglucomutase a unique serine residue is phosphorylated (82),
and for the phosphoglycerate mutases from yeast (83) and from rabbit or chicken muscle (84, 85), a unique histidine residue bears the phosphoryl group. In both cases, the isolated phosphoenzymes are chemically and kinetically competent (86–88). The formal mechanistic scheme for the latter enzyme is shown in Figure 4A.

The extensive studies of Ray and co-workers (81, 89–91) have delineated the origins of the remarkable specificity of phosphoglucomutase toward its substrates. The phosphoryl group of the phosphoenzyme is hydrolyzed 3 $\times 10^{10}$ times more slowly than it is transferred to the 6-hydroxyl group of glucose 1-phosphate, and from the effects of substrate fragments on the dephosphorylation rate, Ray et al (89) have shown how the different parts of the substrate contribute to the observed rate of phosphoryl group transfer. Ma & Ray (91) have recently examined the spectra of the enzyme-substrate complexes individually (e$\sim$P·glucose 6-phosphate, e$\sim$P·glucose 1-phosphate, and e·glucose 1,6-bisphosphate), preventing reaction by using lithium instead of magnesium (92), by omitting the metal ion, or by using the appropriate deoxy sugar phosphates. These workers distinguish between a “minimal motion” pathway, in which there is a unique substrate binding mode and phosphoryl transfer occurs from either the 1- or 6-hydroxyl group to a single active site serine residue, and an “exchange” mechanism, in which there are two alternate substrate binding modes that allow phosphoryl group transfer to the enzyme from either position on the glucose (91). Spectral differences between the two phosphoenzyme complexes favor the exchange pathway. Risking anthropomorphism, one can imagine that the exchange pathway would be favored by enzymes that catalyze such internal rearrangements. If we allow that the construction of a recognition (binding) site is an easier problem than the creation of a catalytic site, at least in terms of the steric, stereochemical, and functional requirements, then we can suggest that phosphoglucomutase has a single phosphoryl group acceptor with a fixed constellation of catalytic groups to allow group transfer to and from it. This hypothesis is supported by the fact that the phosphoryl group in phosphoglucomutase is remarkably immobilized with a correlation time of about $10^{-7}$ s (93), especially when compared with other phosphoenzymes such as that of alkaline phosphatase (94). Glucose 1,6-bisphosphate could bind in one of two mutually exclusive modes, each of which would allow transfer of a different phosphoryl group to the enzyme.

This picture is attractive for other systems too. In the reaction catalyzed byaconitase (53), the dehydration of citrate to cis-aconitate is followed by the regio- and stereospecific rehydration of cis-aconitate in the opposite sense to isocitrate. To suggest two overlapping catalytic hydration-dehydra-
tion sites appears unreasonable, and to propose a set of convolutions of enzyme with respect to substrate [however ingenious: see (95)] seems unnecessary. All we require is a single, fixed catalytic hydration site and the ability of cis-aconitate to bind in this site either way up. Hydration one way up will give citrate, and hydration the other way up will produce iso-citrate. The active site need not be a glove that accepts only one hand, but may instead be a mitten that accepts either. In the case of aconitase, the intermediate (cis-aconitate) may dissociate from the enzyme, whereas for phosphoglucomutase, the intermediate (glucose 1,6-bisphosphate) dissociates into solution only rarely, but can partially dissociate from the active site, undergo a rotation, and then reassociate without being lost to the medium.

The cofactor-dependent phosphoglycerate mutase proceeds by a phosphoenzyme pathway (81) as shown in Figure 4. The flux kinetics approach of Britton et al (96, 97) not only requires a pathway involving a phosphoenzyme, but the lack of induced transport demands that any isomerization of the phosphoenzyme intermediate, including, of course, a migration of the

\[ \text{Figure 4 Schemes for the cofactor-dependent (A) and cofactor-independent (B) phosphoglycerate mutases.} \]
phosphoryl group to another enzyme site, occur with a rate constant larger than $10^6 \text{ s}^{-1}$. This fact has become important with the refinement of the crystal structure of the yeast enzyme to high resolution (98) and the fitting of the recently determined amino acid sequence to the electron-density map (99). The active site evidently contains two almost parallel histidine residues, each pointing to one of the proposed phosphoryl-group binding loci. It has been suggested that either of these histidines may be phosphorylated and that there is a rapid shuttling of the phosphoryl group between them (99). This mechanism is close to the “minimal motion” proposal. There are, however, some problems with what is a superficially seductive postulate. First, if two phosphoenzymes exist, they must interconvert at the unreasonably rapid rate of $> 10^6 \text{ s}^{-1}$. Second, only one phosphoenzyme (in which His-8 is labeled) has been detected, which, after denaturation, hydrolyzes with a single rate constant (100). Third, we have recently shown (67) that the phosphoryl group is transferred with overall retention of configuration (Table 1), which is most economically interpreted in terms of two phosphoryl group transfers and a single phosphoenzyme intermediate. The “exchange” pathway is directly supported by evidence for overlapping multiple binding sites [from salt effects (101) and inhibition of the enzyme by molecules with several anionic groups (Z. B. Rose, private communication)], and by the finding that the ten carboxy-terminal residues of the yeast mutase, which end in the sequence -Gln-Lys-Lys-Gly, are ill-defined in the crystal (S. I. Winn, H. C. Watson, R. N. Harkins, and L. A. Fothergill, private communication). It seems possible that the carboxy terminus acts as a flexible dicationic “lid” that prevents loss of the 2,3-bisphosphoglycerate intermediate to the medium, and allows the operation of the exchange pathway for this enzyme.

In contrast to the animal and yeast enzymes, the phosphoglycerate mutase from wheat germ catalyzes a cofactor-independent (102) intramolecular phosphoryl group transfer (103, 104). No phosphoenzyme has been detected directly, but from flux kinetics (103) and from studies on the “unnatural” phosphoryl group transfer from substrate analogues to glycerate (105), it has been concluded that a phosphoenzyme is an intermediate. To account for the failure of attempts to isolate the phosphoenzyme and for the absence of isotopic exchange between phosphoglycerate and [14C]glycerate, it is presumed that the phosphoenzyme is of relatively high free energy and that glycerate binds extremely tightly to it (105). Stereochemically, the enzyme catalyzes the transfer with overall retention at phosphorus (67), though this outcome is expected both for a pathway involving a single phosphoenzyme and for a mechanism in which the vicinal hydroxyl group of the substrate adds nucleophilically to the phosphoryl group, which then migrates after one pseudo-rotation of the pentacoordinate intermedi-
ate. The probable pathway is shown in Figure 4B, and as far as the group transfer is concerned, the only difference from the yeast and animal systems (Figure 4A) is that the transfer occurs from the phosphoenzyme to glycerate, rather than from the phosphoenzyme to phosphoglycerate. For the cofactor-dependent enzymes the 2,3-bisphosphoglycerate is occasionally lost (106) (the cofactor must, after all, have access to the active site for the priming of the enzyme into its active phosphorylated form, and cofactor loss would simply be the reverse of this process: Figure 4A), whereas for the cofactor-independent enzyme, the corresponding intermediate (glycerate) is never released (105).

It is worth a brief digression to the rearrangement of farnesyl pyrophosphate to nerolidyl pyrophosphate, a mutase-like reaction in which an allylic pyrophosphate group suffers a 1,3 shift (Equation 2):

$$\text{CH}_3$$

Cane & Iyengar (107) have shown that when the C–O–P bridge oxygen is labeled in the substrate, the product contains just one third of the label in the new C–O–P bridge. This result rules out a phospho-Claisen pathway (no product bridge label expected), a direct 1,3-sigmatropic shift (full product label expected), and pyrophosphate tumbling (one sixth of the label expected), and favors an ion pair in which the three oxygens of the proximal phosphate become equivalent.

