30), 247 (100); exact mass 578.2025, calcd for $C_{46}H_{26}$ 578.2034.

Variable-Temperature NMR Measurements on Compound 14. Compound **14** (2 mg) was dissolved in 1,1,2,2-tetrachloro[1,2-$^2H_2$]ethane (0.8 mL), and 250-MHz ^{1}H NMR spectra were recorded at a variety of temperatures from 273 to 333 K. The sample was allowed to equilibrate at each of the chosen temperatures for 15 min prior to the recording of the spectrum. Temperatures were measured by means of a thermocouple mounted in the NMR spectrometer probe. Coalescence of the diastereotopic isopropyl methyl resonances of compound **14** was observed at 300 K (see Figure 5). This temperature was verified by measurement of the separation of the hydroxyl and methylene proton resonances in a spectrum of ethylene glycol recorded under the same conditions.[24] The coalescence temperature is considered to be accurate to ±2 K. Given that the peak separation in the absence of exchange is 1.9 Hz, and assuming a transmission coefficient of 1, $\Delta G_c^{\ddagger}$ for the exchange process is 16.7 kcal/mol.[19,20]

X-ray Crystallography. Single crystals of compound **4** were formed upon slow evaporation of a chromatographic fraction containing **4** (solvent, 97:3 hexane-benzene). Single crystals of compound **7** were obtained by recrystallization of chromatographically purified **7** from CH_2Cl_2-MeOH; similarly, chromatographically purified **8** was crystallized by slow evaporation of a hexane-benzene solution. Crystallographic measurements were made by using graphite monochromated Cu Kα radiation (λ = 1.541 78 Å) on a Nicolet R3m difractometer. The data for compounds **4** and **7** were collected at 175 K, but the data for **8** were obtained at room temperature (295 K). The crystallographic data and details of data collection are reported in Table I.

Acknowledgment. Partial support of this work was from Petroleum Research Fund Grant 17157-AC4 and a gift from Alfred Bader, both of which are gratefully acknowledged. We thank James E. Jackson, Bart Kahr, and Silvio Biali for many useful discussions and the last individual also for assistance with the VT-NMR experiments.

Supplementary Material Available: X-ray data for **4**, **7**, and **8**, including atomic coordinates, bond lengths and angles, and anisotropic thermal parameters (13 pages). Ordering information is given on any current masthead page.

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The Effect of Divalent Metal Ions on the Rate and Transition-State Structure of Phosphoryl-Transfer Reactions[1]

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Contribution No. 1617 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254. Received December 12, 1986

Abstract: The divalent metal ions, Mg^{2+} and Ca^{2+}, catalyze the reaction of *p*-nitrophenyl phosphate dianion (PNPP) with substituted pyridines but do not increase the associative character of the transition state, as indicated by values of β_{nuc} in the range 0.17-0.21 for PNPP and for its metal ion complexes. The reactions of phosphorylated morpholinopyridine (MPP) are inhibited approximately twofold by Mg^{2+} and Ca^{2+}. This shows that there is no electrophilic catalysis from the interaction of a metal ion with the phosphoryl oxygen atoms and is consistent with electron donation from the phosphoryl oxygen atoms in a dissociative transition state. The constant value of $\beta_{lg} = -1.02$ to -1.05 for the hydrolysis of three phosphorylated pyridines in the presence and absence of 0.33 M Mg^{2+} and the constant ratio of the rate constants for the reactions of two pyridines with MPP and MPP·Mg also provide no indication of a significant change in transition-state structure upon binding of the metal ion. The dependence of the observed rate constants on the concentration of the metal ions and substrates at ionic strength 1.0 indicates the formation of 1:1 complexes with K_a(PNPP·Mg) = 14.8 M^{-1} and K_a(PNPP·Ca) = 7.5 M^{-1} at 39.2 °C and K_a(MPP·Mg) = 4.4 and 9.1 M^{-1} at 25.1 and 52.3 °C, respectively. The three- to sixfold catalysis by Mg^{2+} and Ca^{2+} of the reaction of PNPP with pyridines contrasted, with the inhibition of reactions with uncharged leaving groups, suggests that the bound metal ion interacts with the phenoxide ion leaving group as well as the phosphoryl oxygen atoms in the transition state. 2,4-Dinitrophenyl phosphate dianion (DNPP) binds Ca^{2+} and Mg^{2+} with kinetically determined association constants of 6.0 and 6.2 M^{-1}, respectively, at 39.1 °C and ionic strength 1.0, and DNPP·Ca reacts with pyridines sevenfold faster than free DNPP. The constant value of β_{nuc} for the reactions of DNPP and DNPP·Ca with two substituted pyridines again gives no evidence for a significant change in nucleophilic participation upon complexation with a divalent metal ion. The reactions of DNPP·Mg with pyridines are at least eightfold slower than the reactions of free DNPP because of an unfavorable interaction of Mg^{2+} and the ortho nitro group of DNPP in the transition state. The effects of Mg^{2+} and Ca^{2+} on the rate constants for hydrolysis follow the same trends as for the reactions with pyridines, except that the hydrolysis of PNPP·Mg is 20% slower than that of PNPP.

The mechanisms employed by enzymes to catalyze phosphoryl-transfer reactions are not understood. It has often been suggested that complexation with a metal ion could change the mechanism of phosphoryl transfer from the largely dissociative mechanism observed in the absence of a metal ion to an associative mechanism, with more nucleophilic involvement in the transition state.[2-4] A change from a dissociative to an associative mechanism would allow a larger potential advantage from catalysis by induced intramolecularity in an enzymatic reaction because reactions with "tight" transition states have stricter requirements for alignment than reactions with "loose" transition states.[5] In addition, a change to an associative mechanism would give a greater potential advantage from general base catalysis due to the increased bond formation to the nucleophile in the transition state and concomitant charge development on the nucleophile. We have used a nonenzymatic system to test whether or not complexation with a metal

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(1) Supported in part by grants from the National Institutes of Health (GM20888 and AM07251) and the National Science Foundation (PCM81-17816). D.H. was supported by a Fellowship of the Gillette Foundation.

(2) See, for example: Mildvan, A. S.; Fry, D. C. *Adv. Enzymol.* **1987**, *59*, 241-313. Mildvan, A. S. *Adv. Enzymol.* **1979**, *49*, 103-126. Mildvan, A. S.; Grisham, C. M. *Struct. Bond.* **1974**, *20*, 1-21. Benkovic, S. J.; Schray, K. J. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York; 1973; Vol. 8, pp 201-238. Williams, A.; Naylor, R. A. *J. Chem. Soc. B* **1971**, 1973-1979.

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ion causes this change to an associative mechanism for phosphoryl transfer.

Reactions of phosphate monoester dianions and related compounds exhibit a small amount of bond making and a large amount of bond breaking in the transition state, as indicated by small values of β_{nuc}, large negative values of β_{lg}, near zero values of $\Delta S^{\ddagger}$ and $\Delta V^{\ddagger}$, and a large bridge ^{18}O isotope effect.[4,6-12] These results characterize the transition state for phosphoryl transfer as dissociative and metaphosphate-like. However, we are aware of no evidence for the formation of a metaphosphate intermediate in aqueous solution,[13] and there is evidence against the formation of a metaphosphate intermediate in phosphoryl transfer from phosphorylated pyridines and isoquinoline to pyridines.[11,12]

It might have been expected that binding of metal ions would change the transition state for phosphate monoester hydrolysis to the associative transition state that is observed with alkyl substitution on the phosphoryl oxygen atoms. This change toward associative character with alkyl substitution is shown by the values of β_{nuc} of 0.0, 0.3, and 0.6 for reactions of substituted pyridines with a phosphate monoester dianion, a phosphate diester monoanion, and a neutral phosphate triester, respectively, each with 2,4-dinitrophenolate ion as the leaving group.[6,14,15] Bronsted-type correlations for the reactions of dinitrophenyl phosphate and bis-2,4-dinitrophenyl phosphate monoanions with substituted pyridines have slopes of $\beta_{nuc} = 0.56$ and 0.54, respectively.[6] These large, similar values of β_{nuc} suggest that protonation of a monoester can also change the reaction mechanism to one with a more associative transition state that is typical for phosphate diesters.

The rapid hydrolysis of phosphate monoester monoanions relative to the uncharged monoester[16,17] and of monoester dianions relative to diester monoanions[18] suggests that the driving force for the facile phosphoryl transfer from phosphate monoesters is largely electron donation from the phosphoryl oxygen atoms in a transition state with dissociative character. Binding of a metal ion to the phosphoryl oxygen atoms might be expected to influence the transition state for phosphoryl transfer by the following: hindering electron donation from the oxygen atoms, thereby removing this driving force for the dissociative mechanism; facilitating the development of negative charge on the phosphoryl oxygen atoms, thereby stabilizing the transition state for the associative mechanism; and polarizing the phosphoryl P–O bond, thereby rendering the phosphorus atom more susceptible to nucleophilic attack.

