82%) of colorless **1-benzoyl-4-methylhexahydro-4*H*-azepine (2)**: bp 120–128° (0.08–0.09 mm); $\nu_{C=O}$ 1630, $\nu_{C=C}$ 1600, 1575, 1495, $\nu_{C_6H_5}$ 785, 730, 705 cm^{-1} neat; $\delta^{37°}_{CDCl_3}$ 222, 205 (multiplets, 4 H, $-CH_2NCH_2-$), 128–170 (6 H, $-CH_2-$), 59 (doublet, 3 H, J = 5.5 Hz, $-CH_3$).

Bioconversion of 1-Benzoyl-4-methylhexahydro-4*H*-azepine (2). The extracts from bioconversion of **2** (2.0 g, 9.23 mmol) with *S. sulfurescens* were chromatographed on Florisil (3.8 × 35 cm), which was dry packed with Skellysolve B. Elution with 10% (v/v) and with 25% acetone in Skellysolve gave mixtures of ketonic and hydroxylic products as determined by tlc and ir and nmr spectra. Decolorization and crystallization of several later fractions from acetone–Skellysolve B gave 0.442 g of colorless crystals, mp 91–93°. Two recrystallizations from acetone–Skellysolve B gave shiny, colorless crystals, identified by their nmr spectrum as **1-benzoyl-4-hydroxy-4-methylhexahydro-4*H*-azepine (3)**: mp 94–96°; $[\alpha]D$ −4° (*c* 0.747, $CHCl_3$); ν_{OH} 3370, $\nu_{C=O}$ 1600, $\nu_{C=C}$ 1575, 1530, 1505, $\nu_{C_6H_5}$ 740, 715, 705 cm^{-1} in Nujol; $\delta^{37°}_{CDCl_3}$ 235–190 (multiplet, 4 H, $-CH_2NCH_2-$), 102 (multiplet, 6 H, 3-CH_2-), 72.5 (singlet, 3 H, -CH_3) cps.

Anal. Calcd for $C_{14}H_{17}NO_2$ (233.30): C, 72.07; H, 8.21; N, 6.00. Found: C, 71.46, 72.48; H, 8.14, 8.03; N, 6.19.

The remaining chromatography fractions containing products and the filtrates from the above crystallizations were combined in acetone and oxidized with excess Jones reagent.[14] Following work-up, the product mixture (1.02 g) was chromatographed on Florisil (100 g), which was packed with Skellysolve B. The products were eluted with 20% (v/v) acetone in Skellysolve B. Two fractions of ketone, one fraction of mixture (~1:3 ketone to alcohol), and four fractions of alcohol were obtained. Tlc (20% methanol in benzene) showed this separation of products. The ketone (**4**) (crude weight, 0.24 g, 1.03 mmol, 11%) failed to crystallize; $\nu_{C=O}$ 1705, 1605 cm^{-1} on the oil; $\delta^{37°}_{CDCl_3}$ 242 (broad multiplet, 2 H, >CHNCH<), 202 (multiplet, 2 H, >CHNCH<), 160 (multiplet, 3 H, $-CH_2COCH<$), 102 (four-line pattern, 2 H, $-CH_2-$), 66 (doublet, 3 H, J = 6.5 Hz, $-CH_3$) cps. The alcohol-containing fractions (crude weight, 0.37 g) gave 0.182 g of crystalline **3**, mp 93–95°; total yield of **3**, 0.624 g (2.68 mmol, 29%).

Bioconversion of the Mixture, *N*-Benzoyl-*cis*-4-methylcyclohexylamine (5) and *N*-Benzoyl-*trans*-4-methylcyclohexylamine (6). The methylene chloride extracts from bioconversion of this mixture (25.0 g, 0.115 mol, containing about 40% of one component and 60% of the other as determined by vpc analyses on several columns) were chromatographed on a column of Florisil (10.5 × 50 cm) which was dry packed with Skellysolve B. The column was eluted with 2-l. fractions of increasing proportions of acetone in Skellysolve B. Fraction 10 (20% acetone in Skellysolve B, crude weight 0.34 g) was recrystallized from acetone–Skellysolve B, giving 0.227 g (0.975 mmol, 0.8%) of colorless crystals, mp 154–156°. Two recrystallizations from acetone–Skellysolve B gave ***N*-benzoyl-*trans*-4-methyl-4-hydroxycyclohexylamine (8)**: mp 156–158°; $\nu_{OH,NH}$ 3380, 3300, $\nu_{C=O}$ 1635, $\nu_{amide\ II}$ 1540, $\nu_{C_6H_5}$ 700 cm^{-1} in Nujol; δ_{CDCl_3} 385 (1 H, doublet, J = 8 Hz, -NH-), 234 (1 H, broad, -CHN-), 130–190 (8 H, $-CH_2-$), 72 (3 H, singlet, CH_3).

Anal. Calcd for $C_{14}H_{19}NO_2$: C, 72.07; H, 8.21; N, 6.00. Found: C, 71.96; H, 8.24; N, 6.02.