**Reactions of Phosphoric Diesters**

Nucleophilic attack at the phosphorus of phosphoric diesters may occur with all three kinds of nucleophile: water, a cosubstrate, or an enzymic group. Direct water attack occurs with some nucleases, either on the acyclic diester, as in the case of *Staphylococcal* nuclease, or on a five-membered cyclic diester, as in the second step of the reaction catalyzed by ribonuclease A. Attack by a cosubstrate occurs in the nucleotidyl transfer enzymes, with the second substrate attacking the α-phosphorus of a nucleoside oligophosphate. This group includes many enzymes that activate carboxyl groups (e.g. the aminoacyl-tRNA synthetases and the fatty acyl-CoA synthetases) and enzymes that transfer nucleotides, such as the nucleic acid polymerases and the adenyllylating enzymes. Finally, both of these classes may involve the intermediacy of phospho-ester-enzyme intermediates (enzyme-PO$_2$-OR) that derive from initial attack on the diester by an enzyme nucleophile.
Reports of the isolation and kinetic viability of covalent enzyme intermediates are rather few and scattered, even in cases where there are suggestive data from other experiments. The most complete story has been elaborated by Wood and his group for pyruvate phosphate dikinase from *Propionibacterium* (108) and *Bacteroides* (109), which catalyze the gluconeogenic synthesis of phosphoenolpyruvate from pyruvate and ATP. This reaction involves two isolabel phosphorylated enzymes, and proceeds as shown in Equation 3:

\[ \text{enzyme} + \text{ATP} \rightleftharpoons \text{enzyme}^{-\beta} - \text{P} + \text{AMP} \]
\[ \text{enzyme}^{-\beta} - \text{P} + \text{P} \rightleftharpoons \text{enzyme}^{-\beta} - \text{PP} \]
\[ \text{enzyme}^{-\beta} + \text{pyruvate} \rightleftharpoons \text{enzyme} + \text{phosphoenolpyruvate} \]

Steady-state kinetics and product inhibition studies are in accord with this pathway, and the partial exchange reactions are nearly as rapid as the overall reaction. As in the case of phosphoenolpyruvate synthetase from *E. coli* (110), a histidine residue is phosphorylated (111). Covalent enzyme intermediates have also been isolated for a number of nucleotidyl transferases: in the uridylyl-enzyme from galactose-1-phosphate uridylyltransferase (112) the uridylyl group is linked to N-3 of a histidine residue (113), and other nucleotidyl transferases have been shown to use tyrosine (26, 27) or lysine (28) residues as sites of nucleotide attachment.

In general, stereochemical arguments seem to be the most diagnostic of mechanistic pathway for reactions of both phosphoric monoesters (Table 1) and diesters (Table 2), especially for cases where a phosphorylated enzyme intermediate cannot be isolated and characterized. Inversion is evidently the favored stereochemical course for single displacements at phosphorus, and the stereochemistry observed with the enzymes listed in Tables 1 and 2 correlates very well with all that is known about the existence (or nonexistence) of covalent enzyme intermediates for these reactions. Overall reactions (or, as in the case of the ribonucleases, half reactions) for which there is no evidence for any phosphorylated enzyme intermediate all go with inversion, and systems such as alkaline phosphatase or galactose-1-phosphate uridylyltransferase, for which a viable intermediate is known and characterized, go with overall retention. The fact that nucleoside phosphotransferase (Table 1) proceeds with retention (65) therefore suggests a double displacement pathway involving a phosphorylated enzyme for which there is, as yet, no independent evidence. Conversely, the cyclic nucleotide phosphodiesterase from beef heart has been shown to go with inversion (133) (Table 2), and this observation suggests a single displacement reaction with no intermediate. [This enzyme may be contrasted with the 5'-phosphodiesterase from beef intestine, for which an adenylylated enzyme has been isolated (albeit in $< 5\%$ yield on a molar basis) by quenching the
hydrolytic reaction from the steady state (134), and for which the reaction proceeds with overall retention as expected (Table 2).

### REACTION ENERGETICS

One of the early assumptions of mechanistic enzymology was that the covalency changes that occur within the enzyme:substrate complex are slower than the “on-off” steps that characterize the binding of substrates and the release of products. This derived from the view that chemical changes (even catalyzed ones) are slower than the physical steps of association and dissociation, but the facts point increasingly often to product release (in the endergonic direction) as the rate-determining step in enzyme catalysis (7, 8). Moreover, it appears that a common feature of enzyme catalysis is that the rate constants of successive steps are not very different,

### Table 2 Stereochemical course of displacements at phosphoric diesters

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Result</th>
<th>Class&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribonuclease A</td>
<td>Inversion, inversion</td>
<td>A, B</td>
<td>114, 115</td>
</tr>
<tr>
<td>Ribonuclease T&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Inversion</td>
<td>A</td>
<td>116</td>
</tr>
<tr>
<td>Ribonuclease T&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Inversion</td>
<td>A</td>
<td>117</td>
</tr>
<tr>
<td>UDP-glucose pyrophosphorylase</td>
<td>Inversion</td>
<td>A</td>
<td>118</td>
</tr>
<tr>
<td>Galactose-1P-uridylyl-transferase</td>
<td>2 inversions or 2 retentions</td>
<td>A</td>
<td>64</td>
</tr>
<tr>
<td>PRPP synthetase</td>
<td>Inversion</td>
<td>A</td>
<td>120</td>
</tr>
<tr>
<td>RNA polymerase (initiation &amp; exchange)</td>
<td>Inversion</td>
<td>A</td>
<td>121</td>
</tr>
<tr>
<td>RNA polymerase (elongation)</td>
<td>Inversion</td>
<td>A</td>
<td>122, 123</td>
</tr>
<tr>
<td>DNA polymerase</td>
<td>Inversion</td>
<td>A</td>
<td>124</td>
</tr>
<tr>
<td>tRNA nucleotidyl transferase</td>
<td>Inversion</td>
<td>A</td>
<td>125</td>
</tr>
<tr>
<td>Polynucleotide phosphorylase (elongation)</td>
<td>Inversion&lt;sup&gt;b&lt;/sup&gt;</td>
<td>A</td>
<td>126</td>
</tr>
<tr>
<td>Polynucleotide phosphorylase (exchange)</td>
<td>Retention&lt;sup&gt;b&lt;/sup&gt;</td>
<td>A</td>
<td>c</td>
</tr>
<tr>
<td>Acetyl CoA synthetase</td>
<td>Inversion</td>
<td>A</td>
<td>127, 128</td>
</tr>
<tr>
<td>Enterobacter aerogenes phosphatase</td>
<td>Inversion</td>
<td>B</td>
<td>129</td>
</tr>
<tr>
<td>Snake venom phosphodiesterase</td>
<td>Overall retention</td>
<td>B</td>
<td>130, 131</td>
</tr>
<tr>
<td>Bovine intestine 5'-phosphodiesterase</td>
<td>Overall retention</td>
<td>B</td>
<td>c</td>
</tr>
<tr>
<td>Aminoacyl-tRNA synthetases</td>
<td>Inversion</td>
<td>A</td>
<td>132</td>
</tr>
<tr>
<td>Cyclic phosphodiesterase</td>
<td>Inversion</td>
<td>A</td>
<td>133</td>
</tr>
<tr>
<td>Brevibacterium lactofermentum adenylyl cyclase</td>
<td>Inversion</td>
<td>A</td>
<td>119</td>
</tr>
</tbody>
</table>

<sup>a</sup>With the natural unlabeled substrates, Class A involves the conversion of a prochiral center to a prochiral center, and Class B involves the conversion of a prochiral center to a pro-prochiral center (see the section on stereochemistry).

<sup>b</sup>This is in the exchange reaction using ADP<sub>13</sub> and <sup>32</sup>P<sub>j</sub>, which may be a model for the initiation step (as distinct from the elongation steps, which proceed with inversion).

<sup>c</sup>S. J. Benkovic, private communication.
and this must make us wary of the usual simplifying presumption that one transition state in a reaction sequence is cleanly rate limiting.

We have argued earlier (7, 135) that there is a limit to the kinetic effect of accelerating the chemical steps, and that an enzyme is perfect (as far as simple rate enhancement is concerned) once two conditions are met: (a) that all the free energy barriers for the chemical steps are lower than that (a in Figure 5) for the diffusion-limited binding steps of the thermodynamically less stable substrate, and (b) that all the reaction intermediates have free energies just higher than that of the enzyme plus the more stable substrate at its ambient concentration (b in Figure 5) (Isaiah, 40: 4). We have further argued (135) that for high catalytic efficiency, the equilibrium constant for the bound states $K_{\text{int}}$ ($e \cdot s \rightleftharpoons e \cdot p$ for a one-substrate enzyme, or $e \cdot a \cdot b \rightleftharpoons e \cdot c \cdot d$ for a two-substrate enzyme) would be closer to unity than the overall equilibrium constant $K_{\text{eq}}$ ($s \rightleftharpoons p$, or $a + b \rightleftharpoons c + d$) (see Figure 5). These proposals are considered in turn.