The tendency for the transition state to become more associative upon binding of a metal ion can be illustrated by the reaction coordinate-energy diagram shown in Figure 1. The small values of β_{nuc} and the large, negative values of β_{lg} observed for reactions of phosphoryl substrates place the transition state near the upper right corner of Figure 1, with significant diagonal character of the reaction coordinate.[11] Binding of a metal ion should stabilize the lower left corner of the diagram and destabilize the upper right corner relative to reactants and products, as indicated by the bold arrows, because the electron density on the phosphoryl oxygen atoms is increased in the lower left corner and decreased in the upper right corner relative to reactants and products. This perturbation would tend to slide the transition state perpendicular to the reaction path toward the position of lower energy and the associative transition state in the lower left corner, giving an "anti-Hammond" effect as shown by the dotted arrow.[19]

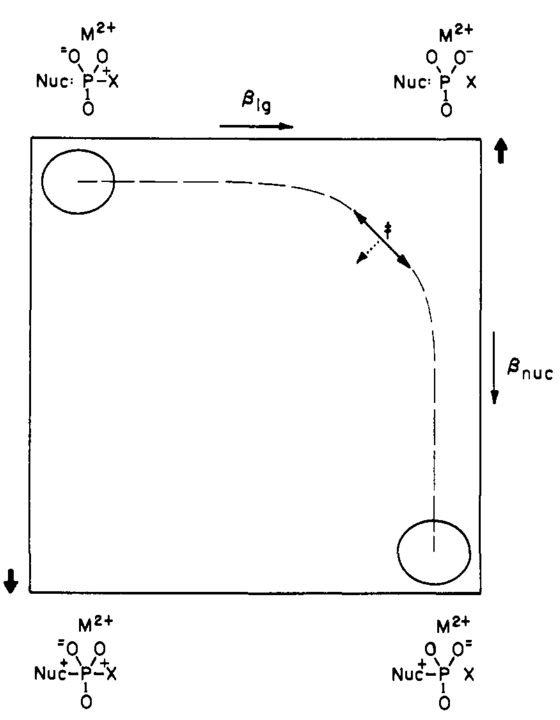
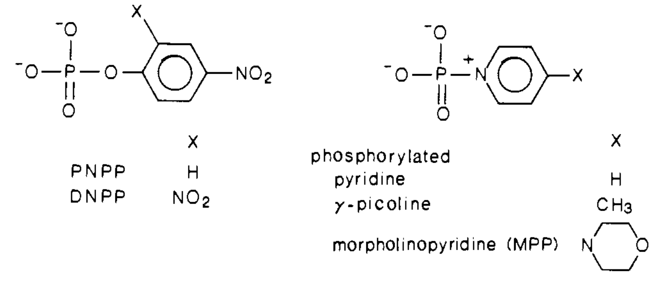


Figure 1. Reaction coordinate–energy contour diagram for phosphoryl transfer. The position of the transition state in the absence of bound metal ion is indicated by ‡. The bold arrows show the perturbation to the energy surface from the binding of a metal ion, and the dotted arrow shows the motion of the transition state that might be predicted from such a perturbation.

We have determined whether or not two biologically relevant divalent metal ions increase the amount of nucleophilic participation in reactions of phosphoryl compounds by measuring β_{nuc} in the presence and absence of the metal ions. Phosphate ester dianions, PNPP and DNPP,[20] and phosphorylated pyridine mo-



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(18) See Figure 3 of ref 14.

(19) (a) Jencks, D. A.; Jencks, W. P. *J. Am. Chem. Soc.* **1977**, *99*, 7948–7960. Jencks, W. P. *Chem. Rev.* **1985**, *85*, 511–527. (b) If metal ions bound more favorably to compounds with tetrahedral geometry than to compounds (or transition states) with trigonal bipyramidal geometry, then the lower left corner of Figure 1 might not be lowered in energy relative to reactants and products upon binding of metal ion. However, the energy of the lower left corner should still be lowered relative to that of the upper right corner because the species in these corners have similar trigonal bipyrimidal geometries and there is more charge density on the phosphoryl oxygen atoms in the lower left corner. This situation would lead to the same predicted change in the structure of the transition state as described in the text.

(20) The following abbreviations are used: PNPP, *p*-nitrophenyl phosphate dianion; DNPP, 2,4-dinitrophenyl phosphate dianion; MPP, phosphorylated morpholinopyridine monoanion; M^{2+}, divalent metal ion; Tris, tris(hydroxymethyl)aminomethane; CHES, 2-cyclohexylaminoethanesulfonic acid; en, 1,2-ethylenediamine.

noanions were studied because the reactions of these simple compounds are analogous to biological phosphoryl-transfer reactions, but the phenoxide and pyridine leaving groups are less likely than leaving groups such as ADP to interfere with the interaction of the metal ion and the phosphoryl group.

We have found that Mg^{2+} and Ca^{2+} have no significant effect on the amount of nucleophilic participation in reactions of these compounds. Furthermore, inhibition by metal ions of the reactions of phosphorylated pyridines is consistent with weaker binding of the metal ion to the transition state than to the ground state as a result of electron donation from the phosphoryl oxygen atoms in the transition state. Catalysis by metal ions of the reactions of aryl phosphate monoesters with uncharged nucleophiles is, therefore, attributed to an interaction of the bound metal ion with the leaving phenoxide anion in the transition state.[21]

Experimental Section

Materials. Substituted pyridines and disodium PNPP were purified by distillation or recrystallization. Morpholinopyridine was a gift from Dr. Mark Skoog, and DNPP was a gift from Prof. David Chipman. Aqueous solutions of MPP and phosphorylated pyridine were prepared as described previously.[11] Aqueous solutions of phosphorylated γ-picoline were also prepared by this method but with the addition of $\sim$3 equiv of $POCl_3$ to a solution of 0.15 M γ-picoline and 1.5 M KOH; addition of this amount of $POCl_3$ is insufficient to achieve a quantitative yield of phosphorylated γ-picoline but avoids the formation of precipitates in reaction mixtures at pH $\sim$9 in the presence of Mg^{2+}.

Kinetic Measurements. Reactions of PNPP at 39.2 ± 0.2 °C and DNPP at 39.1 ± 0.2 °C were followed spectrophotometrically at 410 and 360 nm, respectively. Reactions of MPP at 25.1 ± 0.1 and 52.3 ± 0.1 °C and of phosphorylated pyridine and phosphorylated γ-picoline at 25.1 °C were followed at 303–305, 262, and 256 nm, respectively. Pseudo-first-order rate constants for reactions of PNPP were determined from initial rates; the concentrations of stock solutions of PNPP were determined from the absorbance of p-nitrophenolate ion after acid hydrolysis.[4,22] The reactions of DNPP, MPP, phosphorylated pyridine, and phosphorylated γ-picoline were first order for $>3t_{1/2}$; endpoints were determined after $\geq 10t_{1/2}$. The endpoints for reactions of MPP in the absence of added nucleophile were usually obtained after incubation of the reaction mixture at 100 °C for 25 min; additional incubation did not affect the observed endpoint.

Reactions of 5×10^{-5} M DNPP, 1×10^{-4} M MPP, 5×10^{-4} M phosphorylated pyridine, and 2×10^{-4} M phosphorylated γ-picoline were generally followed by using 1-cm cuvettes; reactions of 1.2×10^{-3} M PNPP and reactions of 2×10^{-5} M MPP in the presence of Ca^{2+} were followed by using 5-cm cuvettes in order to avoid the formation of precipitates. Solutions used in reactions followed with a 5-cm pathlength were passed through 0.45 μ-Millipore filters to remove dust. Precipitates were not observed in reaction mixtures. The ionic strength was maintained at 1.0 or 1.5 with potassium chloride unless otherwise noted. The pH values of reaction mixtures were measured at 25 °C.

Association Constants. Apparent association constants for metal ion and substrate were obtained from the nonlinear least-squares fit of the observed rate constants according to

$$k^{app} = (k_w + k[\mathrm{Nuc}])/(1 + K_a[\mathrm{M}^{2+}]) + (k_w^{M} + k^{M}[\mathrm{Nuc}])K_a[\mathrm{M}^{2+}]/(1 + K_a[\mathrm{M}^{2+}]) \quad (1)$$

This equation was derived from Scheme I and the rate equation

$$v = (k_w + k[\mathrm{Nuc}])[{}^{2-}\mathrm{O_3PXR}] + (k_w^{M} + k^{M}[\mathrm{Nuc}])[\mathrm{M \cdot O_3PXR}] = k^{app}[{}^{2-}\mathrm{O_3PXR}]_{tot}$$

The observed rate constant for the free substrate, $k_w + k[\mathrm{Nuc}]$, was determined in the absence of divalent metal ion; the association constant, $K_a = [\mathrm{RXP \cdot M}]/[\mathrm{RXP}^{2-}][\mathrm{M}^{2+}]$, and the rate constant for the metal-ion–substrate complex, $k_w^{M} + k^{M}[\mathrm{Nuc}]$, were variable parameters. Values for the association constant were determined from the nonlinear least-squares fit of the data obtained with varying metal-ion concentration and a single concentration of each nucleophile. The values were averaged to obtain the association constants reported in Table II. The average value of the standard deviations of the association constants for each metal-ion–substrate complex determined with different nucleophiles was $\sim$10%; the quality of each fit is shown by the calculated lines in Figures 3, 4, 5, and 6. Computer programs for nonlinear least-squares fits to the data were kindly provided by Dr. Neil Stahl.[23]

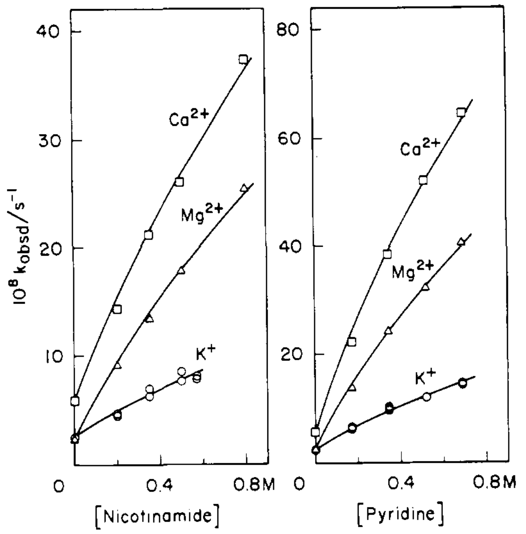
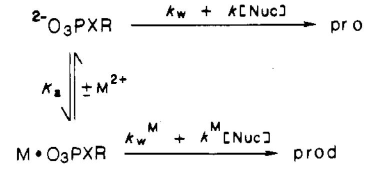


Figure 2. The effect of nucleophile concentration on the metal-ion-catalyzed hydrolysis of PNPP: observed rate constants for the hydrolysis of 1.2×10^{-3} M PNPP in the presence of 1.0 M KCl (○), 0.33 M $MgCl_2$ (△), or 0.33 M $CaCl_2$ (□) with 0.04 M CHES buffer, pH 9.1, at 39.2 °C. The lines are nonlinear least-squares fits to the data that account for the apparent self-association of nicotinamide and pyridine, with self-association constants of 0.35 and 0.5 M^{-1}, respectively, that were determined at 39 °C and ionic strength 1.0 (KCl) (ref 4).