Fractions 13–18 (crude weight, 9.61 g) were combined on the basis of tlc, decolorized, and recrystallized from acetone–Skellysolve B, giving 8.221 g (35.2 mmol, 30%) of colorless crystals, mp 161–163°. Two recrystallizations gave *N*-benzoyl-*cis*-4-methyl-4-hydroxycyclohexylamine (7) as colorless crystals: mp 171–173°; $\nu_{OH,NH}$ 3350, 3310, $\nu_{C=O}$ 1640, $\nu_{amide\ II}$ 1545, $\nu_{C_6H_5}$ 695 cm^{-1} in Nujol; δ_{CDCl_3} 373 (1 H, broad, -NH-), 242 (1 H, broad, -CHN-), 76 (3 H, singlet, $-CH_3$).

Anal. Calcd for $C_{14}H_{19}NO_2$: C, 72.07; H, 8.21; N, 6.00. Found: C, 71.86; H, 8.04; N, 5.84.

Bioconversion of *N*-Benzoyl-*cis*-4-methylcyclohexylamine (5). The extracts from bioconversion of **5** (0.300 g, 1.38 mmol, >95% *cis* isomer of mp 125–128°, lit.[15] mp 130–130.5°) in shake flasks were chromatographed on Florisil (30 g). A single product was isolated. Decolorization and crystallization from acetone–Skellysolve B gave 0.022 g (0.0945 mmol, 7%) of ***N*-benzoyl-*cis*-4-methyl-4-hydroxycyclohexylamine (7)** as colorless crystals, mp 172–174°; infrared spectrum in Nujol is identical with the sample of **7** described above.

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Nucleophilic Attack by Zinc(II)–Pyridine-2-carbaldoxime Anion on Phosphorylimidazole. A Model for Enzymatic Phosphate Transfer[1]

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Abstract: Phosphorylimidazole (PIm) monoanion has been found to transfer rapidly its phosphate group to the anionic oxygen of Zn^{2+}–pyridine-2-carbaldoxime (Zn^{2+}–PCA). The results suggest that transfer proceeds *via* a ternary complex. In the absence of Zn^{2+}, no evidence is found for PCA anion attack on either PIm or *N*-methylphosphorylimidazole (*N*-MePIm). The catalytic role of Zn^{2+} is explained on the basis of its ability to lower the electrostatic barrier toward anionic attack on an anionic center and to provide a template for proper alignment of the oxime oxygen and the phosphorus of PIm. The relevance of these studies to the nucleoside diphosphokinase reaction is discussed.

Nucleophilic attack by anionic oxygen at the phosphorus of phosphate monoester dianions is a common enzyme-mediated reaction, the enzymes responsible generally requiring divalent metal ions for activity.[4]

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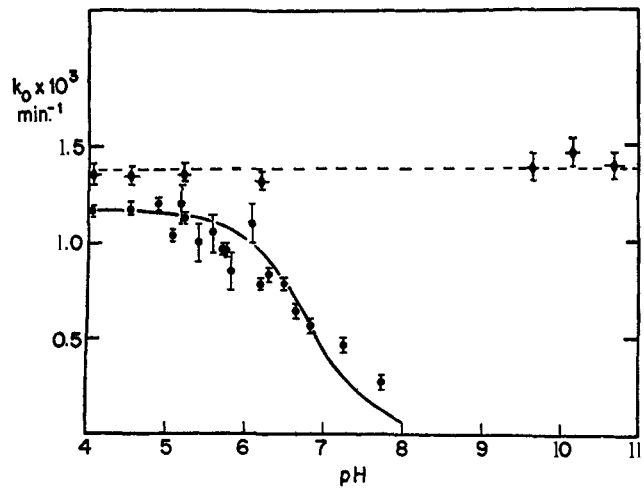

Figure 1. Plot of k_{obsd} for hydrolysis of PIm (●) and of *N*-MePIm (◆) at 40.1° against pH.

Nonenzymatically, attempts to demonstrate such a reaction in the absence of divalent metal ions have met with virtually total failure,[5-7] with the exception of a small reported rate for hydroxide ion attack.[8] In the presence of divalent metal ions phosphate has been shown to attack organic triphosphates,[9,10] and although the conditions under which this reaction is studied are sufficiently complicated as to preclude any detailed mechanistic interpretation of the results, a rate of phosphate transfer substantially greater than that of hydrolysis is indicated.

We wish to report the very rapid nucleophilic attack by the anionic oxygen of Zn^{2+}–pyridine-2-carbaldoxime (Zn^{2+}–PCA) on the dianionic phosphoryl position of monoprotonated phosphorylimidazole (PIm). Our results suggest that the attack proceeds *via* formation of the ternary complex PCA–Zn^{2+}–PIm where the catalytic roles of Zn^{2+} are to (1) overcome the charge repulsion between the phosphoryl group and the oxime anion, and (2) orient the reactive centers properly for reaction to take place. Zn^{2+}–PCA attack on a carbon ester through formation of a ternary complex has been reported previously.[11]