"Off" Rates

It can be shown in a number of ways that product off rates are rate limiting for many enzyme reactions. For one-substrate: one-product systems, the observation that $k_{\text{cat}}/K_m$ is above $10^8 \text{M}^{-1} \text{sec}^{-1}$ in the downhill direction suggests that the highest barrier to catalysis is diffusive, which means that the rate-limiting transition state in the uphill direction involves the release of product. This is evidently the situation for triosephosphate isomerase (136). Values of $k_{\text{cat}}/K_m$ of less that $10^8 \text{M}^{-1} \text{sec}^{-1}$ do not necessarily mean that the slowest step involves covalency changes subsequent to substrate

Figure 5 Free energy profile for an enzyme of high catalytic efficiency. $K_{\text{eq}}$, the overall equilibrium constant; $K_{\text{int}}$, the equilibrium constant for the interconversion of the central complexes; $k_d$, the rate constant for the diffusive substrate binding steps (in either direction).
binding. The steady-state behavior of ordered two-substrate:two-product systems may also indicate that substrate-handling steps are rate limiting, as has been discussed by Cleland (8). Further, transient kinetic studies have shown in a number of cases that catalysis is faster than product loss, or than a conformational change preceding this loss (137), and relatively small primary deuterium isotope effects (e.g. for dehydrogenases) have often been taken as evidence that the catalytic step, in these cases hydride transfer, is not cleanly rate limiting (8). Finally, there are isotope-trapping methods (41, 138) that may demonstrate unambiguously how a particular liganded enzyme form partitions between ligand loss in one direction and the catalytic reaction in the other. For instance, when \([^{14}\text{C}]\) glucose is mixed with hexokinase and then diluted into a solution containing a saturating level of ATP and a large excess of unlabeled glucose, all of the enzyme-bound \([^{14}\text{C}]\) glucose is converted to glucose 6-phosphate (41). This result shows both that the enzyme:glucose complex is functional, and that the rate of loss of glucose from the enzyme is much slower than the rate of conversion of the e-glucose-ATP complex to products (glucose 6-phosphate and ADP). Indeed, the "glucose-off" step is rate limiting for the reaction starting with glucose 6-phosphate and ADP.

In the case of phosphatases, studies on the exchange of \(^{18}\text{O}\) between \(P_i\) and \(H_2^{18}\text{O}\) have recently illuminated the partition behavior of a number of enzyme-\(P_i\) complexes. [A useful new synthesis of \([^{18}\text{O}]P_i\) has been described (142).] On the basis that isotopic exchange occurs primarily by the reversible formation of an anhydro species (involving either the enzyme or a cosubstrate) (20), the rate of appearance of \(^{18}\text{O}\) in reisolated \(P_i\) provides a measure of the ratio of exchange and off rates (Equation 4):

\[
\begin{align*}
\text{exchange} & \quad \text{off} \\
\text{e} \cdot P + H_2O & \overset{\text{exchange}}{\longrightarrow} \text{e} \cdot P_i = e + P_i
\end{align*}
\]

As can be seen from Table 3, the range of partition ratios is very wide. For myosin subfragment 1, only \(P_i\) containing all four oxygens labeled is isolated, which demonstrates that the exchange process (formation of ATP, exchange of the expelled water molecule with the solvent, and cleavage of the ATP) is fast compared with \(P_i\) release (15, 145). Since hydrolysis of \([\gamma-^{18}\text{O}]\)-labeled ATP in \(H_2^{16}\text{O}\) results in essentially unlabeled \(P_i\), it seems that the intermediates involved in the back reaction (from \(P_i\) and ADP) are the same as those involved in ATP hydrolysis (15). This is also true for pyrophosphatase, where the same distribution of partially labeled \(P_i\) is seen from PP\(_i\) hydrolysis as is seen in \(P_i\) exchange experiments (19, 141). It is not true, however, for fructose 1,6-bisphosphatase, where the distribution of labeled \(P_i\) species in the exchange reaction is quite different from that of the \(P_i\) generated in the enzyme-catalyzed substrate hydrolysis (144).
Simplistically, both reactions should proceed via the same ternary complex, e·fructose 6-phosphate·P$_i$, but they clearly do not. Cooperative effects between active sites in this tetrameric enzyme may explain the difference, but the result in any case provides a useful warning. Analogously, the increase in $^{18}$O exchange per ATP formed from ADP and [$^{18}$O]-P$_i$ by chloroplast (146) or mitochondrial (147, 148) ATPase as the ADP and P$_i$ concentrations are lowered, has led to a proposal of an alternating site model in which there is coupling between the subunits of the enzyme.

In contrast to the above systems, the complex of alkaline phosphatase with P$_i$ evidently loses P$_i$ much more rapidly than it forms the phosphoenzyme, since the P$_i$ oxygens are exchanged only one at a time (139, 140). Two caveats are needed here, however. Slow exchange rates may derive from the slow exchange of water between the active site of the phosphoenzyme and bulk solution, or from the inability of enzyme-bound P$_i$ to tumble in the active site. While we may guess that water access to the active site of a hydrolytic enzyme would be facile, this need not be so. The possibility that bound P$_i$ cannot tumble seems unlikely in this case, first because the $^{31}$P NMR of the phosphoenzyme shows that the phosphoryl group is free to rotate (94) [in contrast to the phosphoryl group in phosphoglucomutase (93); see above] and secondly because the cobalt enzyme shows a much higher value of $k_{ex}/k_{off}$ (140) (Table 3), which indicates that the P$_i$ must be at least partially free in this enzyme. The most likely interpretation is therefore that for alkaline phosphatase the P$_i$ “off rate” is faster than the rate of formation of e$\sim$P. [This statement is not in conflict with the earlier one that the rate of phosphate ester hydrolysis may be limited by the P$_i$ off rate. This off rate may indeed be the slowest of the forward steps in phos-

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{ex}/k_{off}$</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn-alkaline phosphatase (pH 7)</td>
<td>$&lt;1$</td>
<td>139</td>
</tr>
<tr>
<td>Zn-alkaline phosphatase (pH 4.4–10)</td>
<td>$&lt;0.3$</td>
<td>140</td>
</tr>
<tr>
<td>Co-alkaline phosphatase (pH 6.8)</td>
<td>$3$</td>
<td>140</td>
</tr>
<tr>
<td>Inorganic pyrophosphatase</td>
<td>$0.7$</td>
<td>19,141</td>
</tr>
<tr>
<td>Acid phosphatases</td>
<td>$&lt;1$</td>
<td>13,143</td>
</tr>
<tr>
<td>Fructose 1,6-bisphosphatase</td>
<td>$\sim1.7^a$</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>$&lt;0.01^b$</td>
<td>144</td>
</tr>
<tr>
<td>Sarcoplasmic reticulum ATPase</td>
<td>$\sim0.08$</td>
<td>18</td>
</tr>
<tr>
<td>Myosin subfragment 1</td>
<td>$&gt;50$</td>
<td>145</td>
</tr>
<tr>
<td>Myosin subfragment 1</td>
<td>$&gt;100$</td>
<td>15</td>
</tr>
</tbody>
</table>

$^a$From an incubation of P$_i$ and fructose 6-phosphate with the enzyme.

$^b$From the P$_i$ generated by enzyme-catalyzed fructose 1,6-bisphosphate hydrolysis.
phate ester hydrolysis, yet much faster than the formation of $e \sim P$ from $e \cdot P_i$. Finally, the use of the $^{18}O$ exchange method has been extended to more complex systems such as glutamine synthetase (149, 150), for which it appears that loss of $^{18}O$ from labeled $P_i$ is some seven times faster than the formation of ATP (from enzyme, ADP, $P_i$, and glutamine).

**Internal Thermodynamics: The Equilibrium Constant of Central Complexes**

In discussing how the catalytic efficiency of an enzyme might be achieved, we argued earlier, on theoretical grounds, that in the ideal catalyst the kinetically significant transition state is flanked by kinetically significant intermediates of equal free energy (135, 151). This means that, for highly efficient enzymes, the free energies of the central complexes ($e'\cdot s$ and $e'\cdot p$ for a one-substrate enzyme; $e' a' b$ and $e' c' d$ for a two-substrate enzyme) should be more nearly equal than the free energies of the unbound species. In terms of Figure 5, $K_{\text{int}}$ should be nearer to unity than $K_{\text{eq}}$. At the time of this proposal, only scattered information was available concerning the internal thermodynamics of enzyme-catalyzed processes, though these data did appear to support the suggestion. [For example, lactate dehydrogenase has $K_{\text{eq}} 4 \times 10^{-5}$ and $K_{\text{int}} 0.25$ (152); myosin ATPase has $K_{\text{eq}} 2 \times 10^5$ and $K_{\text{int}} 10$ (137, 153).] To determine $K_{\text{int}}$ for an enzyme reaction one must either measure the equilibrium position in the presence of a saturating amount of enzyme, or define the relative proportions of the different liganded forms of the enzyme at the steady state. The first approach has been taken by Cohn and her collaborators (154), who have used $^{31}P$ NMR to determine $K_{\text{int}}$ directly for a number of phosphokinases. These results are listed in Table 4. Using the second approach, Wilkinson & Rose (42) have investigated the population of functional complexes of hexokinase by different methods of quenching the steady-state reaction. When hexokinase is rapidly mixed with $[^3H]glucose$ and ATP and the reaction is quenched into acid after a few milliseconds, a burst of glucose 6-phosphate is observed that amounts to about one half of the enzyme on a molar basis, which shows that one half of the enzyme exists in forms where the phosphoryl group has already been transferred to glucose ($e'\cdot glucose$ 6-phosphate$\cdot ADP$ or $e'\cdot glucose$ 6-phosphate). The subsequent rate of production of glucose 6-phosphate (if the quench is delayed) is the same as the steady-state $V$. Other experiments had shown that the steady-state concentration of $e'\cdot glucose$ 6-phosphate is very low (i.e. that the rate of breakdown of $e'\cdot glucose$ 6-phosphate$\cdot ADP$ is limited by the ADP off rate), so at the steady state one half of the enzyme must exist as $e'\cdot glucose$ 6-phosphate$\cdot ADP$. Since both substrates are saturating (i.e. the concentration of $e'\cdot glucose$ is very small), the steady-state
Table 4  Equilibrium constants for free and bound species in phosphoryl group transfer reactions

