ing that Zn^{2+} has little effect on the rates of pyridine or water attack on PIm.[22] Other arguments against models for divalent metal ion catalysis of phosphate ester hydrolysis invoking substantial charge polarization have been summarized recently.[29] The total rate enhancement due to Zn^{2+} is impossible to estimate, since no phosphorylation was detected in its absence, but, from the limits of analytical methods used, the first-order catalytic constant for reaction in the ternary complex can be estimated to be at least 10^4 times greater than the highest possible second-order constant for PCA anion reaction with *N*-MePIm. Further investigation into the relative importance of proximity and charge effects is now in progress.

Rapid phosphorylation of Zn^{2+}–PCA is not limited to PIm. In preliminary experiments phosphoramidate itself was shown to rapidly phosphorylate Zn^{2+}–PCA. It will be of interest to see to what extent phosphate monoesters with poorer leaving groups, such as ATP, can act as phosphate donors.

Nucleoside diphosphokinase, in the presence of Mg^{2+}, catalyzes reaction 10 and is one of several phos-

$$\text{ATP} + \text{GDP} \rightleftharpoons \text{GTP} + \text{ADP} \qquad (10)$$

phate transfer enzymes known to catalyze reactions *via* formation of a phosphorylated enzyme intermediate in which the site of phosphorylation is a ring nitrogen of a histidine side chain.[30] Zn^{2+}–PCA anion attack on monoprotonated PIm can be taken as a model for GDP attack on phosphorylated nucleoside diphosphokinase, since the latter involves attack on a phosphorylated imidazole by an anionic nucleophile of p*K* 6. The rate of the enzymatic reaction[31] is at least 24,000 min^{-1} at 30°, so that the model reaction is 10^4–10^5 times slower, without allowing for either the expected higher reactivity of an α-effect oxime anion as compared with a phosphate, or the replacement of Mg^{2+} with Zn^{2+}. It may thus be necessary to invoke other catalytic processes in addition to those examined in this paper to account for the rate of the enzymatic reaction. However, it should be pointed out that phosphate transfer in the ternary complex, *via* I or II, involves the formation of a five-membered ring, N-O-P-O-Zn^{2+}, which Dreiding models indicate to have substantial strain. An estimate of the strain energy involved can be obtained from the known strain energy of methyl ethylene phosphate, which is about 5.5 kcal/mol.[32] This corresponds to a rate factor of 10^4. If the observed rate of reaction in the ternary complex is multiplied by this factor, a rate close to the enzymatic one results. It is therefore possible that the PCA–Zn^{2+}–PIm complex provides a reasonably complete model for enzymatic catalysis of anionic nucleophilic attack on a phosphorylated imidazole species, with the exception that in the enzymatic case, this attack would not proceed *via* formation of a strained ring structure.

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On the Reactivity of Phosphorylimidazole, an Analog of Known Phosphorylated Enzymes[1]

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Abstract: The reactivity of phosphorylimidazole (PIm) toward various reagents was explored. Unhindered amines were found to attack PIm nucleophilically, but no evidence was found for nucleophilic attack by carboxylic acid anions, or for general base or general acid catalysis of either water or amine attack. Ca^{2+}, Mg^{2+}, and Zn^{2+} were all found to lower slightly observed rates of amine and water attack. The relevance of these results to the problem of enzymatic phosphate transfer is discussed.

Several phosphate-transfer enzymes are now known to function through formation of a phosphorylated imidazole intermediate[4–6] but kinetic studies to date on the hydrolysis of phosphorylimidazole (PIm)[7,8] or phosphorylhistidine[9] have been limited to the determination of pH–rate profiles. For PIm it has been established that above pH 4 the total rate of hydrolysis is due to PIm monoanion.[8] In this paper we report the results of studies on the reactivity of PIm monoanion toward nucleophilic attack, and of the sus-

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Table I. Rate Constants for Nucleophilic Attack on Monoprotonated PIm[a]