Experimental Section

Materials and Equipment. Ca^{2+}–PIm was prepared by an improved method over that previously described.[12a] A 3-g sample of crude diphosphorylimidazole[12b] was suspended in 200 ml of water and 5 *M* KOH was added to adjust the pH to between 12 and 13, as checked by pHydrion papers. The suspension was heated, with stirring, to 95–100° for 30 min, with pH being frequently checked and maintained between 12 and 13 with 5 *M* KOH. The mixture was then cooled in an ice bath, solid calcium phosphate was filtered off, and the pH of the filtrate was adjusted to 8.5 with 5 *M* HCl. A solution of 2 g of $CaCl_2 \cdot 2H_2O$ in 15 ml of water was added, and, after readjustment to pH 8.5 with 5 *M* KOH a small amount of solid was filtered off. To one portion of filtrate was added three portions of absolute ethanol and the resulting solution was stored at 4° for 4 hr, during which time crude Ca·PIm deposited. This material was filtered off, dissolved in the minimum amount of cold water, and reprecipitated by the addition of three parts of absolute ethanol followed by standing at 4° for 3 hr. The purified material was filtered, washed with 75% ethanol, absolute ethanol, and anhydrous ether, dried in a vacuum desiccator, and stored over Drierite at −20°, yield 71%.

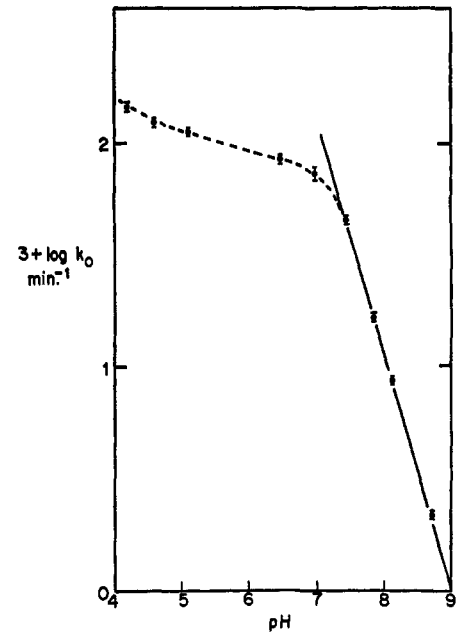

Figure 2. Plot of log k_{obsd} for hydrolysis of PIm at 81.0° against pH.

Paper chromatography showed a single spot whose mobility, relative to inorganic phosphate, was similar to that previously reported.[12a] Phosphate analysis (giving mol wt 227 ± 2), uv spectrum (λ_{max} 210 nm, ϵ_{max} 4.15 × 10^3), and ^{1}H nmr ($\delta_{TSM}^{D_2O}$ 6.99 (d, 1 H), 7.24 (d, 1 H), 7.8 (s, 1 H) are all in accord with the structure Ca·$C_3H_3N_2O_3P \cdot 3H_2O$.

Anal. Calcd: C, 15.01; H, 3.78; N, 11.67; P, 12.90. Found: C, 15.28; H, 3.09; N, 11.56; P, 12.99.

Potassium phosphoramidate was prepared according to the method of Klement and Becht[13] as modified by Chambers and Khorana.[14]

N-Methylphosphorylimidazole (*N*-MePIm) was prepared by incubating *N*-methylimidazole and potassium phosphoramidate as described by Jencks and Gilchrist,[6] and monitoring the extent of reaction with time by the increased lability of aliquots brought to pH 10.7 toward release of inorganic phosphate. In our hands, more concentrated *N*-methylimidazole solutions than those used previously[9] were needed to obtain nearly complete conversion of phosphoramidate to *N*-MePIm.

Pyridine-2-carbaldoxime (PCA) was obtained from Aldrich. Liquid amines were redistilled at atmospheric pressure before use. All other reagents were the purest commercial grade available and were used without further purification. Equipment used is essentially as described previously,[15a] except that ^{31}P nmr spectra were taken on a Varian HA-100 spectrometer.

Since CaPIm is relatively insoluble, concentrated solutions of PIm required prior conversion to the sodium salt, which was accomplished by stirring a solution of CaPIm with Chelex 100 (Na^+ form) (Biorad), filtering off the Chelex, and rotary evaporating the filtrate. Solutions of this material were used for nmr spectra.

Kinetic Procedures. Stock CaPIm solutions (0.03 *M*) were stored at −20° with no detectable hydrolysis after a period of 1 week. Buffer pH values at elevated temperatures were measured by a standard procedure.[15b] Reaction mixtures were generally 0.6 m*M* in PIm and were brought to an ionic strength of 0.5 with KCl. All

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Table I. Relevant Equilibria

	K
$PCAH \rightleftharpoons PCA^- + H^+$	$9 \pm 0.9 \times 10^{-11}$ M,[a] 7×10^{-11} M[c]
$PCAH + Zn^{2+} \rightleftharpoons ZnPCAH^{2+}$	150 ± 15 M^{-1} [a]
$PCA^- + Zn^{2+} \rightleftharpoons ZnPCA^+$	$5 \pm 1.2 \times 10^5$ M^{-1} [a]
$ZnPCAH^{2+} \rightleftharpoons ZnPCA^+ + H^+$	$3 \pm 0.7 \times 10^{-7}$ M,[a] 1×10^{-6} M[d]
$HPIm \rightleftharpoons PIm + H^+$	1×10^{-7} M,[b] 1.6×10^{-7} M[c]

[a] 25°, see ref 11. [b] 39°, $\mu = 1.0$, see ref 6. [c] 40.1°, $\mu = 0.5$, this work. [d] 25°, $\mu = 0.5$, this work.