Scheme I



Results

PNPP. The dependence of the rate constants for hydrolysis of PNPP on the concentration of pyridine and nicotinamide in the presence and absence of 0.33 M Mg^{2+} or Ca^{2+} is curved, as shown in Figure 2. The lines show nonlinear least-squares fits to the data with use of literature values for the apparent self-association of the pyridines[4] that are better than the fits to straight lines obtained without this correction to free nucleophile concentration. The second-order rate constants for reaction of the free nucleophile[4] obtained for nicotinamide and pyridine and data obtained similarly for γ-picoline show that Mg^{2+} and Ca^{2+} catalyze the reactions of PNPP with pyridines (Table I).

The rate constant for the reaction of water with PNPP dianion in the absence of divalent metal ion (Table I) was obtained by extrapolation to 100% dianion in order to correct for the contribution (<30%) of the hydrolysis of PNPP monoanion in the pH range 8.3–9.8. In the presence of 0.33 M Mg^{2+} or Ca^{2+} no correction was made for the contribution (<3%) from the hydrolysis of PNPP monoanion to the observed rate constant for hydrolysis in the pH range 8.8–9.8 for Mg^{2+} and 8.4–9.4 for Ca^{2+}. However, in the presence of divalent metal ion a reaction that is first order in hydroxide ion was observed over these pH ranges, with rate increases of about 300% and 50% for 0.33 M Mg^{2+} and Ca^{2+}, respectively.[24] The rate constants for the water reaction in the presence of 0.33 M Mg^{2+} or Ca^{2+} were obtained by extrapolation to zero hydroxide ion concentration and are listed in Table I. For both metal ions the extrapolation from the observed rate constant at the lowest pH investigated to the pH-independent

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(21) A preliminary account of a portion of this work has been presented: Herschlag, D.; Jencks, W. P. *Fed. Proc.* **1986**, *45*, 1632.

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Table I. Rate Constants for the Reactions of *p*-Nitrophenyl Phosphate Dianion and Its Mg^{2+} and Ca^{2+} Complexes with Substituted Pyridines and Water[a]

metal ion	nucleophile	10^7k^{app},[b] $M^{-1}\ s^{-1}$	10^7k,[c] $M^{-1}\ s^{-1}$
none	water		1.7×10^{-8} [d]
	nicotinamide		1.30
	pyridine		2.63
	γ-picoline		3.9
Mg^{2+}	water	1.4×10^{-8} [d]	1.4×10^{-8} [d]
	nicotinamide	4.0	4.5
	pyridine	8.0	9.1
	γ-picoline	11.3	12.8
Ca^{2+}	water	3.9×10^{-8} [d]	4.8×10^{-8} [d]
	nicotinamide	5.3	6.9
	pyridine	12.3	16.2
	γ-picoline	18.4	24.2

[a] At 39.2 °C, ionic strength 1.0 (KCl) with 0.04 M CHES, pH 9.1, except for the water reactions, for which the pH was varied to extrapolate to pH-independent rate constants (see text). Rate constants for reactions with substituted pyridines were corrected for the apparent self-association of the pyridine using K_{sa} = 0.35, 0.5, and 3 M^{-1} for nicotinamide, pyridine, and γ-picoline, respectively, determined at 39 °C and ionic strength 1.0 (ref 4). [b] Observed second-order rate constants in the presence of 0.33 M Mg^{2+} or Ca^{2+}, except as noted. [c] Second-order rate constants for reaction of PNPP or PNPP·M; the rate constants for PNPP·M were obtained from the observed rate constants in the presence and absence of 0.33 M divalent metal ion and the K_a values for PNPP and M^{2+}. [d] First-order rate constants, with units s^{-1}.

rate constant was <10%. The rate constants in Table I for reactions without metal ion agree satisfactorily with literature values.[4]

The dependence of the rate constant for hydrolysis of PNPP on the concentration of Mg^{2+} and Ca^{2+} in the presence of 0.50 M nicotinamide, 0.35 M pyridine, and 0.30 M γ-picoline is shown in Figure 3. The association constants in Table II for complexation of PNPP with metal ions were obtained from these data prior to normalization, as described in the Experimental Section; the lines in Figure 3 are nonlinear least-squares fits for binding of a single metal ion with use of these association constants. These association constants are consistent with less precise estimations from small changes in the absorbance of PNPP at 400 nm in the presence of varying concentrations of metal ion (data not shown). Varying the initial concentration of PNPP over a fourfold range, (0.6–2.4) × 10^{-3} M, was found to have no effect on the observed rate constant in the presence of 0.35 M pyridine and 0.33 M Ca^{2+} (data not shown); this shows that the reaction is first order in PNPP. The observed rate constants were found to be independent of the concentration of CHES buffer.

The first-order rate constants for hydrolysis and second-order rate constants for the reaction of substituted pyridines with the 1:1 metal-ion–PNPP complexes, k^M, were calculated according to Scheme I and eq 1 from the association constants in Table II and the rate constants in the presence and absence of 0.33 M divalent metal ion, k^{app} and k. The rate constants for the complexes as well as those in the absence of metal ion are given in the last column of Table I.

DNPP. Second-order rate constants for the reactions of DNPP with nicotinamide and pyridine in the presence and absence of 0.33 M Mg^{2+} or Ca^{2+}, which have been corrected for the apparent self-association of the nucleophile,[4] are listed in Table III. The rate constant for hydrolysis was found to be independent of pH in the range pH 8.0–10.7 in the presence of 0.33 M Ca^{2+} with Tris and CHES buffers and in the range pH 7.5–9.4 in the presence of 0.33 M Mg^{2+} with the same buffers; first-order rate constants for hydrolysis are also listed in Table III. Calcium ion catalyzes reactions of DNPP whereas Mg^{2+} inhibits these reactions. The first-order rate constant and uncorrected second-order rate constants for reactions with pyridines in the absence of divalent metal ion agree with literature values;[6,7] the second-order rate constants corrected for apparent self-association of the pyridine nucleophiles are about 30% larger.

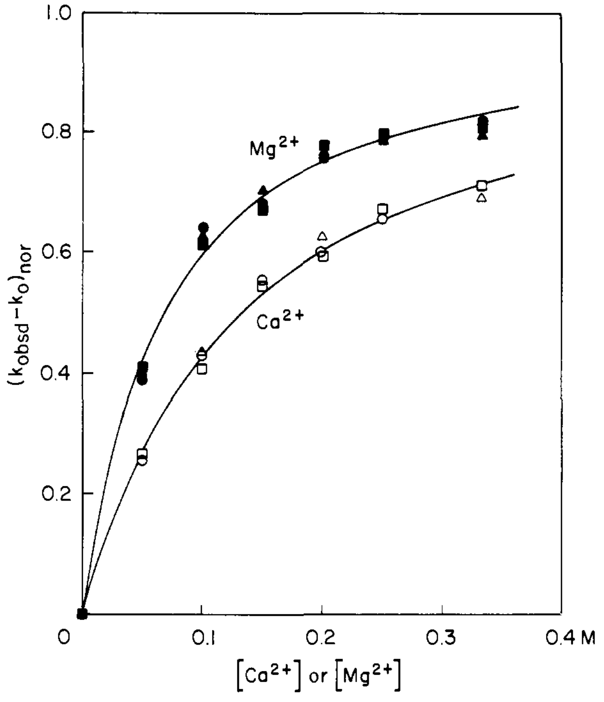


Figure 3. The effect of divalent metal-ion concentration on the observed rate constant for hydrolysis of PNPP: normalized rate constants for the hydrolysis of 1.2 × 10^{-3} M PNPP with varying concentrations of $CaCl_2$ (open symbols) and $MgCl_2$ (closed symbols) in the presence of 0.50 M nicotinamide (△, ▲), 0.35 M pyridine (○, ●), or 0.30 M γ-picoline (□, ■) with 0.04 M CHES buffer (pH 9.2 with Ca^{2+} and pH 9.0 with Mg^{2+}) at ionic strength 1.0 (KCl) and 39.2 °C. The rate constant k_0 is the observed rate constant in the absence of added divalent metal ion and a value of 1.0 for $(k_{obsd} - k_0)_{nor}$ corresponds to the rate constant for the PNPP·M complex. The rate constants for reaction of the PNPP·M complexes with each nucleophile were estimated from nonlinear least-squares fits of the dependence of k_{obsd} on the concentration of divalent metal ion by using the association constants in Table II, and the lines are these theoretical fits to the data. The increases in the observed rate constants from 0 to 0.33 M divalent metal ion were approximately fourfold for Ca^{2+} and greater than twofold for Mg^{2+}. The contribution of the hydroxide-ion-catalyzed reaction was <5% in all cases.

Table II. Kinetically Determined Association Constants for Phosphorylated Substrates with Mg^{2+} and Ca^{2+} [a]

substrate	metal ion	K_a,[b] M^{-1}
PNPP[c]	Mg^{2+}	14.8 (0.7)
PNPP[c]	Ca^{2+}	7.5 (0.8)
DNPP[d]	Mg^{2+}	6.2 (1.1)
DNPP[e]	Ca^{2+}	6.0 (0.6)
MPP[f]	Mg^{2+}	4.6[g] (0.8)
MPP[h]	Mg^{2+}	9.1[i]

[a] Ionic strength 1.0 (KCl) and 39 °C, unless otherwise noted. [b] Determined as described in the Experimental Section. The values are averages of the association constants determined with several nucleophiles; the values in parentheses are standard deviations. [c] Reactions with 0.50 M nicotinamide, 0.35 M pyridine, and 0.30 M γ-picoline were used for the determination of K_a; conditions as in Figure 3. [d] Reactions with 0.27 M pyridine and no added nucleophile were used for the determination of K_a; conditions as in Figure 5. [e] Reactions with 0.25 M nicotinamide, 0.27 M pyridine, and no added nucleophile were used for the determination of K_a; conditions as in Figure 4. [f] Reactions with 0.33 M nicotinamide and 0.35 M pyridine were used for the determination of K_a; conditions as in Figure 6. [g] at 25.1 °C. [h] Reaction with 0.35 M pyridine was used for the determination of K_a; conditions as in Figure 6. [i] At 52.3 °C.