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_{eq}$ (free species)</th>
<th>$K_{int}$ (bound species)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine kinase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.1</td>
<td>1.2</td>
<td>154</td>
</tr>
<tr>
<td>Creatine kinase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.1</td>
<td>~1</td>
<td>153, 154</td>
</tr>
<tr>
<td>Adenylate kinase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.4</td>
<td>1.6</td>
<td>153, 154</td>
</tr>
<tr>
<td>Pyruvate kinase&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pyruvate reaction</td>
<td>$3 \times 10^{-4}$</td>
<td>~1</td>
<td>154</td>
</tr>
<tr>
<td>glycolate reaction</td>
<td>$\geq 50$</td>
<td>~1</td>
<td>154</td>
</tr>
<tr>
<td>Phosphoglycerate kinase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>$3 \times 10^{-4}$</td>
<td>0.8</td>
<td>154</td>
</tr>
<tr>
<td>Hexokinase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>$2 \times 10^{3}$</td>
<td>~1</td>
<td>42, 153</td>
</tr>
<tr>
<td>Phosphoglucomutase&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17</td>
<td>0.4</td>
<td>90, 155</td>
</tr>
<tr>
<td>Myosin ATPase&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>137, 153</td>
</tr>
<tr>
<td>Methionine tRNA synthetase&lt;sup&gt;d&lt;/sup&gt;</td>
<td>$2 \times 10^{5}$</td>
<td>10</td>
<td>137, 153</td>
</tr>
</tbody>
</table>

<sup>a</sup> $K$ values are in the direction [donor] [ADP]/[acceptor] [ATP].
<sup>b</sup> [ADP] [P<sub>i</sub>] [ATP] [H<sub>2</sub>O].
<sup>c</sup> [methionyl-AMP] [PP<sub>i</sub>] [methionine] [ATP].
<sup>d</sup> M. Cohn, private communication.

concentrations of e·glucose·ATP and e·glucose 6-phosphate·ADP must be roughly equal, and because the phosphoryl transfer is relatively fast, $K_{int}$ must be near unity (42).

It is evident from the results in Table 4 that even when the overall equilibrium constant for a reaction is 10<sup>4</sup> or 10<sup>5</sup>, the forces of differential binding (that distinguish between substrates and products) do ensure that the transition state for the catalytic step is flanked by complexes of essentially equal free energy. In our original proposal (135), we suggested that this could be one way in which the free energy of substrate binding may be turned to good catalytic effect, and it seems from the results in Table 4 that this occurs. Most impressive are the data for pyruvate kinase. When the natural substrate-product pair (pyruvate-phosphoenolpyruvate) is replaced by the unnatural pair (glycolate-phosphoglycolate), the overall equilibrium constant changes by more than 10<sup>5</sup>, yet the value of $K_{int}$ is constant at around unity (154). It is possible that the $K_{int}$ for the natural materials actually relates to e·enolpyruvate·ATP = e·phosphoenolpyruvate·ADP, and that replacement of the methylene group by two hydrogens (on going from enolpyruvate to glycolate, and phosphoenolpyruvate to phosphoglycolate) affects each side of the equilibrium equally, so that $K_{int}$ remains unchanged. This change would, in our earlier terminology, be a change in "uniform binding" (135), which would not affect $K_{int}$ even if there were adverse consequences for the overall catalytic efficiency.
THE NATURE OF THE ELEMENTARY STEP

In this section we consider the mechanism of phosphoryl group transfer at the level of the elementary step and review the evidence that bears upon the problem of how a single transfer could be catalyzed enzymically.

**Associative versus Dissociative Pathways**

From the physical-organic literature (1, 2), there are four formal mechanistic extremes for reactions at the phosphorus of a phosphoric ester. There is one dissociative path and three associative reactions, as shown in Figure 6. The dissociative pathway involves the transient formation of the highly reactive intermediate monomeric metaphosphate, which is then captured by the acceptor group. For phosphoric monoester dianions, for example, the sensitivity of reaction rate to the pK_a of the entering nucleophile is low (β_nuc 0–0.1), activation entropies are close to zero (for a bimolecular process the activation entropy would more likely be around ~20 e.u.), in mixed nucleophilic solvents the product ratio is usually quite close to the molar ratio of nucleophiles (implying the intermediacy of a rather indiscriminate species), and there is a substantial ^18O isotope effect for the P–O bond being cleaved (1, 2, 156). This and other evidence [including the generation and trapping of methylmetaphosphate in solution by Satterthwaite & Westheimer (157)] has established the importance of the dissociative reaction. As the degree of esterification or of protonation of the monoester dianion is increased, however, one observes an increase in β_nuc (for 2,4-dinitrophenyl phosphate monoanion, or for methyl-2,4-dinitrophenyl phosphate, β_nuc is up to 0.3–0.4), and the transition state evidently begins to assume some of the characteristics of an associative process (2). For triesters, the reaction rates are equally sensitive to the pK_a's of the nucleophile and the leaving group, and these reactions appear to be associative, having trigonal bipyramidal transition states with the nucleophile and leaving group at the apical positions (1, 2). The pentacoordinate oxyphosphorane need not be a transition state, however, but rather may be an intermediate (especially for cyclic esters in which steric crowding is reduced) with a sufficient lifetime to allow pseudorotation (158, 159). This possibility provides the third and fourth mechanistic extremes, in which addition of the nucleophile at phosphorus leads to a pentacoordinate intermediate that may undergo one or more pseudorotations before the leaving group departs. Recent evidence places these pathways on firm ground even for acyclic systems. Sigal & Westheimer (160) have demonstrated that the phosphoryl oxygen of diphenyl methylphosphonate exchanges with H_2^{18}O at about 8% the rate of hydrolysis, this observation being most reasonably interpreted in terms of an
oxyphosphorane intermediate whose lifetime is enough for at least one pseudorotation and the necessary protonation-deprotonation steps.

How relevant are these mechanistic extremes to phosphoryl transfer processes catalyzed by enzymes? At the active site of an enzyme, where we have a fixed disposition of attacking, leaving, and charge-stabilizing groups, we may assume that the reaction will have a defined stereochemical course. The dissociative pathway (Figure 6 A) can in principle proceed with inversion or retention, the “in-line” associative pathways B and C will go with inversion, and the adjacent associative path D will result in retention. The apparent universality of inversion as the preferred stereochemical course of these reactions (see Tables 1 and 2, and the next section) allows us to focus on Pathways A (of in-line geometry), B, and C (Figure 6). For an enzyme reaction it is difficult to distinguish between Pathways B and C, since even if the pentacoordinate species is a reaction intermediate of some lifetime, it cannot signal its existence by a pseudorotation, which might be possible

![Diagram](image)

Figure 6 Pathways for nucleophilic displacements on a phosphoric monoester. For an enzyme-catalyzed reaction, the acceptor group is always present, though this is not shown above for Pathway A.
if it were in free solution. Mildvan (161) has suggested two criteria that may bear upon the distinction between dissociative and associative in-line displacements. First, he has proposed that the distance between the nucleophile $N$ and the attacked phosphorus would be shorter (say $\leq 3.3\,\text{Å}$) for an associative path than for a dissociative reaction (say $\geq 4.9\,\text{Å}$). Secondly, if one assumes that compared to the ground state, the peripheral oxygen atoms bear more charge in an associative transition state and less charge in a dissociative transition state, it is expected that coordination of the ubiquitous divalent metal cation to oxygen(s) of the transferred phosphoryl group would inhibit the dissociative pathway but accelerate an associative process. Data on both of these questions from NMR measurements (161) favor the dissociative pathway for DNA polymerase and the associative route for phosphoribosylpyrophosphate synthetase and pyruvate kinase, though the likelihood of protein conformational changes and the possibility of ligand exchange processes makes these results only suggestive. Jencks has argued, indeed, that for an enzyme-catalyzed process there is little meaningful distinction amongst Pathways $A$, $B$, and $C$ (Figure 6) (W. P. Jencks, private communication). As we have seen earlier, the acceptor nucleophile is always present in an enzymic phosphoryl transfer (either as an enzyme group or as a bound cosubstrate), and Jencks suggests that there will be little difference in steric or electronic terms between a dissociative pathway containing a "spectator" nucleophile (which may do rather more participating than spectating) poised to accept metaphosphate, and an associative pathway having a loose transition state with substantial bond breaking and little bond formation.