Table II. k_{obsd} for PIm and N-MePIm Hydrolysis

Compound	pH	Temp, °C	Buffer[a]	$k_{obsd} \times 10^2$, min^{-1}
PIm	2.66	40.1	A	0.26 ± 0.01[b]
	3.10	40.1	A	0.18 ± 0.01[b]
	4.05	40.1	B	0.117 ± 0.002
	4.54	40.1	B	0.119 ± 0.002
	4.88	40.1	C	0.121 ±0.002
	5.07	40.1	B	0.104 ± 0.002
	5.18	40.1	D	0.12 ± 0.001[b]
	5.22	40.1	C	0.113 ± 0.003
	5.41	40.1	E	0.10 ± 0.01[b]
	5.58	40.1	D	0.105 ± 0.010[b]
	5.70	40.1	C	0.097 ± 0.003
	5.75	40.1	C	0.095 ± 0.001
	5.82	40.1	F	0.085 ± 0.010[b]
	6.09	40.1	D	0.110 ± 0.041[b]
	6.19	40.1	C	0.080 ± 0.001
	6.31	40.1	C	0.083 ± 0.001
	6.66	40.1	G	0.064 ± 0.001
	6.85	40.1	G	0.056 ± 0.001
	7.30	40.1	G	0.047 ± 0.001
	7.77	40.1	G	0.026 ± 0.0003
PIm	4.15	81.6	B	14.5 ± 0.7
	4.54	81.6	B	12.5 ± 0.3
	5.07	81.6	B	11.2 ± 0.2
	6.44	81.6	G	8.5 ± 0.3[b]
	6.94	81.6	G	7.5 ± 0.5[b]
	7.41	81.6	G	4.8 ± 0.2[b]
	7.82	81.6	H	1.69 ± 0.03
	8.11	81.6	H	0.88 ± 0.02
	8.71	81.6	H	0.217 ± 0.005
N-MePIm	4.05	40.1	B	0.136 ± 0.005
	4.54	40.1	B	0.136 ± 0.005
	5.22	40.1	C	0.136 ± 0.005
	6.19	40.1	C	0.132 ± 0.005
	9.64	40.1	H	0.139 ± 0.005
	10.15	40.1	I	0.146 ± 0.005
	10.66	40.1	I	0.140 ± 0.005

[a] Buffers used: A triethylenediamine, B 0.1 M acetate, C 0.1 M succinate, D 0.1 M piperazine, E 0.1 M trifluoroethylamine, F 0.1 M 1,2-diamino-2-methylpropane, G 0.1 M N-methylmorpholine, H 0.1 M borate, I 0.1 M PCA. [b] Extrapolated to zero buffer concentration.

runs obeyed first-order or pseudo-first-order kinetics, and were generally followed for two half lives.

Rates of PIm and N-MePIm hydrolysis were measured either spectrophotometrically [by the decrease in absorption at 228 nm (pH 2.5–6.5) or at 230 nm (pH 7–9],[16] or by phosphate appearance, using the modified Martin–Doty procedure.[17] Several checks indicated that both methods gave the same results. pH–rate profiles were determined by extrapolation at each pH to zero buffer concentration.

Rates of phosphate transfer to Zn^{2+}–PCA were determined by measuring the decrease in the quantity, inorganic phosphate plus acid labile phosphate, using the modified Martin–Doty procedure of Jencks and Gilchrist,[17] with the following change. The water–isobutyl alcohol mixture containing the reaction aliquot was not shaken for 5 sec immediately after addition of the 2% ammonium molybdate solution in 2 N sulfuric acid, but rather allowed to stand for 50 min at room temperature with occasional shaking. After this standing period, a final shaking was performed, and the analysis for inorganic phosphate proceeded in the usual manner.[17] Suitable control studies showed that during the standing period all PIm present in the aliquot is fully hydrolyzed, whereas phosphorylated Zn^{2+}–PCA is entirely stable. Thus this procedure provides a measure of inorganic phosphate plus acid-labile phosphate.

Equilibrium constants relevant to this work are summarized in Table I. Constants determined in this study are in reasonable accord with previously reported constants, considering the differences in conditions employed.

Results

PIm and N-MePIm Hydrolysis. The rate of PIm hydrolysis (Table II, Figures 1 and 2) in the pH range 4–9 reflects the amount of PIm monoanion in solution. The decrease in rate above pH 6 is what is to be expected for a PIm monoanion pK of 6.8 and the plot of log k *vs.* pH, with a slope of −1 down to pH 9, is evidence the PIm dianion is at least three orders of magnitude less reactive than the monoanion. Below pH 4 there is a rapid rise in rate, but the possible contributions to this rise (acid catalysis, formation of neutral PIm) have not been resolved.