Figure 4 shows the dependence on Ca^{2+} concentration of the rate constant for DNPP hydrolysis in the absence of added nucleophile and in the presence of 0.25 M nicotinamide or 0.27 M pyridine; Figure 5 shows the dependence on Mg^{2+} concentration in the presence and absence of 0.27 M pyridine. The association constants for complexation of DNPP with Ca^{2+} and Mg^{2+} determined from the data of Figures 4 and 5 are listed in Table II,

Table III. Rate Constants for the Reactions of 2,4-Dinitrophenyl Phosphate Dianion and Its Mg^{2+} and Ca^{2+} Complexes with Substituted Pyridines and Water[a]

metal ion	nucleophile	10^3k^{app},[b] M^{-1} s^{-1}	10^3k,[c] M^{-1} s^{-1}
none	water		1.90×10^{-4}[d]
	nicotinamide		0.77
	pyridine		0.86
Mg^{2+}	water	5.7×10^{-5}[d]	$<2 \times 10^{-5}$[d]
	nicotinamide	0.27	<0.1
	pyridine	0.31	<0.1
Ca^{2+}	water	8.2×10^{-4}[d]	11.4×10^{-4}[d]
	nicotinamide	4.2	5.9
	pyridine	4.4	6.2

[a] At 39.1 °C, ionic strength 1.0 (KCl) with 0.05 M Tris, pH 8.0. Rate constants for reactions with nicotinamide and pyridine were corrected for their apparent self-association by using $K_{sa} = 0.35$ and 0.5 M^{-1}, respectively, which were determined at 39 °C and ionic strength 1.0 (ref 4). [b] Observed rate constant in the presence of 0.33 M Mg^{2+} or Ca^{2+}. [c] Second-order rate constant for the reaction of DNPP or DNPP·M; the rate constants for DNPP·M were obtained from the observed rate constants in the presence and absence of 0.33 M divalent metal ion and the values of K_a for DNPP and M^{2+}. [d] First-order rate constants, with units s^{-1}.

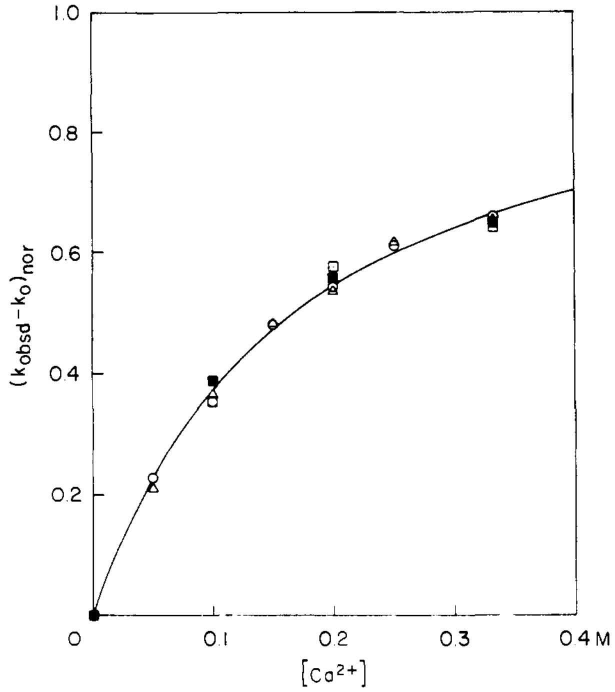


Figure 4. The effect of Ca^{2+} concentration on the observed rate constants for hydrolysis of DNPP: normalized rate constants for the hydrolysis of 4×10^{-5} M DNPP in the presence of varying concentrations of $CaCl_2$ and 0.25 M nicotinamide (△), 0.27 M pyridine (○), or no added nucleophile (□ and ■ at pH 7.6) with 0.05 M Tris buffer pH 8.0, at ionic strength 1.0 (KCl) and 39.1 °C. Rate constants were normalized as described in Figure 3, and the line is a nonlinear least-squares fit to the data with use of the association constant in Table II. The observed rate constants increased by greater than fourfold from 0 to 0.33 M Ca^{2+}.

and the lines in Figures 4 and 5 are nonlinear least-squares fits to the data with use of these association constants. Rate constants for the reactions of the metal-ion–DNPP complexes, determined according to Scheme I and eq 1 as for the analogous PNPP complexes, are listed in Table III. Only upper limits of the rate constants for reactions of the DNPP·Mg complex could be determined because of the large decrease in the observed rate constants in the presence of Mg^{2+}.

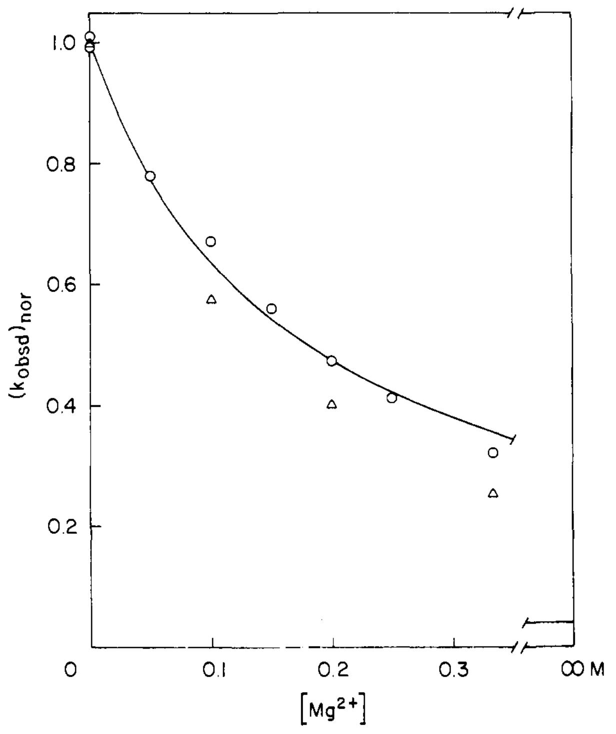


Figure 5. The effect of Mg^{2+} concentration on the observed rate constants for hydrolysis of DNPP: normalized rate constants for the hydrolysis of 4×10^{-5} M DNPP in the presence of varying concentrations of $MgCl_2$ and 0.27 M pyridine (○) or no added nucleophile (△) with 0.05 M Tris buffer, pH 8.0, at ionic strength 1.0 and 39.1 °C. Rate constants were normalized differently than in Figures 3 and 4 because of the inhibition by Mg^{2+}: the rate constant observed at each concentration of Mg^{2+} was divided by the rate constant observed in the absence of added Mg^{2+}, so that $(k_{obsd})_{nor} = 1$ corresponds to the rate constant in the absence of Mg^{2+} and $(k_{obsd})_{nor} = 0$ corresponds to an observed rate constant of 0. The line is a nonlinear least-squares fit to the observed rate constants in the presence of pyridine (○) with use of the association constant in Table II. The fit to the data in the absence of added nucleophile is not shown.

First-order kinetic plots were found to be linear for $>3t_{1/2}$ in the presence of Mg^{2+} and Ca^{2+}, which shows that the reactions are first order in DNPP. The following suggest that the buffers do not affect reactions of the metal-ion–substrate complex: the observed rate constants for the hydrolysis of DNPP in the presence of 0.33 M Ca^{2+} or Mg^{2+} were found to be the same in 0.05 M Tris buffer and in 0.04 M CHES buffer at several ratios of buffer components; the observed rate constant of the pyridine-catalyzed reaction in the presence of 0.33 Mg^{2+} was found to be the same in CHES and pyridine buffers; and the observed association constant for DNPP and Ca^{2+} was found to be the same at two different ratios of buffer components, Tris/TrisH$^+$ = 0.85 and 0.25 (Figure 4, open and closed squares). The absence of a significant difference between the association constants for DNPP and metal ion determined from the reaction with pyridines and from the reaction with water (Figure 4; Table II) suggests that there is no significant coordination of the pyridines with Ca^{2+} and Mg^{2+} that affects the reaction.

Phosphorylated Pyridines. Mg^{2+} and Ca^{2+} inhibit the reactions of phosphorylated pyridines with pyridines and with water, in contrast to the catalysis seen for most of the reactions of aryl phosphates. The rate constant for hydrolysis of MPP in the presence of 0.35 M pyridine decreases sharply with increasing concentration of Mg^{2+} (Figure 6, squares). This decrease is significantly more marked than that observed with increasing concentration of potassium chloride, even after dividing the concentration of potassium chloride by three to account for the difference in ionic strength (Figure 6, circles). This suggests that Mg^{2+} binds to MPP and inhibits hydrolysis. The steeper rate decrease with increasing concentration of Mg^{2+} when ionic strength is not held constant than at ionic strength 1.0 (Figure 6, triangles and squares) is consistent with stronger binding of Mg^{2+} to MPP at lower ionic strength, as expected for an ionic interaction.