In an effort to distinguish between an associative and a dissociative mechanism for pyruvate kinase, Lowe & Sproat (162) have applied the positional isotope exchange method of Midelfort & Rose (52), and found that the enzyme catalyzes the randomization of $^{18}\text{O}$ between the $\beta,\gamma$-bridge and $\beta$-nonbridge positions of ATP in the absence of cosubstrate. The randomization reaction occurs about 20 times more slowly than when pyruvate is present. It was suggested that the catalysis of isotope randomization indicated a dissociative pathway, since there is no evidence for the intermediacy of a phosphoenzyme (69, 163). Catalysis of positional isotope exchange is not, however, a general phenomenon with phosphokinases [hexokinase catalyzes no such exchange even in the presence of lyxose (I. A. Rose, private communication)], and the pyruvate kinase results can also be rationalized by the action of an enzyme nucleophile or of water as a poor surrogate for the hydroxyl group of enolpyruvate in an associative displacement. Since isotope exchange between the $\gamma$-oxygen of ATP and $\text{H}_2^{18}\text{O}$ is not observed, any rationalization involving nucleophilic attack by water requires that $\text{P}_i$ at the active site does not tumble, and that $\text{P-O}$ bond rotations are only possible about the bonds indicated in Figure 7. We have
found (W. A. Blättler, unpublished work) that when chiral \( [\gamma^{16}\text{O},^{17}\text{O},^{18}\text{O}]\text{-ATP} \) is incubated with pyruvate kinase in the absence of cosubstrate, the \( \gamma \)-phosphoryl group racemizes (if at all) at least 300 times more slowly than the \( \beta \)-phosphoryl group undergoes positional isotope exchange. This is not a surprise: even if monomeric metaphosphate were formed at the active site, it would surely be tightly bound, and any rotation would be very slow.

Experiments with two kinds of substrate analogues have been done in efforts to cast light on the dissociative-associative question, though it must be admitted that the results have not been very helpful. Replacement of a peripheral oxygen by sulfur to give a phosphorothioate reduces the rate of nucleophilic attack on triesters (for which an associative pathway can be assumed) by more than an order of magnitude, yet increases the rate of reaction of monoesters by the metaphosphate path by a similar factor. The finding that, compared to the all-oxy compounds, phosphorothioates are handled more slowly by kinases, much more slowly by alkaline phosphatase, and undetectably by a phosphomutase, has led to the suggestion (164, 165) that an associative mechanism is preferred in enzyme-catalyzed reactions. Conversely, methyl phosphonic esters are about ten times more susceptible to nucleophilic associative displacements than the analogous phosphoric esters, and it is expected that the methyl substitution would disfavor heterolysis in the dissociative reaction. The finding that the \( \gamma \)-methyl phosphonate analogue of ATP (adenosine 5'\([\beta\text{-methylphosphonyl}]\text{diphosphate}\) is not a substrate for seven phosphokinases or for alkaline phosphatase, could therefore be interpreted as evidence for the dissociative mechanism (166). In both of these cases, however, steric effects, metal binding effects, and changes in H-bond donor-acceptor properties could all interfere with the enzymes' normal catalytic processes, and these considerations severely compromise the interpretation of the observed rates.

The binding of oxovanadium (IV) and vanadium (V) ions and their uridine complexes to ribonuclease (167), and the fact that vanadate, molybdate, and tungstate bind very tightly to phosphatases (168, 169) and Na,K-ATPase (170) has suggested that such species may act as structural and electronic analogues of the transition states for associative displacements on
these enzymes. Certainly the structural differences are small (e.g. P–O in phosphate is 1.52 Å; Mo–O in molybdate is 1.76 Å), yet the $K_1$ values for phosphate and molybdate with acid phosphatase are $1 \times 10^{-3}$ M and $6 \times 10^{-8}$ M respectively (168). Whether these data can really be used to distinguish between associative and dissociative transition states is, however, another matter. Analogously, the cooperative binding by some kinases of ADP, the unphosphorylated cosubstrate, with such planar ions as nitrate or formate in quaternary complexes (171, 172), can be taken as evidence for any of the first three pathways shown in Figure 6. Even if there are real structural and electronic differences between the dissociative and associative mechanisms, more subtle approaches will be required to distinguish between them.

**Stereochemistry**

From a stereochemical point of view, there are three kinds of displacement at phosphoric monoesters and diesters. Each of these is illustrated below in a conceptual outline that exemplifies the experimental approaches without attempting to describe in detail the chemistry and biochemistry involved.

**PROCHIRAL SUBSTRATE $\rightarrow$ PROCHIRAL PRODUCT**  In this class, the phosphorus at which the displacement occurs is, in the natural unlabeled substrate, a prochiral center, and the reaction results in the creation of a new prochiral center at that phosphorus. An example is the reaction catalyzed by the enzyme UDP-glucose pyrophosphorylase, in which the phosphoryl group of glucose 1-phosphate displaces PP$_i$ by nucleophilic attack at P$_a$ of UTP (Equation 5):

$$\text{UTP} + \alpha-\text{d-glucose 1-phosphate} \rightleftharpoons \text{UDP-glucose} + \text{PP}_i$$

Figure 8 shows schematically the experiments of Sheu & Frey (118) that have allowed the stereochemical course of this reaction to be established. The prochiral phosphorus at which displacement occurs is made into a chiral center by substitution of one of the two peripheral oxygens with sulfur. [For recent reviews on the nucleoside phosphorothioates, see (173, 174)] To interpret the experiment shown in Figure 8, it is unnecessary to know the absolute configuration at phosphorus in the phosphorothioates, since the diastereoisomers of the substrate (UTPaS) can be related directly to the diastereoisomers of the derived product (UDPaS). Other examples of this class are included in Table 2. In one of these, the reaction catalyzed by phosphoribosylpyrophosphate synthetase, the prochiral phosphorus was made chiral not by replacement of one of the two peripheral oxygens by sulfur, but by ligation of one of the oxygens to an exchange-inert cation, Co (III). The relative configuration at phosphorus in the substrate and the
product was then established from the sign of the circular dichroic spectra of these materials (120).

**PROCHIRAL SUBSTRATE → PRO-PROCHIRAL PRODUCT** In this class, a prochiral phosphorus suffers attack by water, thereby creating a phosphate monester that is pro-prochiral at phosphorus. An example is the reaction catalyzed by cyclic diesterase, in which the prochiral phosphorus center in cyclic AMP is hydrolyzed to give a pro-prochiral phosphorus center in 5'-AMP. Stec & Eckstein and their collaborators have solved the stereochemical course of this reaction by synthesis of the $S_p$ diastereoisomer of the phosphorothioate of cyclic AMP (175) (see Figure 9). Reaction with the diesterase in $H_2^{18}O$ produces a chiral sample of $AMPaS^{18}O$. The phosphorus configuration of this material was determined by treatment

![Diagram](https://example.com/diagram.png)

**Figure 8** Evaluation of the stereochemical course of UDP-glucose pyrophosphorylase$^a$ (118). $a,$ Urd, uridine; $P,$ the chiral phosphorus; $b,$ the $^{31}P$ NMR chemical shifts of the $S_p$ isomers are always downfield relative to the $R_p$ isomers; $c,$ the unused isomer turns out to be $S$ (based on its NMR spectrum), so the $R$ isomer of UTPaS is the one that the enzyme accepts; $d,$ this reaction does not affect any bonds involving the chiral phosphorus; $e,$ this is the $S$ isomer, so the pyrophosphorylase reaction proceeded with inversion at $P_a$ of UTPaS.
with adenylate kinase/ATP and pyruvate kinase/phosphoenolpyruvate, which produces only the \( S_p \) diastereoisomer of ATP\( \alpha \)S. The configuration of the AMP\( \alpha \)Sa\(^{18}O \) was then established by determining whether the \(^{18}O \) in the ATP\( \alpha \)Sa\(^{18}O \) was in a peripheral \( \alpha \) position or in the \( \alpha,\beta \)-bridge (133). Other examples of this class of stereochemical problem are included in Table 2.