N-MePIm has a pH-independent rate of hydrolysis in the range 4–10 (Table II, Figure 1), similar in magnitude to the pH-independent rate of PIm hydrolysis. This agrees with earlier results of Jencks and Gilchrist,[6] and constitutes strong evidence that the kinetically important tautomer of PIm monoanion is protonated on an imidazole nitrogen, rather than a phosphoryl oxygen. Evidence that this tautomer is also the dominant one in aqueous solution comes from the observation (Table III) that the ^{31}P chemical shift undergoes hardly any change in the pH range 5–9, whereas protonation of a phosphoryl group is known to result in marked changes in chemical shift.[15,18]

Table III. ^{31}P Chemical Shift in PIm

pH	δ, ppm
9.3	+4.76
7.15	+4.73
5.45	+4.69
4.93	+4.64
2.3	+6.82

Activation parameters for PIm monoanion hydrolysis were obtained from the data in Table IV. $\Delta H^{\ddagger} = +23.5$ kcal/mol and $\Delta S^{\ddagger} = -5.2$ eu. These results are similar to those found for phosphoramidate monoanion hydrolysis.[6]

Phosphate Transfer to Zn^{2+}–PCA. When PIm is dissolved in Zn^{2+}–PCA buffers the phosphoryl group is rapidly transferred to the oxime oxygen. Two lines

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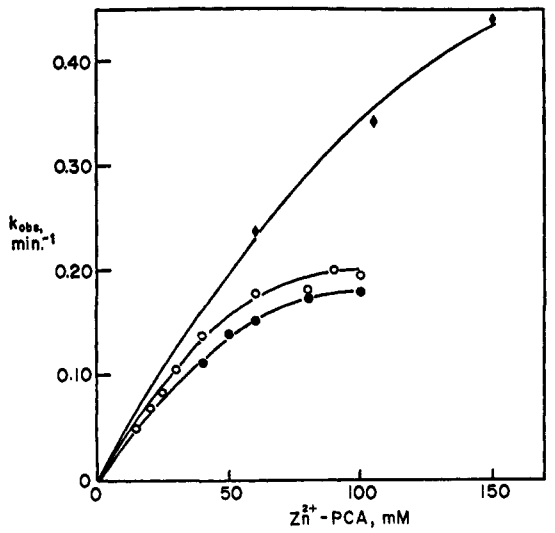


Figure 3. Plot of k_{obsd} for phosphate transfer to Zn^{2+}–PCA from PIm, pH 6.06 (○) and pH 5.73 (●), and from *N*-MePIm, pH 5.63 (◆), against Zn^{2+}–PCA concentration, T = 29.2°. Values of k_{obsd} are accurate within ±5%.

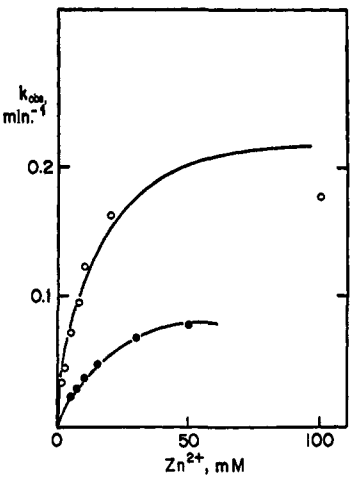


Figure 4. Plot of k_{obsd} for phosphate transfer to Zn^{2+}–PCA from PIm against Zn^{2+} concentration at $[PCA]_t$ = 0.1 *M*, T = 29.2°: ○, pH 6.37 in 0.02 *M* 2,6-lutidine added to maintain pH constant at low Zn^{2+}; ●, pH 4.8, in 0.05 *M* *N*,*N*′-dimethylpiperazine, added to maintain pH constant at low Zn^{2+}. Values of k_{obsd} are accurate within ±5%.

of evidence support this statement. First, the quantity inorganic phosphate plus acid-labile phosphate is found to decrease to zero. Of the possible nucleophiles in solution only phosphorylation of the oxime oxygen could give this result. Second, when PIm and Zn^{2+}–PCA are combined in a ratio of 2:1, it is found that the uv spectrum of an aliquot is virtually unchanged between neutral pH and 0.1 *N* NaOH (Table V). PCA itself undergoes a large change in this range, which is similar to the change between phenol and phenolate ion. The lack of pH dependence and the similarity of the spectrum to that of protonated PCA constitute strong evidence for phosphorylation of the oxime oxygen by PIm.

Table IV. PIm Monoanion Hydrolysis

T, °C	$k_0 \times 10^3$, min^{-1}
40.1	1.19 ± 0.03
60.0	12.1 ± 0.1
81.0	112 ± 2

Table V. Uv Spectral Data

Species	pH	λ_{max}, nm	$\epsilon_{max} \times 10^3$
PCAH	7.41	239	10.1
		277	7.7
PCA	13	290	15.6
Phosphorylated PCA	7.41	240	9.3
		279	7.8
Phosphorylated PCA	13	242	8.8
		278	8.2

For kinetic studies, Zn^{2+}–PCA was always in large excess over PIm so that rates of phosphate transfer were pseudo first order in PIm. Unless otherwise indicated, the reaction mixture was buffered by Zn^{2+}–PCA. Although phosphorylated Zn^{2+}–PCA hydrolyzes faster than PIm,[19] the rate of phosphate transfer to Zn^{2+}–PCA is sufficiently rapid that the hydrolysis could be ignored in calculating rate constants (but see below).