The association constants for the complex of MPP with Mg^{2+} at 25.1 and 52.3 °C given in Table II were determined from the

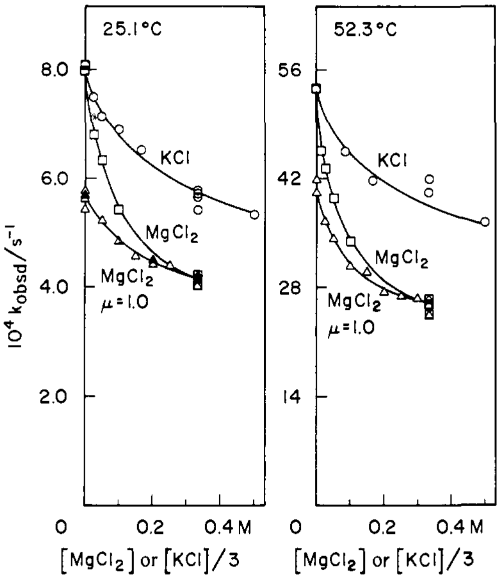


Figure 6. The effect of Mg^{2+} concentration on the observed rate constants for hydrolysis of MPP: rate constants for hydrolysis of 1×10^{-4} M MPP with varying concentrations of $MgCl_2$ in the presence of 0.35 M pyridine and 0.05 M Tris, pH 7.7 (open symbols) or 8.1 (closed symbols), at ionic strength 1.0 (KCl) (△, ▲), without control of ionic strength (□), and with varying concentrations of KCl in the absence of Mg^{2+}, without control of ionic strength (○) at 25.1 and 52.3 °C. The bottom lines are nonlinear least-squares fits to the data with use of the association constants in Table II.

data of Figure 6 at ionic strength 1.0 and from similar data with 0.33 M nicotinamide at 25.1 °C, as described in the Experimental Section; the bottom lines in Figure 6 are least-squares fits to the dependence on Mg^{2+} concentration with use of these association constants. Values of $\Delta H = +5$ kcal/mol and $\Delta S = +20$ eu for the association of Mg^{2+} and MPP were calculated from these association constants. Positive values of ΔS for association of ions are expected as a result of the release of water of solvation from the ions[25] and have been observed, for example, in the binding of Mg^{2+} to methyl phosphate and sulfate dianions and to AMP^{2-}, ADP^{3-}, and ATP^{4-}.[26] In contrast to the temperature dependence of the kinetically determined association constant for Mg^{2+} and MPP, the effect of potassium chloride concentration on the rate of MPP hydrolysis is the same at 25.1, 45.1, and 52.3 °C (Figure 6 and data not shown).

Saturation behavior was also observed for catalysis by Mg^{2+} of the reactions of anionic nucleophiles with MPP; the same association constant, within error, was obtained from these data at 25.1 °C and ionic strength 1.5 as from the rate inhibition data of Figure 6.[24] We conclude that the inhibition by Mg^{2+} at constant ionic strength results from binding of Mg^{2+} to MPP.

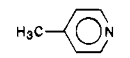
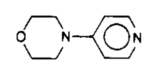
Second-order rate constants for the hydrolysis of MPP catalyzed by pyridine or nicotinamide in the presence and absence of 0.33 M Mg^{2+} and by pyridine in the presence of 0.33 M Ca^{2+} were determined at 25.1 °C and ionic strength 1.0 (Table IV). These rate constants were derived directly from the observed rate constant at a single concentration of 0.35 M pyridine or 0.33 M nicotinamide. No correction was made for the contribution of <1% to the observed rate constant from the pyridine-independent reaction and from catalysis by Tris buffer and hydroxide ion. Second-order rate constants for the pyridine-catalyzed reaction in the presence and absence of 0.33 M Mg^{2+} at 52.3 °C were derived similarly (Table IV). These rate constants provide a direct measure of the effect of metal ions. The rate constants obtained at 25.1 °C in the absence of divalent metal ion agree with literature values.[11]

Table IV. Rate Constants for Reactions of Phosphorylated Morpholinopyridine and Its Mg^{2+} and Ca^{2+} Complexes with Substituted Pyridines and Water at 25.1 and 52.3 °C[a]

metal ion	nucleophile	$10^4 k^{app}$,[b] M^{-1} s^{-1}	$10^4 k$,[c] M^{-1} s^{-1}
	25.1 °C		
none			
	nicotinamide		5.6
	pyridine		21
Mg^{2+}			
	nicotinamide	3.8	2.7
	pyridine	15.7	12.0
Ca^{2+}			
	pyridine	16.7	*d*
	52.3 °C		
none			
	pyridine		149
Mg^{2+}			
	pyridine	94	77

[a] Ionic strength 1.0 (KCl), with 0.05 M Tris buffer, pH 7.7 and 8.1. Rate constants for reactions with nicotinamide and pyridine were corrected for their apparent self-association by using $K_{sa} = 0.35$ and 0.5 M^{-1}, respectively, which were determined at 39 °C and ionic strength 1.0 (ref 4). [b] Observed second-order rate constants in the presence of 0.33 M Mg^{2+} or Ca^{2+}. [c] Second-order rate constants for reaction of MPP or MPP·M; the rate constants for MPP·M were obtained from the observed rate constants in the presence and absence of 0.33 M divalent metal ion and the value of K_a for MPP and M^{2+}. [d] A value of K_a for MPP and Ca^{2+} was not determined so that rate constants for the reaction of MPP·Ca could not be calculated.

Table V. Rate Constants for the Hydrolysis of Phosphorylated Substituted Pyridines at 25.1 °C and Ionic Strength 1.5

		k_{obsd}, s^{-1}	
leaving group	pK_a	no Mg^{2+} [a]	0.33 M Mg^{2+} [b]
	5.51[c]	10.7×10^{-3}	8.0×10^{-3} [d]
	6.33[c]	1.59×10^{-3}	1.11×10^{-3} [e]
	9.01[f]	3.00×10^{-6}	1.77×10^{-6} [g]

[a] In the presence of 1.5 M KCl and 0.05 M CHES buffer, pH 8.2. [b] In the presence of 0.33 M $MgCl_2$, 0.5 M KCl, and 0.05 M CHES buffer, pH 7.7–8.6, the rate constants were extrapolated to zero hydroxide ion concentration (see text). [c] At 25 °C in 1 M KCl, from Satterthwait and Jencks (Satterthwait, A. C.; Jencks, W. P. *J. Am. Chem. Soc.* **1974**, *96*, 7031–7044). [d] The extrapolation corrected for a contribution of ~2–25% to the observed rate constant from the hydroxide-ion-catalyzed reaction. [e] The extrapolation corrected for a contribution of ~10–50% to the observed rate constant from the hydroxide-ion-catalyzed reaction. [f] At 25 °C in 1 M KCl, from ref 11. [g] The extrapolation corrected for a contribution of ~50–300% to the observed rate constant from the hydroxide-ion-catalyzed reaction.

A rate constant of 12.4×10^{-4} M^{-1} s^{-1} for the reaction of the MPP·Mg complex with pyridine at 25.1 °C was calculated from the rate constants in the presence and absence of 0.33 M Mg^{2+} (Table IV) and K_a (Table II) according to Scheme I and eq 1. A rate constant of 11.6×10^{-4} M^{-1} s^{-1} for this reaction was determined from the least-squares fit to eq 1 of the dependence of the observed rate constant on Mg^{2+} concentration shown in Figure 6. The rate constant given in Table IV is the average of these values. The second-order rate constants in Table IV for the reactions of MPP·Mg with nicotinamide at 25.1 °C and with pyridine at 52.3 °C were determined similarly.

The rate constant for the hydroxide ion-independent hydrolysis of MPP in the presence of 0.33 M Mg^{2+} at 25.1 °C and ionic strength 1.5 (Table V) was obtained by extrapolation to zero hydroxide ion concentration to correct for the contribution from reaction with hydroxide ion of ~50–300% in the pH range 7.8–8.5.[24] Table V also gives first-order rate constants for the hydrolysis of phosphorylated pyridine and phosphorylated γ-picoline in the presence and absence of 0.33 M Mg^{2+}; the rate

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(25) Kauzmann, W. *Adv. Prot. Chem.* **1959**, *14*, 1–63.

(26) Phillips, R. *Chem. Rev.* **1966**, *66*, 501–527. Izatt, R. M.; Eatough, D.; Christensen, J. J.; Bartholomew, C. H. *J. Chem. Soc. A* **1969**, 47–53. Brintzinger, H. *Helv. Chim. Acta.* **1965**, *48*, 47–54. Khan, M. M. T.; Martell, A. E. *J. Am. Chem. Soc.* **1967**, *89*, 5585–5590. Belaich, J. P.; Sari, J. C. *Proc. Natl. Acad. Sci. U.S.A.* **1969**, *64*, 763–770.

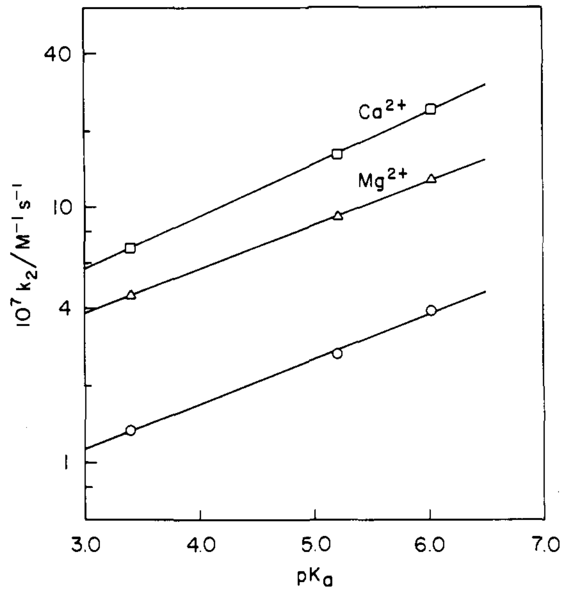

Figure 7. Bronsted-type plots showing the dependence on pyridine basicity of the logarithm of the second-order rate constants for the pyridine-catalyzed hydrolysis of PNPP (○), PNPP·Mg (△), and PNPP·Ca (□) at 39.2 °C and ionic strength 1.0. Rate constants are from Table I, and values of the pK_a for nicotinamide, pyridine, and γ-picoline of 3.4, 5.2, and 6.0 are from ref 4.