\[ \begin{align*}
\text{AMP\( \alpha \)Sa}^{{18}O} & \rightarrow \text{Adenylate kinase (ATP) + ATP} \\
& \rightarrow \text{Pyruvate kinase (PEP) + PEP} \\
\end{align*} \]

**Figure 9** Evaluation of the stereochemical course of cyclic diesterase\(^a\) (133). \( a \), A, adenine; Ad, adenosine; A', \( N,N \)-dibenzoyladenine; \( b \), the configuration at phosphorus is known from the method of synthesis (175): the phosphoranilidate precursor was separated from its phosphorus epimer, and the configuration at phosphorus deduced by \(^{31}P \) NMR. The sulfuration proceeds with retention of configuration (176, 177); \( c \), this reaction is known to produce the \( S_p \) isomer of ADP\( \alpha \)S (which is phosphorylated in situ to the \( S_p \) isomer of ATP\( \alpha \)S). The absolute configuration of these species has been reported independently by three groups (123, 130, 178, 179); \( d \), since the absolute configuration at \( P_\alpha \) is known, evaluation of whether the \(^{18}O \) label in the ATP\( \alpha \)Sa\(^{18}O \) occupies the \( \alpha,\beta \)-bridge position or is in the peripheral position on \( P_\alpha \) (as shown), determines the configuration of the chiral phosphorus in the AMP\( \alpha \)Sa\(^{18}O \).
PRO-PREOCHIRAL SUBSTRATE → PRO-PREOCHIRAL PRODUCT This class contains all those enzymes that catalyze phosphoryl group transfer reactions, where both substrate and product are phosphoric monoesters. An example is the reaction catalyzed by glycerol kinase (Equation 6):

\[ \text{ATP} + \text{glycerol} \rightleftharpoons \text{sn-glycerol 3-phosphate} + \text{ADP} \]

To determine the stereochemical course of such reactions, the transferred phosphoryl group has been made chiral, the three formally equivalent peripheral oxygen atoms being distinguished either by using \(^{16}\text{O}, ^{18}\text{O}\) and sulfur, or by using \(^{16}\text{O}, ^{17}\text{O}, \text{and} ^{18}\text{O}\). Glycerol kinase has been studied by both methods (59, 60) (the fact that both approaches give the same answer suppresses lingering fears that the much more slowly reacting phosphorothioates may follow a different mechanistic and stereochemical course). For each, ATP of known chirality at the \(\gamma\)-phosphorus was synthesized (59, 180), and the absolute configuration of the product established independently. The scheme for the \([^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}]\) case is summarized in Figure 10.

Although the germinal stereochemical experiment in the field of enzyme-catalyzed reactions at phosphorus was done in 1970 by Usher & Eckstein (114), it is only in the last two years or so that the data in Tables 1 and 2 have been obtained. Synthetic approaches to chiral phosphorothioate diesters (182, 183), chiral \([^{18}\text{O}]\)phosphorothioate monoesters (58, 62, 63, 178), and chiral \([^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}]\)phosphoric monoesters (59, 180, 184) have been devised, and essentially any labeling pattern of any of the phosphate groups in ATP can now be achieved. Moreover, methods for the analysis of the location of isotopic labels in such molecules are now well developed. Mass spectrometry still has the edge if material supply is a problem (52, 139, 144, 181), but the effect of \(^{18}\text{O}\) substitution on the \(^{31}\text{P} \)NMR chemical shift (185, 186) (even bridge and nonbridge \(^{18}\text{O}\) can be distinguished with high resolution instruments) and of \(^{17}\text{O}\) substitution on the line-width of the \(^{31}\text{P} \)NMR signal (128, 187) are nondestructive methods that are especially attractive when enough product is available.

The usefulness of phosphorothioate analogues of phosphates in both mechanistic and stereochemical studies must not blind us to the fact that the rates of many enzyme-catalyzed reactions are often much slower with phosphorothioate substrates. Thus, although nine of the ten glycolytic enzymes (from hexokinase down to pyruvate kinase) will accept phosphorothioates with varying decreases in rate compared with the all-oxygen substrates (58), phosphoglycerate mutase appears not to tolerate the sulfur substitution (or more precisely, phosphorothioate isomerization by this enzyme is undetectably slow compared with the background rate arising from wash-out of sulfur from the substrate) (G. Orr and G. Chin, unpub-
lished experiments). Further, the relative rates of reaction of diastereoisomeric phosphorothioate diesters vary over a wide range. Snake venom phosphodiesterase hydrolyzes the $R_p$ isomer of a dinucleoside monophosphorothioate [Up(S)dT] more than 1000 times more rapidly than the $S_p$ isomer (188), yet ribonuclease T2 shows almost no discrimination between the diastereoisomers of Up(S)A (117). Hexokinase utilizes only the $S_p$ isomer of ATPαS (189), yet phosphofructokinase shows no discrimination between the $S_p$ and $R_p$ diastereoisomers either as substrates or as allosteric effectors (190). Even the observed steady state kinetic characteristics can change with sulfur substitution, the cyclic AMP-dependent protein kinase from beef heart showing intersecting double-reciprocal plots with Mg·ATP,

![Diagram](image)

Figure 10 Evaluation of the stereochemical course of glycerol kinase (59). a, $\Phi$, $^{17}O$; $\bullet$, $^{18}O$; b, from asymmetric chemical synthesis (59, 180); c, transfer of the chiral phosphoryl group with retention of configuration to propanediol (9); d, "in-line" ring closure with loss of one of the three peripheral oxygens gives three cyclic diesters in equal amounts; e, methylation of all six exocyclic oxygens, separation of "syn" and "anti" diastereoisomers; f, these steps effectively isolate the species containing both $^{17}O$ and $^{18}O$, and establish which of these two isotopes bears the methyl group "label." This establishes the absolute configuration of the $[^{14}O,^{17}O,^{18}O]$phosphoryl group in the phosphopropanediol (180, 181) and therefore in the product of the kinase reaction, sn-glycerol 3-phosphate.
but parallel plots with Mg·ATPβS (E. T. Kaiser, private communication). The effect of varying the essential divalent metal ion can also be dramatic and is considered in the next section. In general, however, the consistency of the picture that emerges from the data listed in Tables 1 and 2—that each enzyme-catalyzed displacement at phosphorus leads to inversion at phosphorus—suggests that phosphorothioates are well-behaved probes of the stereochemical aspects of phosphoryl transfer reactions. The in-line geometry that has now been repeatedly proved by direct stereochemical study was anticipated by some distance-mapping investigations using NMR (191, 192), and has been shown to be the preferred mode of attack at phosphorus in a “prebiotic” displacement on a cyclic nucleoside phosphorothioate in the solid state (193).

**Metal Ions**

Most enzymes that catalyze displacements at phosphoric esters have a requirement either for a loosely bound divalent cation [e.g. Mg for all kinases, Ca for *Staphylococcal* nuclease (194)] or a tightly-bound cation of a metalloenzyme [e.g. Zn for alkaline phosphatase (5) or for *E. coli* DNA polymerase I (195)]. In this section we focus on the role of the ubiquitous Mg in the kinase-catalyzed reactions. Despite the existence of multiple cation binding sites for some enzymes [for instance, RNA polymerase binds one Mn (II) tightly and another six or so more loosely (196), and inorganic pyrophosphatase binds two Mn per subunit (197)], it is generally assumed that phosphokinases require at least one divalent cation that complexes directly to phosphoryl group oxygens. A number of studies on model systems have failed to indicate an obviously preferred ligation pattern or to distinguish amongst the various possible catalytic functions of the essential dication. For example, the metal ion of a phosphokinase could coordinate with the α and β phosphoryl groups of ATP, thereby enhancing the effectiveness of ADP as a leaving group. Alternatively, the development of negative charge on the oxygens of the transferring phosphoryl group could be stabilized by metal ligation. In this case there would be a necessary change in the metal ligation pattern during catalysis. Thirdly, the stereochemical demands of ligation symmetry could act as a template in the precise positioning of the oligophosphate chain with respect to other catalytic functionalities. Finally, coordination to two oxygens of the transferred phosphoryl group could result in ground state distortion that would be relieved on going to the trigonal bipyramidal transition state. In order to decide what the cation may be doing, the obvious prerequisite is to discover where it is, and there has been considerable recent activity in this area.
One may naively suppose that the chemical shift of the $^{31}\text{P}$ NMR signal of a metal-ATP complex indicates whether or not a particular phosphate is a metal ligand. Unfortunately, this is not true. Jaffe & Cohn (198) have shown that even the large chemical shift on the $P_\beta$ resonance of ATP that occurs on complexation with Mg cannot be used as evidence that the $\beta$-phosphate oxygen acts as a magnesium ligand. When the $\gamma$-phosphoryl group is protonated, the $P_\gamma$ of $\beta,\gamma$-methylene-ATP moves downfield by 1.4 ppm, yet $P_\beta$ moves upfield by 2.7 ppm. In contrast, when the $\gamma$-phosphoryl group protonates in $\beta,\gamma$-imido-ATP, $P_\gamma$ moves upfield by 1.4 ppm, and $P_\beta$ moves upfield by 4.0 ppm. Analogously unpredictable chemical shifts are seen for the Mg complexes of the various phosphorothioates of ATP (198). Changes in the phosphorus-phosphorus coupling constants also occur on metal ligation of the oligophosphate chain (199), and while useful, these changes are again not independently predictable. The finding, however, that complexation with Mg causes the $^{17}\text{O}$ NMR signal of $[\beta^{17}\text{O}]$-ATP to broaden but has no effect on that of $[\alpha^{17}\text{O}]$-ATP$\alpha$S (M.-D. Tsai, private communication), suggests that $^{17}\text{O}$ NMR may provide a direct method for defining the coordination pattern of nucleotides to cations.