Nucleophile	pK_a	(Amine)$_t$, M	pH	No. of runs	k_c, min^{-1} M^{-1}	Kinetic method
Triethylenediamine monocation	3.1	0.01–0.1	5.2[b]	6	0.161 ± 0.003	Uv
Trifluoroethylamine	5.41	0.01–0.5	5.4	9	0.014 ± 0.001	Uv
Piperazine monocation	5.58	0.01–0.1	5.2, 5.6, 6.1	20	0.245 ± 0.006	Uv, phosphate analysis
Aminoacetonitrile	5.8[d]	0.08–0.2	5.8[d]	3	0.058 ± 0.003	Uv
1,2-Diamino-2-methylpropane monocation	6.32	0.04–0.2	5.8	5	0.083 ± 0.003	Uv
2-Aminomethylpyridine monocation	2.3	0.12	4.54[b]	2	0.0040 ± 0.0004	Phosphate analysis
4-Aminomethylpyridine monocation	4.2	0.2	4.7	2	0.157 ± 0.003	Phosphate analysis
Pyridine	5.2	0.06–0.2	5.2	5	0.196 ± 0.003	Phosphate analysis
Water				20	0.00120 ± 0.00003[c]	Uv

[a] Ionic strength brought to 0.5 with KCl. [b] In acetate buffer. All other runs are self-buffered. [c] First-order rate constant, min^{-1}. [d] Measured at 25°. All other pK_a's and pH's are measured at 40.1°.

ceptibility of PIm monoanion hydrolysis to general acid, general base, and divalent metal ion catalysis.

Experimental Section

Materials, methods, and kinetic procedures are as previously described.[8] Rates of PIm disappearance were measured by uv difference or, for pyridines, by phosphate analysis using the modified Martin–Doty procedure of Jencks and Gilchrist.[10] This procedure does not distinguish between phosphorylated pyridines and inorganic phosphate (although it does distinguish between PIm and inorganic phosphate). All kinetic runs were performed at 40.1 ± 0.1°. Rate constants were reproducible within 3%.

Results

Reaction with Amines. The rate of PIm disappearance was markedly accelerated in unhindered primary, secondary, and tertiary amine buffers, as shown in Figure 1 for the case of piperazine. The results were

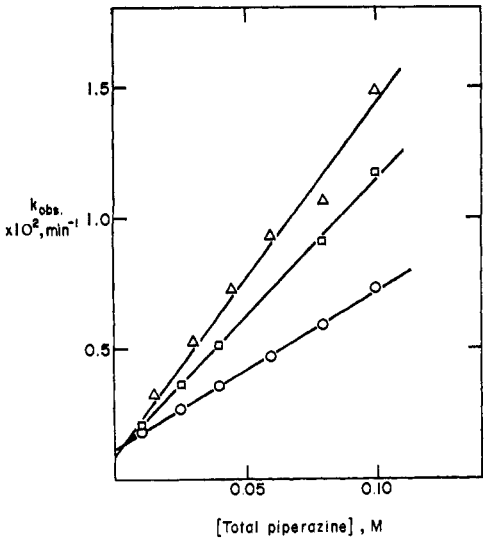

Figure 1. Plot of k_{obsd} for PIm disappearance against total piperazine concentration at pH 5.18 (○), pH 5.58 (□), and pH 6.09 (△).

analyzed according to eq 1–4. In no case was evidence

$$\text{rate} = k_{obsd}[\text{PIm}]_t \quad (1)$$

$$[\text{PIm}]_t = [\text{PIm}^{2-}] + [\text{HPIm}^-] \quad (2)$$

$$K = a_{H^+}[\text{PIm}^{2-}]/[\text{HPIm}^-] \quad (3)$$

$$k_{obsd} = \frac{k_0 + k_c'[\text{total buffer}]}{1 + K/a_{H^+}} \quad (4)$$

found for a term in [buffer]^2 over the concentration ranges studied.

That catalysis is due solely to the basic form of the buffer is shown by the following observations.