constants in the presence of Mg^{2+} were obtained by extrapolation to hydroxide ion independent rate constants, as for MPP. The rate constants for hydrolysis of phosphorylated pyridines were found to be independent of the concentration of hydroxide ion in the absence of metal ion and independent of the concentration of CHES buffer in the presence and absence of divalent metal ion. First-order kinetic plots for reactions of phosphorylated pyridines are linear for $>3t_{1/2}$, which shows that the reactions are first order in substrate. The rate constants in the absence of Mg^{2+} are similar to literature values obtained at different ionic strengths.[11,27]

Discussion

Changes in Transition-State Structure. The data of Figures 2 and 3 show that Mg^{2+} and Ca^{2+} catalyze the reaction of PNPP with substituted pyridines. A plot of the logarithms of the second-order rate constants for these reactions (Table I) against the pK_a of the pyridine nucleophile (Figure 7) gives slopes of β_{nuc} = 0.18, 0.17, and 0.21 for the reactions of substituted pyridines with PNPP, PNPP·Mg, and PNPP·Ca, respectively. These values of β_{nuc} are the same within experimental uncertainty, and a difference of 0.04, even if it were real, would not represent a significant difference in the structure of the transition state. The value of β_{nuc} = 0.18 for PNPP is not significantly different from a literature value of 0.15.[28] It should be noted that the variation in the values of β_{nuc} in the presence and absence of bound metal ion is a smaller percentage of the values after correction for desolvation of the attacking pyridine;[29] $\beta_{nuc(cor)}$ = 0.32, 0.31, and 0.34 for the reactions with PNPP, PNPP·Mg, and PNPP·Ca, respectively, with use of the formula $\beta_{nuc(cor)} = (\beta_{nuc} - \beta_{desolv})/(1 - \beta_{desolv})$ with $\beta_{desolv} = -0.2$. The fact that there is no significant change in the value of β_{nuc} upon complexation with Mg^{2+} or Ca^{2+} strongly suggests that catalysis by these metal ions does not increase nucleophilic participation.

Similar results are obtained for catalysis by Ca^{2+} of reactions of DNPP. The ratio of the rate constants for reaction with two pyridines, which differ in pK_a by 1.7 units, is 1.1 for both DNPP and DNPP·Ca (Table III), again showing no significant change in sensitivity to nucleophilicity upon complexation with metal ion. Results with a series of four quinuclidines also reveal no significant change in the value of β_{nuc} for DNPP·Ca compared to that for DNPP, with β_{nuc} = −0.10 and −0.11 for DNPP and DNPP·Ca, respectively.[29] The negative values of β_{nuc} have been attributed to a requirement for desolvation of the nucleophile before reaction, as noted above; the corrected values of β_{nuc} are 0.1.[29]

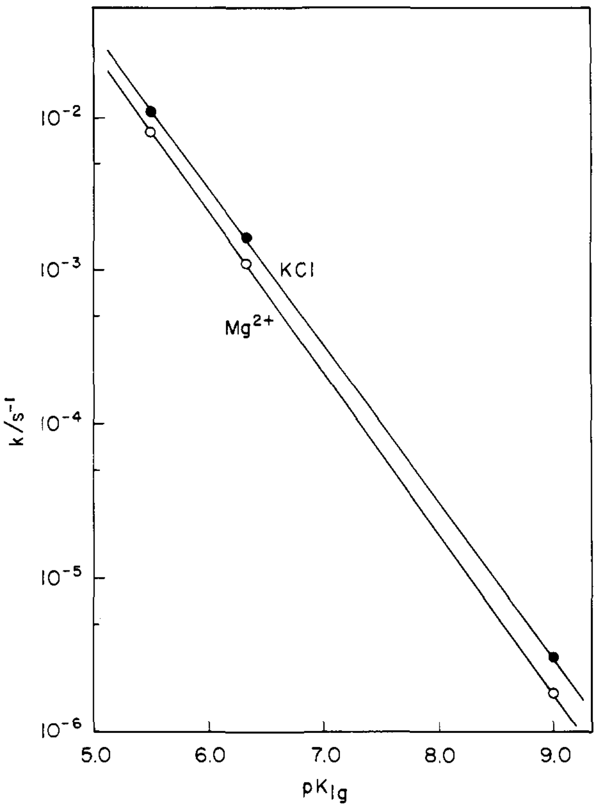

Figure 8. Bronsted-type plot showing the dependence on the basicity of the leaving pyridine of log k for hydrolysis of phosphorylated pyridines in the presence of (○) and absence (●) of 0.33 M $MgCl_2$ at ionic strength 1.5 (KCl) and 25.1 °C (Table V).

The data of Figure 6 and Tables IV and V show that Mg^{2+} inhibits the hydrolysis of MPP. The ratio of the rate constants for the reactions with two pyridines, which differ in pK_a by 1.7 units is 3.8 and 4.4 for MPP and MPP·Mg, respectively. These ratios are the same within experimental uncertainty; the difference corresponds to a difference in the value of β_{nuc} of 0.04. The absence of a significant change in the small value of β_{nuc} upon complexation with Mg^{2+} is evidence that the symmetrical transition state for the concerted phosphoryl transfer between pyridines remains dissociative and metaphosphate-like.[11]

Activation parameters for the reaction of MPP and pyridine were calculated from the rate constants at 25.1 and 52.3 °C (Table IV). The values of $\Delta H^{\ddagger}$ = 13 kcal/mol and $\Delta S^{\ddagger}$ = −26 eu for free MPP and $\Delta H^{\ddagger}$ = 13 kcal/mol and $\Delta S^{\ddagger}$ = −30 eu for MPP·Mg also provide no indication of a change in the nature of the transition state upon complexation with Mg^{2+}.

The Bronsted-type plot of log k against the pK_a of the leaving group for the hydrolysis of phosphorylated pyridines (Figure 8) gives slopes of β_{lg} = −1.05 and −1.02 in the presence and absence of 0.33 M Mg^{2+}, respectively. This provides no evidence for a significant change in the dissociative character of the transition state. It should be noted that the value of β_{lg} for the substrate–metal-ion complexes may be slightly less negative than the value observed in the presence of 0.33 M Mg^{2+}, because the association constants for phosphorylated pyridines and Mg^{2+} may decrease with decreasing pK_a of the pyridine. The large negative value of β_{lg} for hydrolysis of phosphorylated pyridines is equivalent to a small value of β_{nuc} for the reverse reaction with water as the leaving group.

We conclude that there is no significant increase in the amount of nucleophilic participation for phosphoryl-transfer reactions in

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[27] Jameson, G. W.; Lawlor, J. M. *J. Chem. Soc. B* **1970**, 53–57.

[28] From the rate constants for the same nucleophiles reported in ref 4.

[29] Jencks, W. P.; Haber, M. T.; Herschlag, D.; Nazaretian, K. L. *J. Am. Chem. Soc.* **1986**, *108*, 479–483.

the presence of bound Mg^{2+} or Ca^{2+}; the transition state remains dissociative and metaphosphate-like upon complexation with a divalent metal ion.

Analysis of a reaction coordinate–energy contour diagram and less formal approaches had suggested that metal ions might bring about a change to a more associative transition-state structure (see Figure 1 and Introduction). The absence of a detectable change indicates that replacing solvating water molecules at the phosphoryl oxygen atoms by a metal ion provides too small a perturbation relative to the curvature of the surface to cause significant movement of the transition state perpendicular to the reaction coordinate (Figure 1). The amount of movement of the transition state is inversely proportional to the curvature of the surface.[19] Small changes in transition-state structure for phosphoryl transfer with large changes in the basicity of the nucleophile or leaving group have been observed;[11,29] however large changes, such as a change from a predominantly dissociative to a predominantly associative transition state, are not observed.

It has been suggested, based on data from X-ray diffraction and vibrational spectroscopy, that binding of a metal ion increases the P–O bond length and single bond character for the bonds of the oxygen atoms complexed with the metal ion, but that the P–O bond of the oxygen atom which is not complexed with the metal ion compensates by decreasing in length and increasing in double bond character.[30] Such compensation may also ameliorate the effects of a bound metal ion in the transition state for phosphoryl transfer. This compensation may account for the fact that binding of Mg^{2+} to methyl 2-oxo-1-phosphonopropane monoanion enhances the rates of ketone enolization at C-1 and C-3 by the same extent, compared to the rates in the absence of metal ion.[31]

The value of $\beta_{nuc} = 0.15$ observed for the reaction of pyridines with the Zn^{2+}–pyridine-2-carbaldoximyl phosphate dianion complex is also consistent with little nucleophilic participation for phosphoryl transfer in the presence of a bound metal ion.[32]

One exception to the dissociative transition state for reactions of substituted phosphates in the presence of bound metal ions has been observed with Co(III). The metal-ion–phosphate ester complex of *cis*-$Co^{III}(en)_2(OH)$PNPP reacts by intramolecular attack of the coordinated hydroxide ion to form $Co(en)_2PO_4$ with the incorporation of oxygen atoms from the solvent over the course of the reaction, which suggests that a pentavalent phosphorane is formed through an associative transition state in the presence of bound Co(III).[33] The different nature of coordination to Co(III) compared with Mg^{2+} and Ca^{2+} and the intramolecular nature of the Co(III) complex reaction compared with the intermolecular reactions with Mg^{2+} and Ca^{2+} could account for the difference in the transition states. The slow dissociation rates of the Co(III) complex suggest that the triply charged Co(III) interacts strongly with the phosphoryl oxygen atom, possibly with some covalent character, and the geometry of the intramolecular reaction may favor an associative transition state.