**CATION-DEPENDENT DIASTEREISOISOMER PREFERENCE** In a novel approach to the problem of the structure of metal-nucleotide complexes, Jaffe & Cohn (200, 201) found that whereas the $R_\beta$ diastereoisomer of ATP$\beta$S was preferred by hexokinase when Mg (II) was the cation ($V$ for $R/V$ for $S \sim 600$), the rate ratio was reversed ($R/S \sim 0.03$) when Cd (II) was used as the essential dication. The ligand preference (oxygen versus sulfur) of Mg and Cd is well-known, and it is reasonable that hexokinase should prefer only one geometrical arrangement: Mg·ATP$\beta$S $R$ ($\beta$-oxygen as a ligand) or Cd·ATP$\beta$S $S$ ($\beta$-sulfur as a ligand). This proposal was confirmed by using Co (II) (which will chelate either oxygen or sulfur), with which hexokinase handles both diastereoisomers of ATP$\beta$S. These findings initially suggested that a metal-dependent stereospecificity reversal (e.g. for the diastereoisomers of ATP$\beta$S) is evidence for the involvement of that phosphoryl group in metal ligation during catalysis. Thus the fact that hexokinase prefers the $S_\alpha$ diastereoisomer of ATP$\alpha$S regardless of the activating metal, suggested that in the hexokinase reaction the oxygens of $P_\alpha$ are not ligands for the metal (201). Using this criterion, DNA polymerase I also seems to bind $P_\beta$ and not $P_\alpha$ (124), phosphoglycerate kinase appears to bind $P_\alpha$ and not $P_\beta$, and pyruvate kinase shows metal-dependent diastereoisomer preference at both $P_\alpha$ and $P_\beta$ (202). The picture is not so simple, however, and some interpretative caution is evidently necessary. With RNA polymerase, the $S_\beta$ epimer of ATP$\alpha$S is preferred regardless of the activating metal ion, yet both the $S_\beta$ and $R_\beta$ epimers of ATP$\beta$S are
substrates with Mg (203). Such behavior could be explained if the metal is not bound to the oxygens of $P_a$ and if the binding site of this enzyme is unusually accommodating in terms of the ATP$\beta$S diastereoisomers. But with Cd (II) as the cation, only one isomer of ATP$\beta$S (the $R_p$ isomer) is a substrate (203)! In order to embrace this finding one has to postulate an enzyme-binding locus for the nonliganded peripheral $P_{\beta}$ atom that (for Mg) just balances the Mg preference for oxygen over sulfur. These possibilities are illustrated in Figure 11.

A further surprise in this area has emerged from a study of the interaction of the ATP$\beta$S diastereoisomers with valyl-tRNA synthetase from E. coli (204). In the PP$_i$ exchange reaction (Equation 7a) the $S_p$ isomer is preferred by a factor of about 90, whereas in the aminoacylation reaction (Equation 7a plus b) $R_p$ is preferred by 3.5-fold:

$$e + \text{amino acid} + \text{ATP} \rightleftharpoons e\cdot \text{aminoacyladenylate} + \text{PP}_i \quad 7a.$$  
$$e\cdot \text{aminoacyladenylate} + \text{tRNA} \rightleftharpoons \text{aminoacyl-tRNA} + \text{AMP} + e \quad 7b.$$  

It transpires that the enzyme catalyzes an interchange reaction between ATP$\beta$S $S$ and ATP$\gamma$S, the equilibrium being heavily towards the latter (204). This finding shows that an apparent diastereoisomer preference can be misleading if an interchange reaction intervenes in the reaction of one of the diastereoisomers.

SUBSTITUTION-INERT COMPLEXES OF NUCLEOTIDES  Although the intracellular concentration of free Mg is well above the dissociation constants for Mg·ATP and Mg·ADP so that these nucleotides are mainly present as their metal complexes, the kinetic stability of the complexes is

![Figure 11](https://example.com/f11.png)  

Figure 11  Notional interaction of ATP$\beta$S isomers with RNA polymerase. $\beta$-$\gamma$ metal ligation is assumed. The arrow represents an enzyme-binding interaction that favors O over S (thus balancing the ligand preference of Mg for O over S).
low, $K_{\text{exchange}}$ being around $10^4$ sec$^{-1}$. To overcome this problem Cleland and his group have exploited the kinetically stable aquo-chromium (III) and cobalt (III) ammine complexes to probe the ligation pattern of the cation in phosphokinase reactions (205). Reaction of Cr (III) with ATP produces a $\gamma$-monodentate complex, four isomeric $\beta,\gamma$-bidentate complexes that can be separated from each other on columns of $\beta$-cyclodextrin, and a number of as yet incompletely separated $\alpha,\beta,\gamma$-tridentate complexes (206). There are two diastereoisomeric (bidentate) cobalt tetraammine complexes with ATP (207), and Cornelius & Cleland (208) showed that one of these is a quasi-substrate for hexokinase, though the reaction rate is more than a thousand times slower than with Mg·ATP. The products from an incubation of the pair of Co(NH$_3$)$_4$·ATP complexes with hexokinase and glucose are a product complex of Co(NH$_3$)$_4$·ADP·glucose 6-phosphate and the “inactive” nonutilized diastereoisomer of Co(NH$_3$)$_4$·ATP. These materials can be separated, and addition of hexokinase to the product complex then regenerates the “active” isomer of Co(NH$_3$)$_4$·ATP. This species has a positive circular dichroic spectrum at 550 nm, and it is degraded by periodate (cleaving the ribose ring) and then aniline at pH 5 (eliminating the adenosine) to a diastereoisomeric crystalline complex of trimetaphosphate [Co(NH$_3$)$_4$·PPP] that shows the same circular dichroic behavior (208). Crystallographic analysis (209) (actually carried out on the more accessible nonutilized isomer, after degradation to Co(NH$_3$)$_4$·PPP) showed that hexokinase uses the so-called $\Lambda$ (as distinct from $\Delta$) diastereoisomer. On the basis that the Co (III) takes the place of Mg in the normal reaction, the geometrical arrangement of ATP at the active site of hexokinase would be as shown in Figure 12. Studies with both bidentate Co (III) and bidentate Cr (III) complexes show that hexokinase, glycerol kinase, creatine kinase, and arginine kinase are specific for the $\Lambda$ isomers (210), while pyruvate kinase, phosphofructokinase, adenylate kinase (210), cyclic AMP-dependent protein kinase (211), and phosphoribosylpyrophosphate synthetase (120) preferentially handle the $\Delta$ forms. No kinase handles the $\alpha,\beta,\gamma$-tridentate Cr (III) complexes, though this mixture often inhibits kinases competitively and may induce the binding of the cosubstrate [as with creatine kinase (212)] or a partial reaction [as with pyruvate kinase (199, 199a)]. These results have led to the suggestion (205) that $\beta,\gamma$-bidentate coordination in Mg·ATP results, after phosphoryl transfer, in an enzyme ternary complex where ADP and the phosphorylated cosubstrate are each monodentate ligands for Mg. The metal coordination would then change to give $\alpha,\beta$-bidentate ADP [except with hexokinase and glycerol kinase, where $\beta$-monodentate Mg·ADP is probably the immediate product (210)] allowing the departure of the phosphorylated cosubstrate and the ADP.
The two approaches outlined above, namely the preference for \( \Delta \) or \( \Delta \) isomers of substitution-inert ATP complexes and the metal-dependent preference for phosphorothioate diastereoisomers, each suggest the coordination pattern and the configuration of \( \text{Mg} \cdot \text{ATP} \) at the enzyme's active site. Thus an enzyme that uses the \( \Delta \) isomer of \( \text{Co(NH}_3\text{)}_4 \cdot \text{ATP} \) should prefer the \( \text{Mg} \) (II) complex of \( \text{R}_p \text{ATP}\beta \text{S} \) and the \( \text{Cd} \) (II) complex of \( \text{S}_p \text{ATP}\beta \text{S} \). Since the absolute configurations of the diastereomers of \( \text{ATP}\beta \text{S} \) have been determined independently (62), this prediction can be tested. Gratifying agreement has been found in all those cases for which both the \( \Delta \) versus \( \Delta \) preference of the \( \text{Co(NH}_3\text{)}_4 \cdot \text{ATP} \) or \( \text{Cr} \cdot \text{ATP} \) complexes, and the \( \text{Mg} \cdot \text{ATP}\beta \text{S} \) and \( \text{Cd} \cdot \text{ATP}\beta \text{S} \) preferences, have been determined. [Creatine kinase had seemed to be an exception (205), but more recent work has pulled it into line (P. M. J. Burgers and F. Eckstein, private communication).] It is clear that in such experiments, care must be taken to measure initial rates, and only a true stereospecificity reversal (i.e. a real change in preference for the \( \beta \)-phosphorus epimer of \( \text{ATP}\beta \text{S} \) on changing from \( \text{Mg} \) to \( \text{Cd} \)) can be used in evidence. Further, in experiments with the substitution-inert cations, we are assuming that the trivalent ions occupy the same position as the catalytic \( \text{Mg} \) in the normal reaction, and not some other "spectator" locus. Multiple cation requirements are quite common, and while many kinases accept one isomer of \( \text{Co(NH}_3\text{)}_4 \cdot \text{ATP} \) in the absence of any other metal ion, there are exceptions. Phosphoribosylpyrophosphate synthetase uses the \( \Delta \) isomer only in the presence of \( \text{Mg} \) or \( \text{Mn} \) (120), and although pyruvate kinase (which normally requires two divalent metal ions) binds \( \text{Cr} \cdot \text{ATP} \), this complex cannot catalyze the enolization of pyruvate except in the presence of added \( \text{Mg} \) or \( \text{Mn} \) (213). The picture that has emerged so far, however, is a coherent one, and the assumptions made appear to be valid.