(i) At constant piperazine dication concentration, k_c' was proportional to added piperazine monocation. At constant piperazine monocation concentration (0.02 M) and in a range where rate was independent of pH, raising piperazine dication concentration up to 0.11 M had no effect of k_c'.

(ii) In acetate buffer at pH 5.18, k_c' was proportional to added triethylenediamine monocation.

(iii) N-Methylmorpholinium ion, up to 0.35 M, had no effect on k_{obsd} for hydrolysis in a piperazine buffer of pH 5.18.

Equation 4 can thus be rewritten as eq 5. Values

$$k_{obsd} = \frac{k_0 + k_c[\text{buffer in basic form}]}{1 + K/a_{H^+}} \quad (5)$$

of k_c are given in Table I.

Evidence that k_c refers to nucleophilic attack rather than general base catalysis comes from the following observations.

(i) Pyridine has a fairly large k_c term, whereas 2,6-lutidine fails to show any catalysis up to 0.2 M. This type of behavior has been previously suggested[11] to be diagnostic of nucleophilic catalysis.

(ii) No catalysis is observed up to 0.2 M imidazole. In this case nucleophilic attack is a virtual reaction. If general base catalysis were an important catalytic pathway, one would have expected to see a catalytic term for imidazole.

(iii) Acetate, succinate, and N-methylmorpholine show no significant catalysis of PIm hydrolysis. Borate shows no significant catalysis of N-methyl PIm hydrolysis.

No effort was made to measure the accumulation of the phosphoramidates which would be the initial products of nucleophilic attack. However, previous studies have shown phosphoramidate zwitterions[7,12,13] to hydrolyze at least an order of magnitude faster than PIm monoanion, so it is likely that relatively little accumulation takes place.

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Table II. k_{obsd} as a Function of Added Divalent Metal Ion[a]

Metal ion	M	Buffer[b]	$k_{obsd} \times 10^3$ min^{-1}
None		A	1.04
Mg^{2+}	0.050	A	0.92
	0.084	A	0.86
	0.117	A	0.71
Ca^{2+}	0.025	A	1.01
	0.050	A	0.93
	0.100	A	0.87
None		B	5.3
Mg^{2+}	0.017	B	4.9
	0.033	B	4.8
	0.050	B	4.7
	0.067	B	4.5
	0.117	B	4.0
Ca^{2+}	0.017	B	4.8
	0.033	B	4.7
	0.050	B	4.6
	0.067	B	4.4
	0.100	B	4.3
	0.117	B	4.3
None		C	2.9
Mg^{2+}	0.033	C	2.6
	0.067	C	2.4
	0.100	C	2.15
None		D	2.7
Mg^{2+}	0.017	D	2.35
	0.033	D	2.3
	0.084	D	1.9
None		E	12.7
Zn^{2+}	0.016	E	12.2
	0.032	E	10.1
	0.050	E	9.1
	0.080	E	8.0
None		F	1.2
Zn^{2+}	0.12	F	0.87

[a] Ionic strength brought to 0.5 with KCl. The error in k_{obsd} is ±3%. [b] A, acetate, pH 5.07; B, 0.03 M piperazine, pH 6.09; C, 0.03 M piperazine, pH 5.18; D, 0.25 M trifluoroethylamine, pH 5.41; E, 0.12 M pyridine, pH 5.20; F, acetate, pH 4.55.

Metal Ion Effects. The effect of Ca^{2+}, Mg^{2+}, and Zn^{2+} on PIm hydrolysis was studied in several different buffer systems and the results are summarized in Table II. Acetate buffer shows no catalysis of PIm hydrolysis, so the small rate decreases seen on addition of metal ions reflect a reduced reactivity toward hydrolysis of M^{2+}–PIm complexes, as compared with free PIm.

The decelerating effects of divalent metal ions in amine buffers are difficult to interpret exactly, since both M^{2+}–amine complexes[14] and M^{2+}–PIm complexes have to be considered, and decreases in k_{obsd} may simply be due to decreases in unprotonated amine concentration. Nevertheless, the results in Table II definitely show that there is no significant catalysis of nucleophilic amine attack on PIm.