The rate of phosphate transfer reached a saturating value with respect to Zn^{2+}–PCA and, at 0.1 *N* PCA, with respect to Zn^{2+} (Figures 3 and 4). The results of Figure 4, when analyzed according to eq 1–5, using

$$K_{1(app)} = \frac{(Zn\text{–}PCA)_t}{(Zn^{2+})(PCA)} \quad (1)$$

$$K_{2(app)} = \frac{(PCA\text{–}Zn\text{–}PIm)_t}{(PIm)_t(ZnPCA)_t} \quad (2)$$

$$(PIm)_t = (HPIm)^- + (PIm)^{2-} \quad (3)$$

$$(ZnPCA)_t = (ZnPCAH)^{2+} + (ZnPCA)^+ \quad (4)$$

$$(PCA\text{–}Zn\text{–}PIm)_t = (H_2PCA\text{–}Zn\text{–}PIm)^{2+} + (HPCA\text{–}Zn\text{–}PIm)^+ + (PCA\text{–}Zn\text{–}PIm) \quad (5)$$

the values for equilibrium constants listed in Table I, gave values for $K_{2(app)}$ of 62 M^{-1} at pH 6.37 and 53 M^{-1} at pH 4.8. The results of Figure 3 are consistent with these values, but were not used to derive values of $K_{2(app)}$ because of changes in pH on Zn^{2+}–PCA dilution.

Phosphorylated Zn^{2+}–PCA hydrolysis was found to be accelerated as the pH was increased above six, and by pH 6.5 was rapid enough that the kinetic method used to measure the rate of phosphate transfer to Zn^{2+}–PCA gave deviations from first-order dependence at times greater than one half-life. A possible explanation for the increased rate is the formation of a highly reactive hydroxy Zn^{2+} complex,[20] but this point was not pursued in detail because of the formation of precipitates in Zn^{2+}–PCA solutions at pH values >7. Reliable values of k_{obsd} for phosphate transfer could thus be obtained only at pH values below 6.5.

The pH–rate profile at 0.1 *N* Zn^{2+}–PCA is shown in Figure 5 and the data are presented in Table VI. At this concentration of Zn^{2+}–PCA virtually all of the PIm in solution is present as the ternary PCA–Zn^{2+}–

(19) In 0.1 *M* Zn^{2+}–PCA at 40°, pH 5.7, k_{obsd} for phosphorylated PCA hydrolysis is 5.7 × 10^{-3} min^{-1}.

(20) The pK_a for $[H_2O\text{–}Zn\text{–}PCA]^+ \rightleftharpoons HOZnPCA + H^+$ is 7.7 (ref 11).

Table VI. k_{obsd} for Phosphate Transfer from PIm and *N*-MePIm to Zn^{2+}–PCA[a]

Compound	pH	$-k_{obsd} \times 10^2$, min^{-1}— 19.5°	29.2°
PIm	3.96[b]		1.6
	4.51[b]		4.0
	4.88[b]		5.7
	5.08[b]		6.6
	5.40		14.0
	5.58		16.0
	5.75		17.8
	5.95		19.5
	6.0[c]		19.5
	6.06		19.5
	6.21		18.3
	6.32		17.8
	6.37[d]		17.8
	6.57		17.8
N-MePIm	4.09[b]		3.5
	4.62[b]		7.4
	4.89[b]		11.4
	5.15[b]		12.9
	5.65		32
	5.83		41
	5.95	24	42[e]
	6.03	31	45[e]
	6.06		45
	6.21	31	
	6.55	34	54[e]

[a] At 0.1 *N* Zn^{2+}–PCA. Runs are self-buffered unless otherwise indicated. Rate constants are accurate within ±5%. [b] Added buffer 0.1 *M* *N*,*N*′-dimethylpiperazine. [c] In presence of 0.05 *M* dimethylpiperazine. [d] Added buffer 0.1 *M* 2,6-lutidine. [e] Estimated (see text).

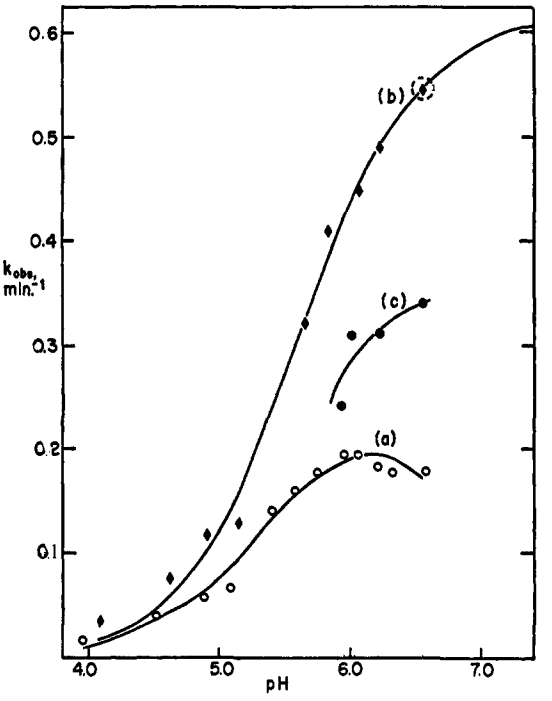


Figure 5. Plot of k_{obsd} for phosphate transfer to Zn^{2+}–PCA from PIm (a) (○) and *N*-MePIm (b) (◆) at 29.2°, and from PIm (c) (●) at 19.5° at 0.1 *N* Zn^{2+}-PCA. Lines are theoretical (see text). The point at pH 6.55 in curve (b) is estimated, not measured.