**Catalysis**

Even when the preferred mechanistic route of an enzyme is clear, we are still faced with ascertaining the reasons for the catalytic rate enhancement.

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**Figure 12** Arrangement of substrates at the active site of hexokinase based upon (a) the diastereoisomer of \( \text{Co(NH}_3\text{)}_4 \cdot \text{ATP} \) that is handled by the enzyme, (b) the \( \text{ATP}\beta \text{S} \) diastereoisomer preference in the presence of different divalent metal cations, and (c) the fact that the transferred phosphoryl group suffers inversion of configuration.
First, the facts that phosphokinases are very poor ATPases and that phosphomutases are very poor phosphatases suggest that the catalytic apparatus of these phosphoryl transfer enzymes is not assembled until the acceptor nucleophile is in place and water is absent. The thermodynamic driving force for phosphoryl transfer (say from ATP or from a phosphoenzyme) is considerable, and the phosphoryl group donor must be kinetically protected from the 55 M ambient water until the acceptor binds. This requirement suggests that these enzymes are likely to suffer conformational changes of a classical "induced fit" type (214) on the binding of their substrate(s). That such changes occur is well-documented from both kinetic and structural work, the most detailed structural results being those on hexokinase. Steitz and his group have examined three independent crystal forms of hexokinase: the A isozyme as a glucose complex (215); the unliganded B isozyme dimer (216); and the B isozyme monomer containing bound α-toluoyl-2-glucosamine (217) or bound 8-bromo-ATP (218). The first two structures differ markedly in that one lobe of the bilobed monomer has rotated by some 12° with respect to the other as a result of glucose binding. The glucose molecule is all but engulfed by the protein, leaving a shallow depression in which the ATP may bind. Certainly this model makes ordered substrate addition (glucose leading) attractive, and suggests that the conformational change is necessary catalytically (219). There may, indeed, have to be a further conformational change when ATP binds, since the position of the γ-phosphoryl group of ATP [deduced, it must be said, from model building based on the position of 8-bromo-ATP in the complex with the B isozyme, and the knowledge that it is the A isomer of Co(NH₃)₄·ATP that is a partial substrate for the enzyme (208, 209)] is still 6 Å from the 6-hydroxyl group of glucose (218). In fact, all kinases that have been subjected to crystallographic investigation [in addition to hexokinase, those at high resolution include phosphoglycerate kinase (220), pyruvate kinase (221, 222), phosphofructokinase (223), and adenylate kinase (224, 225), as well as phosphorylase a (226) and b (227)] have marked bilobed structures, and phosphoglycerate kinase (220, 228) has also been shown to undergo a substrate-induced cleft closure (229).

The necessary presence of the phosphoryl group acceptor, whether this be a cosubstrate or an enzymenucleophile, before catalysis can occur, clouds the distinction between pathways A (with the acceptor present), B, and C (Figure 6). For reactions in free solution, there are, of course, detectable and characteristic differences between these paths, and there are different catalytic elements that could accelerate each path (2, 230). But for an enzyme-catalyzed reaction, the problem reduces to one of accelerating a reaction that proceeds via a transition state something like that shown in Figure 13.
Following our earlier formalism (135), the problem of rate enhancement can be divided into two steps: (a) equalization of the internal thermodynamics, and (b) acceleration of the phosphoryl transfer itself (see Figure 14). If the free energies of the bound species are made more nearly equal by differential binding interactions with the enzyme, then by linear free energy relationship arguments, the kinetic barrier to interconversion will be lowered. The fact that the known equilibrium constants for enzyme-bound species are so close to unity (Table 4) supports this view. Now that we have a rather symmetrical transition state flanked by complexes of roughly equal free energy, any further rate-enhancement (i.e. increase in $k_f$) can only be effected by stabilizing the symmetrical transition state and/or by destabilizing both of the flanking intermediates. How this may be done must remain conjectural until more phosphoryl transfer enzymes have been studied at very high resolution crystallographically, but some clues exist. To stabilize the transition state pictured in Figure 13, we clearly need a general acid and a general base, and up to three electrophilic loci to stabilize the peripheral phosphoryl oxygens. The catalytic benefits of general acid catalysts have been well-established in model systems (e.g. 231), and suggestive evidence for the importance of carboxyl groups in a number of phosphoryl transfer enzymes has been obtained (e.g. 217, 232, 233). Candidates for the electrophilic centers shown in Figure 13 are the metal cation, and arginine, lysine, and other hydrogen-bond donors of the protein. An intriguing role for the divalent metal cation has been suggested by Sargeson and co-workers (234), who have proposed, on the basis of model work with a Co (III) complex of $p$-nitrophenyl phosphate, that the much faster hydrolysis rate of the complex than of the free phosphoric ester may derive from the relief of strain that occurs on going from the tetrahedral ground state to the trigonal bipyramidal transition state. This proposal leans on the early experiments of Westheimer's group on 5-membered cyclic phosphoric diesters and tri-

![Figure 13](image.png)

Figure 13 Putative transition state for an in-line displacement (by $R'OH$) at a phosphoric monoester. HA, a general acid; B$, a general base; $\circledast$, positive charges or hydrogen-bond donors.
esters (158, 235–237), and has been called pseudocyclization by Benkovic & Schray (2). There is much evidence from protein modification experiments for the involvement of arginine residues in phosphoryl-transfer enzymes (238–241) and direct interaction with both bridge and peripheral phosphoryl group oxygens is clear from at least two high resolution crystallographic studies (225, 242). Simple complexation of a phosphoric diester with guanidinium ions has been shown to have a modest catalytic effect [(243), though see (244)], and we can expect that enzymes will exploit these bidentate electrophiles to stabilize the transition state for group transfer. Finally, theoretical considerations have led to suggestions that the antiperiplanar orientation of filled oxygen orbitals may assist $P-O$ bond formation and cleavage processes (245, 246), and it will be interesting to see if this phenomenon, which has been so nicely exemplified in carbon chemistry (247), contributes to the chemistry and enzymology of the phosphoric esters.

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916 KNOWLES

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