Mechanisms of Catalysis and Inhibition by Metal Ions. The reactions of the MPP·Mg complex with pyridines are about twofold slower than those of free MPP (Table IV). Similar rate decreases of ~30% were observed for the reactions of MPP with pyridines in the presence of 0.33 M Mg^{2+}, for the reactions of MPP, phosphorylated pyridine, and phosphorylated γ-picoline with water in the presence of 0.33 M Mg^{2+}, and for the reaction of MPP with pyridine in the presence of 0.33 M Ca^{2+} (Tables IV and V). Inhibition of 15–40% by 0.08–0.12 M Mg^{2+}, Ca^{2+}, and Zn^{2+} of reactions of phosphorylated imidazole with water and with amine nucleophiles has been observed previously,[34] and 0.2 M Mg^{2+} and Ca^{2+} inhibit the hydrolysis of phosphoramidate.[35,36]

This inhibition represents stronger binding of the metal ion to the ground state than to the transition state. This is consistent with a mechanism in which there is electron donation from the phosphoryl oxygen atoms in going from the ground state to a metaphosphate-like transition state. Changes in geometry between the ground state and the transition state also could lead to inhibition by metal ions. However, the fact that there is similar inhibition by Mg^{2+}, Zn^{2+}, and Ca^{2+}, which have different ionic radii of 0.65, 0.74, and 0.99 Å, respectively, does not support this explanation. The decrease in the rate constant for the reaction of MPP with pyridine with increasing ionic strength (Figure 5, upper lines) is also consistent with greater electrostatic stabilization of the ground state than the transition state. Prigodich and Haake have previously suggested that the lack of catalysis by metal ions of *N,N*-dimethyl-*N'*-phosphoroguanidine hydrolysis is consistent with a predominantly dissociative transition state.[37]

The catalysis of the reactions of PNPP with pyridines by factors of three- to sixfold, contrasted with the inhibition of reactions of substrates with uncharged leaving groups, suggests that the metal ion interacts with the developing negative charge of the *p*-nitrophenolate ion leaving group in the transition state. The large negative values of β_{lg} observed for phosphoryl-transfer reactions suggest that there is a large amount of negative charge development on an anionic leaving group in the transition state.[7,8] Metal-ion catalysis by an interaction with the anionic leaving group is an example of general acid catalysis by a Lewis acid.

Values of $\Delta G° = -2.4$ kcal/mol and $K_a = 50\ M^{-1}$ (39 °C and ionic strength 1.0) for Ca^{2+} binding to the transition state for the reaction of PNPP with pyridine were calculated from the rate constants for the reactions of PNPP and PNPP·Ca (Table I) and K_a for PNPP and Ca^{2+} in the ground state (Table II).[38] The association is stronger than that in the ground state and is stronger than expected for Ca^{2+} binding to *p*-nitrophenolate anion.[39] This suggests that the metal ion interacts with both the phosphoryl and phenolate oxygen atoms in the transition state. For the reverse reaction, nucleophilic attack of *p*-nitrophenolate anion on phosphorylated pyridine, the metal ion would interact electrostatically with both the nucleophile and substrate and thereby provide a template for the reaction.

There is precedent for catalysis of phosphoryl-transfer reactions through interaction of metal ions with the leaving group.[40] Coordination of Zn^{2+} to pyridine-2-carbaldoximyl phosphate results in an increase in the rate of reaction with pyridine nucleophiles and with water; Zn^{2+} is known to coordinate to the free leaving group and lower the pK_a of its conjugate acid. Hsu and Cooperman have concluded that this decrease in the leaving group pK_a can account for catalysis by Zn^{2+}.[32] Coordination of Cu^{2+} to 2-(4(5)-imidazolyl)phenyl phosphate results in an increase in the rate of hydrolysis by $>10^4$-fold. The observed rate of the catalyzed reaction corresponds to that for a substrate with a leaving group that has a pK_a 6 units lower than the free substrate, ac-

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(31) Kluger, R. In *Bioorganic Chemistry*; van Tamelen, E. E., Ed.; Academic Press: New York, 1978; Vol. 4, pp 277–291. Kluger, R.; Wong, M. K.; Dodds, A. K. *J. Am. Chem. Soc.* **1984**, *106*, 1113–1117.

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(34) Lloyd, G. J.; Hsu, C.-M.; Cooperman, B. S. *J. Am. Chem. Soc.* **1971**, *93*, 4889–4892.

(35) Gilchrist, M.; Jencks, W. P., unpublished results.

(36) It has been shown that there is no large effect of divalent metal ions on the rate of hydrolysis of *N,N*-dimethyl-*N'*-phosphoroguanidine (ref 37); however, substitution of dications for monocations at ionic strength 1.0 suggests that the dications Mg^{2+}, Ca^{2+}, and Mn^{2+} inhibit hydrolysis when compared to monocations such as Li^+, Na^+, K^+, and Cs^+. The extent of inhibition is similar to that observed with other neutral amine leaving groups.

(37) Prigodich, R. V.; Haake, P. *J. Org. Chem.* **1984**, *49*, 2090–2093.

(38) Calculated by using transition state theory; see, for example: Jencks, W. P. *Catalysis in Chemistry and Enzymology*; McGraw Hill: New York, 1969 p 606.

(39) Although we are not aware of published values for association constants for Mg^{2+} or Ca^{2+} with phenolate anions, the association constant for Ca^{2+} and hydroxide ion of ~10–30 M^{-1} at ionic strength 0–0.04 and 0–25 °C (*Stability Constants. Part II Inorganic Ligands*; The Chemical Society: London, Spec. Publ. No. 7, 1958; p 3) provides an upper limit. Increasing the ionic strength to 1.0 would be expected to decrease this limiting association constant. For example, the association constant for PNPP and Mg^{2+} at ionic strength 0.01–0.02 of 170 M^{-1} (Robinson, J. D. *Biochemistry* **1969**, *8*, 3348–3355) is 11-fold larger than the value of 14.8 M^{-1} at ionic strength 1.0 and 39 °C (Table II).

(40) Cooperman, B. S. In *Metal Ions in Biological Systems*, Sigel, H., Ed., Marcel Dekker: New York, Vol. 5, pp 79–125.

cording to a structure-reactivity correlation; Cu^{2+} could be coordinated by the leaving group in a planar six-membered ring.[3] The change in the value of β_{lg} upon the addition of Zn^{2+} from -1.2 to -0.7 for the intramolecular hydrolysis reaction of the phosphate diesters, salicylate O-aryl phosphate dianions, provides evidence for electrophilic catalysis by Zn^{2+} that involves neutralization of some of the negative charge on the leaving phenoxide ion in the transition state.[41] Other metal ions, including Mg^{2+} and Ca^{2+}, also catalyze this reaction.

It has been suggested that inhibition of PNPP hydrolysis by guanidinium ions results from association with the phosphoryl oxygen atoms and destabilization of the dissociative transition state relative to the ground state.[42] Two guanidinium ions bind to PNPP in 95% aqueous ethanol, and the hydrolysis of PNPP is inhibited fourfold by addition of 1 M guanidinium cation in 85% aqueous ethanol.[42] The difference between this inhibition of hydrolysis and the catalysis by Ca^{2+} and the intermediate effect of Mg^{2+} (Table I) is consistent with the absence of a favorable interaction of the guanidinium ion with the anionic leaving group in the transition state. The small inhibition of PNPP hydrolysis by Mg^{2+} is consistent with a competition between catalysis and inhibition.

The observed effects of metal ions on the rate of phosphoryl-transfer reactions with neutral leaving groups suggest that there is no electrophilic catalysis of these reactions from an electrostatic interaction of the metal ion with the phosphoryl oxygen atoms, in agreement with several previous conclusions.[30,37,40,43] Electrophilic catalysis has often been suggested as a mechanism for metal-ion catalysis of phosphoryl-transfer reactions,[44,45] and such catalysis would be predicted for an associative mechanism, in which there is an increase in electron density on the phosphoryl oxygen atoms in going from the ground state to the transition state. However, the constant slopes of linear free energy relationships show that the transition states have dissociative character both in the absence and in the presence of metal ions. As noted above, the effects of metal ions on the rate of phosphoryl transfer are consistent with this dissociative transition state even though there is nucleophilic participation in the transition state of these in-line phosphoryl transfer reactions.

The effect of an alkyl group or a proton that forms an additional covalent bond to a phosphoryl oxygen atom of a phosphate monoester is much larger than the effect of ionic complexation with metal ions. The addition of a covalent bond to give a phosphate diester results in a change to a more associative transition state and a slower hydrolysis reaction, which is consistent with inhibition of electron donation compared with the transition state of the monoester reaction.[15,18] For example, the value of β_{nuc} for methyl 2,4-dinitrophenyl phosphate monoanion is 0.3 greater than that for DNPP dianion, and the rate constant for hydrolysis is 600-fold smaller for the diester.[6,15] There is a much smaller effect from the ionic interaction with a metal ion: no significant change in transition-state structure and only a twofold rate decrease are observed, as noted above. In addition, the ionic interaction of a metal ion with both the phosphoryl and leaving group oxygen atoms in the transition state for PNPP dianion hydrolysis can be contrasted with the covalently bonded (P)O–H proton of PNPP monoanion that is completely or nearly completely transferred from the phosphoryl to the leaving group oxygen atom in the transition state.[46] This proton transfer accelerates the reaction greatly by neutralizing the large negative charge on the leaving group and by facilitating electron donation from the phosphoryl oxygen atoms in the transition state; the corresponding catalytic and inhibitory effects in the reactions catalyzed by Mg^{2+} and Ca^{2+} are much smaller.

A charge–dipole interaction between the substrate and the dipole of the nucleophile does not appear to influence the linear free-energy relationships under the conditions of these experiments, because complexation with Mg^{2+} and Ca^{2+} does not significantly increase the observed value of β_{nuc}. An electrostatic interaction between the dipole of nucleophiles and the negative charges of the phosphoryl oxygen atoms should be more unfavorable with nucleophiles of higher pK_a because of the larger electron density at the nucleophilic atom. This interaction might be expected to decrease the observed rate constants for basic nucleophiles and the value of β_{nuc} for phosphoryl transfer, in the absence of a change in the amount of bond formation in the transition state. Binding of a divalent metal ion should neutralize the negative charges of the phosphoryl oxygen atoms and lessen or eliminate a charge–dipole effect, so that an increase in β_{nuc} would be expected if this interaction were significant.