PIm complex. In the pH range of interest there will be four differently protonated forms of the ternary complex. Taking X as this symbol for the ternary complex, these are XH_2, in which both oxime oxygen and imidazole rings are protonated, XH, in which only the imidazole ring is protonated, HX, in which only the oxime oxygen is protonated, and X, in which neither group is protonated. Curve (a) of Figure 5 is a theoretical curve derived from the assumption that only XH or its kinetic equivalent (see Discussion) is reactive. The relevant equations are

$$\text{rate} = k(XH^+) = k_{obsd}(X)_t \quad (6)$$

$$(X)_t = (H_2X^{2+}) + (XH^+) + (HX^+) + (X) \quad (7)$$

$$k_{obsd} = \frac{kK_1}{K_1 + K_2 + (a_{H^+}) + [K_1K_{12}/(a_{H^+})]} \quad (8)$$

$$K_1 = (a_{H^+})(XH^+)/(H_2X^{2+})$$

$$K_2 = (a_{H^+})(HX^+)/(H_2X^{2+}) \quad (9)$$

$$K_{12} = (X)(a_{H^+})/(XH^+)$$

A computerized least-squares fit to the data[21] gave values for kK_1, $K_1 + K_2$, and K_1K_{12}, and, making the assumption that $K_2 = K_{12}$, gave $k = 0.28 \pm 0.05$ min^{-1}, $K_1 = 3.80 \pm 0.8 \times 10^{-6}$ *M*, and $K_2 = 1.5 \pm 0.3 \times 10^{-7}$ *M*.

The pH–rate profile for phosphate transfer from *N*-MePIm to Zn^{2+}–PCA, at 0.1 *N* Zn^{2+}–PCA, is also shown in Figure 5 and Table VI. If X′ is taken as the symbol for the ternary complex PCA–Zn^{2+}–NMePIm, then there are two forms of the complex in the pH range of interest, HX′, where the oxime oxygen is protonated, and X′, where it is not. Curve (b) of Figure 5 was calculated on the basis that only X′ is reactive. A computerized least-squares fit to the data[21] gave a value for $K = (X')(a_{H^+})/(HX')$ of $2.57 \pm 0.30 \times 10^{-6}$ which is similar to the value for K_1 reported above. k, the rate constant for phosphate transfer in X′, was determined as 0.61 ± 0.07 min^{-1}. Since, from Figure 3, 0.1 *N* Zn^{2+}–PCA is insufficient to saturate *N*-MePIm this value of k is a factor of 2–3 less than the true rate constant in the ternary complex. At pH's above 6.1 rates became too rapid for accurate measurement at 29.2° and instead were measured at 19.5° (Figure 5, curve (c)). The data at the lower temperature are important because they show that k_{obsd} begins to level off at high pH. A comparison of curves (b) and (c) below pH 6.1 leads to a calculated rate increase of 1.60 ± 0.15-fold on going from 19.5 to 29.2°. This factor was then multiplied by the measured k_{obsd} at pH 6.55, 19.5°, to yield an estimated k_{obsd} at pH 6.55, 29.2° (Table VI).

As controls, *N*-MePIm and PIm were hydrolyzed in PCA buffers at pH 10.2 and 10.6 in the absence of Zn^{2+}. The quantity inorganic phosphate plus acid-labile phosphate remained constant during the run, indicating a total lack of PCA phosphorylation at the oxime oxygen. Similarly, the rate of phosphate appearance was unaffected by PCA which rules out phosphorylation at the pyridine nitrogen, since phosphorylated pyridines show up in the modified Martin–Doty assay as inorganic phosphate. This leaves phosphorylation of the oxime nitrogen as a possibility, but only if it both hydrolyzes at a rate virtually identical with that of *N*-MePIm, and is analyzed as acid-labile phosphate in the modified Martin–Doty assay.

(21) M. H. Lietzke, "A Generalized Least Squares Program for the IBM 7090 Computer," ORNL-3259, Office of Technical Service, U. S. Department of Commerce, Washington, D. C.

However, the central finding of the control experiment, that the oxygen of PCA anion does not attack zwitterionic phosphoramidate (whether *N*-MePIm or phosphorylated oxime nitrogen) at a measureable rate in the absence of Zn^{2+} is independent of the extent of phosphorylation of the oxime nitrogen.

PIm hydrolysis in the present of 0.12 *M* Zn^{2+} actually showed a slight rate depression.[22]

The variation of k_{obsd} at pH 6.0 with temperature for transfer to ZnPCA is shown in Table VII. From eq 8

Table VII. Variation of k_{obsd} for Phosphate Transfer from PIm to Zn^{2+}–PCA with Temperature at pH 6.0, 0.1 *N* Zn^{2+}–PCA

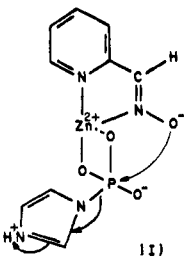
Temp, °C	k_{obsd}, min^{-1}
29.0	0.22 ± 0.02
17.8	0.089 ± 0.008
8.6	0.045 ± 0.004

the variation of k_{obsd} with temperature may reflect changes in both the rate constant and several ionization constants. However, over the limited temperature range employed it is doubtful that the changes in the ionization constants could result in a shift of the pH–rate profile by more than a few tenths of a pH unit. From the broadness of the peak near pH 6.0, it is a reasonable assumption that the observed temperature dependence of k_{obsd} reflects principally the temperature dependence of the rate constant. Thus, the data in Table VII can be used to calculate approximate values for activation parameters, giving $\Delta H^{\pm} = +12.6$ kcal/mol, $\Delta S^{\pm} = -28.3$ eu.