Salt Effects. The effect of varying ionic strength between 0.25 and 1.25 on both uncatalyzed and piperazine monocation catalyzed hydrolysis of PIm is shown in Table III. The uncatalyzed rate (succinate buffer) shows a positive salt effect of about 35% in this range. By contrast, the piperazine monocation catalyzed rate (column 3) has a negative salt effect of about 40%. It is doubtful that this represents a specific effect of K^+ complexation, since there is a lack of saturation behavior at high KCl concentration, and replacement of KCl by tetramethylammonium chloride makes little difference in the rate.

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Table III. Salt Effects on PIm Hydrolysis

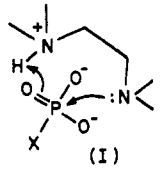
Added salt, μ	k_{obsd}[d] $\times 10^3$ min^{-1} Piperazine buffer	k_{obsd} Succinate buffer[b]	k_{obsd} (piperazine) − k_{obsd} (succinate) $\times 10^3$ min^{-1}
KCl, 0.25	7.4[a]	0.88	6.5
0.35	6.2[a]		
0.45	6.4[a]		
0.50	6.2[a]	0.92	5.3
0.65	5.9[a]		
0.85	5.5[a]		
1.00		1.12	
1.25	5.3[a]	1.19	4.1
0.50	9.3[c]		
$Me_4N^+Cl^-$, 0.50	9.9[c]		

[a] 0.03 M piperazine, pH 5.58. [b] 0.05 M succinate, pH 5.75. [c] 0.06 M piperazine, pH 6.09. [d] The error in k_{obsd} values is ±3%.

Discussion

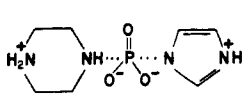
This study has shown PIm monoanion to be quite reactive toward nucleophilic attack by amines but rather insensitive toward general acid and general base catalysis of hydrolysis. The reactivity of PIm monoanion is thus quite similar to what has been found for other phosphoryl dianions (phosphate monesters and phosphoramidates) with good leaving groups.[7,12,15-17]

The results in Table I show an enhanced reactivity of monocationic alkylamines over neutral amines of approximately equal or high pK_a, and this phenomenon has also been noted previously for compounds similar to PIm.[7,12,16,17] For the case of 4-nitrophenylphosphate dianion, Kirby and Jencks[17] have argued reasonably that an explanation involving intramolecular general acid catalysis (I) is unlikely since the rigid



(I)

triethylenediamine monocation is sterically prevented from forming such an activated structure, yet is also unusually reactive. They therefore assign the observed enhanced rate to simple electrostatic attraction between the cationic amine and the phosphoryl dianion. The data presented in Table III support this interpretation, since they are consistent with there being a favorable charge interaction in the transition state for piperazine monocation attack on PIm which is not present in the transition state for water attack. Moreover, assuming the former transition state to be as shown below, it can be argued that the energy to be expected from such an interaction is of sufficient magnitude to



account for the observed rate enhancements. This conclusion comes from the following considerations. Ammonium substitution lowers the pK_a of carboxylic,

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phosphonic, and phosphoric acids,[18-26] and assuming the increased acidity to be due solely to the favorable interaction between the ammonium substituent and the acid anion provides a model for evaluating the interaction term in the transition state for piperazine monocation attack on PIm monoanion. From Dreiding models, in a fully formed trigonal bipyramid with both attacking piperazine and leaving imidazole in the axial positions,[27] the average distance between the cationic nitrogen and the anionic oxygens is 4.5 Å. However, the low β values found for amine attack on phosphoramidates suggest that the transition state occurs well before full bond formation between attacking amine and phosphorus,[28] so that a transition state distance of 5.0–5.5 Å is perhaps more reasonable. In 3-aminocyclohexanecarboxylic acid, the cationic nitrogen is at an average distance of 5.3 Å (cis) and 4.9 Å (trans) from the anionic oxygens. The pK_a's (cis, 3.70; trans, 3.85)[18] are an average of 1.1 log units lower than the pK_a of cyclohexanecarboxylic acid (4.9),[19] assuming that the differences in conditions under which the pK_a determinations were made are not of great consequence. This leads to a calculated energy of interaction of the order of 1.4 kcal/mol for a monocation–monoanion interaction through an organic molecule at a distance of approximately 5 Å. Although the reactive species of PIm is formally a monoanion, the interaction of piperazine monocation with PIm is at least partially monocation–dianion, so that an interaction energy of the order of 2–2.5 kcal/mol is expected. Applying all of this energy to stabilizing the transition state would lead to a rate acceleration of 25- to 50-fold. The rate accelerations seen in Table I (for example, piperazine monocation *vs.* trifluoroethylamine) are clearly within this range.