Although Ca^{2+} catalyzes the reactions of DNPP with pyridines and water by factors of seven- and sixfold, respectively, Mg^{2+} inhibits these reactions at least eightfold (Table III). The absence of such inhibition with PNPP (Table I) shows that Mg^{2+} does not bind strongly to the transition state because of an unfavorable interaction with the ortho nitro group of DNPP. The geometry of this interaction is not understood. The inhibition cannot be explained by sequestering of DNPP by Mg^{2+} in a different unreactive ground-state conformation because the observed association constant for Mg^{2+} and DNPP is not larger than expected in the absence of the ortho nitro group. This is shown by the fact that the binding of PNPP with Mg^{2+} is approximately twofold stronger than with Ca^{2+} while the binding of DNPP and Mg^{2+} and Ca^{2+} is almost the same (Table II).

Implications for Enzymatic Catalysis. Data from enzymatic phosphoryl transfer reactions have been interpreted in terms of both associative and dissociative mechanisms; however, none of the data exclude either mechanism.[47] It has been suggested that binding of a phosphoryl group in the active site of an enzyme will involve interactions with positively charged amino acid side chains or metal ions and that such binding will favor an associative transition state.[48] In contrast to these suggestions, our data on the nonenzymatic reaction provide precedent for a largely dissociative transition state in the presence of bound Mg^{2+} and Ca^{2+}; furthermore, there is no significant increase in associative character upon binding of these metal ions.

Although it is not known how enzymes catalyze phosphoryl-transfer reactions, there are mechanisms that could provide considerable catalysis of phosphoryl transfer through a largely dissociative transition state. The possible roles of metal ions in this catalysis have been reviewed:[30,40,45] our results provide precedent for or evidence against several of these mechanisms in nonenzymatic reactions.

Metal ions are known to catalyze reactions of carbonyl compounds by polarization of the C=O bond.[49] The inhibition by

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[41] Steffens, J. J.; Siewers, I. J.; Benkovic, S. J. *Biochemistry* **1975**, *14*, 2431–2440.

[42] Cotton, F. A.; laCour, T.; Hazen, E. E., Jr.; Legg, M. J. *Biochim. Biophys. Acta* **1977**, *481*, 1–5.

[43] A suggestion that Mg^{2+} does not increase the electrophilicity of phosphorus in phosphoryl-transfer reactions was based on the resistance to solvolysis of both disubstituted pyrophosphates and their complexes with Mg^{2+}; however, rate constants were not determined, and this result is not necessarily applicable to reactions of monosubstituted phosphates. Ramirez, F.; Marecek, J. F. *Pure Appl. Chem.* **1980**, *52*, 1021–1045. Ramirez, F.; Marecek, J. F.; Chan, Y. F.; McCaffrey, T. *Synthesis* **1978**, 519–521.

[44] See, for example: Cohn, M. *J. Cell Comp. Physiol.* **1959**, *54*, 17–31. Wieker, W.; Thilo, E. *Z. Anorg. Chem.* **1962**, *313*, 296–308. Schwartz, J. H. *Proc. Natl. Acad. Sci. U.S.A.* **1963**, *49*, 871–878. Bruice, T. C.; Benkovic, S. J. *Bioorganic Mechanisms*; W. A. Benjamin: New York, 1966; Vol. 2, Chapter 7. Khan, M. M. T.; Mohan, M. S. *Ind. J. Chem.* **1976**, *14A*, 945–950, 951–954. Coleman, J. E.; Chlebowski, J. F. In *Advances in Inorganic Biochemistry*; Eichhorn, G. L., Marzilli, L. G., Eds.; Elsevier: New York, 1979; Vol. 1, pp 1–66.

[45] Jencks, W. P. *Catalysis in Chemistry and Enzymology*; McGraw Hill: New York, 1969; pp 111–115.

[46] For the hydrolysis of phosphate monoester monoanions, complete or nearly complete proton transfer to the oxygen atom of the leaving group in the transition state is suggested by the solvent deuterium isotope effects of near unity and the much smaller (negative) values of β_{lg} for hydrolysis of the monoanions compared with the dianions (ref 7, 8, 10, and 16).

[47] Knowles, J. R. *Ann. Rev. Biochem.* **1980**, *49*, 877–919.

[48] Chlebowski, J. F.; Coleman, J. E. *J. Biol. Chem.* **1974**, *249*, 7192–7202. Reed, G. H.; Barlow, C. H.; Burns, R. A., Jr. *J. Biol. Chem.* **1978**, *253*, 4153–4158. Hassett, A.; Blättler, W.; Knowles, J. R. *Biochemistry* **1982**, *21*, 6335–6340. See, also ref 2.

[49] See, for example: Fife, T. H.; Przystas, T. J. *J. Am. Chem. Soc.* **1986**, *108*, 4631–4636, and references therein.

metal ions of reactions of phosphorylated pyridines described here as well as earlier work on other systems shows that there is no electrophilic catalysis of phosphoryl transfer through an electrostatic interaction with the phosphoryl oxygen atoms;[30,37,40] the dissociative transition state is destabilized relative to the ground state, rather than stabilized, by an electrophilic interaction with the phosphoryl oxygen atoms. Thus, an enzymatic reaction that proceeds through a largely dissociative transition state is not subject to catalysis via this electrostatic interaction.

The catalysis by Mg^{2+} and Ca^{2+} of reactions of PNPP suggests that an enzymatic reaction could be catalyzed by an electrostatic interaction with the leaving group that stabilizes the development of electron density on the leaving group in the transition state. Larger rate enhancements have been reported with Zn^{2+} and Cu^{2+} when the leaving group can chelate the metal ion.[3,32] The precise positioning obtainable at the active site of an enzyme could increase the metal-ion–leaving group interaction in the transition state and result in greater rate enhancements than those observed nonenzymatically. From analysis of the crystal structure of alkaline phosphatase Sowadski et al. have suggested that an enzyme-bound Zn^{2+} could interact with an anionic leaving group of a monosubstituted phosphate compound in the transition state;[50] the small negative value of β_{lg} observed in the alkaline phosphatase reaction is consistent with such an interaction.[51] The Mg^{2+} bound to ATP in kinase reactions may similarly decrease the basicity of the leaving ADP anion.[45]

In the reverse of the metal-catalyzed hydrolysis of PNPP the interaction with the metal ion in the transition state will stabilize an attacking phenolate ion and will facilitate deprotonation of a *p*-nitrophenol molecule to form the stronger nucleophile, *p*-nitrophenolate ion. It has been suggested that the Zn^{2+} near the nucleophilic serine residue of alkaline phosphatase could provide such catalysis by favoring deprotonation of the attacking serine residue to give a high concentration of the stronger nucleophile. The second Zn^{2+} at the active site could reduce the electron density on the leaving group during the formation of the covalent enzyme–phosphate intermediate as described above and could facilitate deprotonation of the nucleophilic water molecule during the breakdown of this intermediate.[52]

The simultaneous interaction of a metal ion with the phosphoryl oxygen atoms and anionic leaving group of PNPP in the reaction with pyridine shows that the metal ion will provide a template for the reverse reaction of nucleophilic attack by an anion on a phoshorylated pyridine. Similarly, a metal ion on an enzyme could help align substrates in the active site for catalysis by induced intramolecularity between two substrates or between a substrate and enzymatic catalytic groups.[5]

Screening of multiple negative charges by a metal ion could facilitate phosphoryl transfer to an anionic nucleophile and allow an anion of high charge density, such as ATP, to bind in a hydrophobic active site. Electrostatic repulsion slows reactions of anionic nucleophiles with anionic phosphate compounds.[53] Nucleophilic attack by pyridine-2-carbaldoxime anion on phosphorylated imidazole is observed only if the nucleophile is complexed with zinc ion[54] and nonenzymatic formation of pyrophosphate in aqueous solution from inorganic phosphate and ATP or acetyl phosphate anions is greatly facilitted by divalent metal ions or high concentrations of monovalent cations.[8,13,55]

Finally, an enzyme could preferentially stabilize the transition state through the positioning of functional groups or metal ions so that there is a more effective interaction with the trigonal bypyrimidal geometry of the transition state than with the tetrahedral geometry of the ground state.[56] A metal ion can provide catalysis through the mechanisms discussed in this section when the catalytic advantage is large enough to overcome the small inhibition that is seen in the nonenzymatic reaction. For strain to provide catalysis, the change in geometry in going from the ground state to the transition state must be energetically more important than this unfavorable effect of the metal ion on electron donation from the phosphoryl oxygen atoms.

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(50) Sowadski, J. M.; Handschumacher, M. D.; Murthy, H. M. K.; Foster, B. A.; Wyckoff, H. W. *J. Mol. Biol.* **1985**, *186*, 417–433.

(51) Hall, A. D.; Williams, A. *Biochemistry* **1986**, *25*, 4784–4790.

(52) See, for example: ref 33 and 50.

(53) See, ref 13 and references therein.

(54) Lloyd, G. J.; Cooperman, B. S. *J. Am. Chem. Soc.* **1971**, *93*, 4883–4889.

(55) Vieyra, A.; Meyer-Fernandes, J. R.; Gama, O. B. H. *Arch. Biochem. Biophys.* **1985**, *238*, 574–583. Lowenstein, J. M. *Nature* (*London*) **1960**, *187*, 570–573. Lowenstein, J. M. *Biochem, J.* **1958**, *70*, 222–230. Tetas, M.; Lowenstein, J. M. *Biochemistry* **1963**, *2*, 350–357. Lowenstein, J. M. *Biochem. J.* **1960**, *75*, 269–274.

(56) Farrell, F. J.; Kjellstrom, W. A.; Spiro, T. G. *Science* (*Washington, DC*) **1969**, *164*, 320–321.