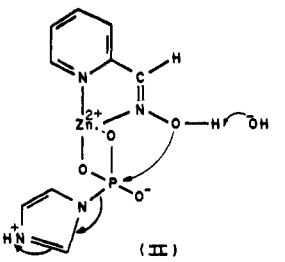
Discussion

The results presented are in accord with the mechanisms for phosphate transfer shown in I or II.[23a]

The formation of a ternary complex, with Zn^{2+} serving as a bridge between the PCA and PIm, is indicated by the observation of hyperbolic saturation kinetics (Figures 3 and 4) as well as by the lack of a transfer reaction in the absence of Zn^{2+}. The very



(II)



(III)

small difference in $K_{2(app)}$ at pH's 4.8 and 6.4 strongly suggests that the imidazole moiety of PIm is not bound to Zn^{2+} in the ternary complex. Furthermore, the magnitude of $K_{2(app)}$ (62 M^{-1}) is quite similar to what has been found for the analogous binding of Zn^{2+}–2,2-bipyridyl to phosphate monoesters dianions (200 M^{-1}),[24] especially considering that $K_{2(app)}$ is determined at high KCl concentrations. Thus, it seems reasonable to suppose that PIm is bound to Zn^{2+}–PCA through its phosphoryl dianion moiety. The extreme stability of PIm dianion to hydrolysis suggests the monoprotonated form is the reactive species in the transfer reaction, and this is supported by the downturn in rate above pH 6.1 for transfer from PIm, which is absent for transfer from *N*-MePIm. Unfortunately solubility problems and the increased reactivity of phosphorylated PCA above pH 6.6 prevented a clearer demonstration of this point. The downturn in rate below pH 6, seen for both PIm and *N*-MePIm, can be reasonably assigned to the need for an anionic oxime oxygen, as in I, or to specific base catalysis, as in II. Mechanisms I and II are kinetically equivalent, and there is as yet no basis for a firm choice between them. Lack of general base catalysis by 2,6-lutidine or *N*,*N*′-dimethylpiperazine monocation (Table V) is evidence against II, but might merely imply that base catalysis proceeded with a high Brønsted β. Also II would require a very fast second-order rate constant, about 10^6 M^{-1} sec^{-1} at 29.2°,[25] but one that is clearly within the diffusion controlled limit. On the other hand, II would be expected to have a less unfavorable charge–charge repulsion interaction. The difference in activation parameters for hydrolysis and phosphate transfer are also consistent with I or II. The lower $\Delta H^{\pm}$ for transfer is as expected for attack of the more powerful oxime anion nucleophile, while the more negative $\Delta S^{\pm}$ for transfer is reasonable in view of the requirement for precise orientation of groups in I and II.

Using the $\Delta H^{\pm}$ for PIm hydrolysis to extrapolate the rates of PIm hydrolysis and *N*-MePIm hydrolysis to 29° leads to calculated rates at this temperature of 0.3×10^3 min^{-1} for PIm hydrolysis and 0.35×10^3 min^{-1} for *N*-MePIm hydrolysis. Compared to hydrolysis then, the transfer reaction to Zn^{2+}–PCA in the ternary complex is 1×10^3 times faster for PIm and approximately 4×10^3 faster for *N*-MePIm.

We believe that the high rate of phosphate transfer in the ternary complexes X and X′ supports a double catalytic role for Zn^{2+}. First, as a template, to bring the phosphoryl group and oxime anion together with a favorable orientation for intramolecular reaction (the so-called proximity effect[26]), and second, probably more importantly, to shield the negative charge of the phosphoryl group and thus lower the electrostatic barrier toward attack by the negatively charged oxime anion. The importance of this second effect is emphasized by the almost total lack of evidence for anionic oxygen attack at phosphoryl dianions.[5] Although similar mechanisms for metal ion catalysis of such reactions have been proposed previously[9,27,28] this study represents the first example where there is both good evidence for transfer through a ternary complex and a very large catalysis by divalent metal ion. A catalytic role for Zn^{2+} emphasizing its ability to increase the positive character of phosphorous through coordination to phosphoryl oxygen is quite unlikely in view of the find-

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(23) (a) We have no evidence that PIm acts as a bidentate ligand for Zn^{2+}, but this mode of binding has been found in similar complexes.[23b] (b) F. Lincoln and D. R. Stranks, *Aust. J. Chem.*, 21, 57 (1968).

(24) H. Sigel, D. B. McCormick, and K. Becker, *Biochem. Biophys. Acta.*, 148, 655 (1967).

(25) Calculated by setting $k(XH^+)$ in eq 5 equal to $k_2(H_2X^{2+})(OH^-)$.

$$k_2 = k(XH^+)/(H_2X^{2+})(OH^-) = kK_1/K_w$$

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(28) A. E. Martell, *Advan. Chem. Ser.*, 37, 161 (1963).

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 pK 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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