The low value of k_c for 1,2-diamino-2-methylpropane monocation as compared with piperazine and triethylenediamine monocations can be rationalized if it is assumed that the 1-amino position is slightly more basic than the 2-amino position, since it is expected that the 2-amino nitrogen will, on steric grounds, be a poor nucleophile. Lack of an enhanced rate for 4-aminomethylpyridine monocation as compared to pyridine is probably not due to its decreased basicity, since the β value for substituted pyridine attack on phosphoramidate monoanion is low.[7] Instead, it likely reflects the large distance between the cationic site and the phosphoryl oxygens in the transition state. The very slow rate for 2-aminomethylpyridine monocation attack is presumably due to the stringent steric requirements previously noted for pyridine attack on phosphoryl dianions.[13]

Zn^{2+}, Mg^{2+}, and Ca^{2+}, which are all cofactors for at least some phosphate transfer enzymes, have only slight effects of both water and amine attack on monoprotonated PIm. This is similar to what has been found for other phosphoryl dianions with good leaving groups.[7,29,30] Lack of strong catalytic effects by divalent metal ions on hydrolysis of molecules of this type has been disappointing, in view of the known requirement of most phosphate transfer enzymes for divalent metal ions, and the strong catalytic effects of divalent metal ions on processes such as amino acid ester hydrolysis.[31] However, these results can be understood in a perfectly straightforward manner when the differences in sensitivity to charge polarization effects of these processes are considered. Thus, protonated glycine ethyl ester hydrolyzes 10^{11} times faster than the neutral species,[31] whereas $^+NH_3PO_3H^-$ hydrolyzes only twice as fast as $^+NH_3PO_3^{2-}$.[13] Divalent metal ions are less effective than protons in polarizing charge,[32] but for carboxylic esters even a partial effect would result in strong catalysis, whereas the same is not true for phosphoramidates.

Metal ions could also catalyze hydrolysis of phosphoramidates by overcoming the charge barrier toward hydroxide ion attack. Similar mechanisms have been proposed for metal ion catalysis of acetyl phosphate hydrolysis,[30] and, in an accompanying paper, for Zn^{2+} catalysis of pyridine-2-carbaldoxime anion attack on PIm.[8] Unfortunately, the extreme kinetic stability of PIm dianion restricted kinetic studies to a region of very low hydroxide ion concentration and this perhaps accounts for the lack of catalysis observed.

Amine attack on PIm, where there is no charge barrier to overcome, also shows no catalysis by divalent metal ions. This does not preclude a direct catalytic participation by divalent metal ions in enzymes catalyzing similar reactions, but it should be pointed out that the rates of amine attack are sufficiently rapid that such participation, except perhaps for purposes of correct alignment, may not be necessary. Thus, multiplication of the values of k_c (Table I) by 10^3 for conversion of a bimolecular reaction into an enzymatic intramolecular reaction with correct orientation of the two reacting centers[33] gives hypothetical rate constants of 2×10^2 min^{-1} at 40°.[34] This rate is for imidazole as a leaving group, and for phosphate transfer from donors with poorer leaving groups, such as adenosine triphosphate, the achievement of rapid rates would require enzymatic participation in increasing leaving group ability. Although divalent metal ions could in principle aid in this process, previous studies on a model system designed to test this possibility gave completely negative results.[29]

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