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Catalysis by Imidazolium Ion and Metal Ions in the Reaction of a Phosphate Diester

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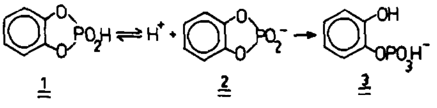
The rates of reaction of catechol cyclic phosphate in water and in acetonitrile–water demonstrate that imidazolium ion and metal ions (Na^+, Mg^{2+}, Zn^{2+}) cause significant accelerations. These studies provide models for the potential role of cations in catalysis of reactions of phosphate anions by enzymes. In catalysis by Zn^{2+}, we find that two to three imidazoles are required for coordination to Zn^{2+} for most effective catalysis. Enough water must be present to solvate imidazole and coordinate to Zn^{2+}, indicating that a coordinated H_2O is the nucleophile in Zn^{2+} catalysis. Product analysis also supports this conclusion.

INTRODUCTION

The importance of phosphate esters in biological systems has led us to investigate the fundamental principles involved in reactions at phosphorus (*1*). X-ray structures and catalytic models exist for the enzymes ribonuclease (*2*, *3*) and staphylococcal nuclease (*4*, *5*), and there are many other enzymes which hydrolyze phosphate esters and need to be understood, such as the phosphodiesterase which inactivates cyclic AMP. This report is part of continuing research on the fundamental chemistry of catalysis including understanding catalysis and reaction pathways in systems containing limited amounts of water. Under these conditions, we hope to mimic the conditions in enzymes with active sites which contain little or no water. Since much of our fundamental knowledge of biologically relevant reactions comes from studies in aqueous solution, we need to understand the perturbations caused by limiting the amount of water if we are to understand completely the functional roles of the various structural features of enzymic active sites (*6*).

We have studied the reaction of catechol cyclic phosphate (**2**) in water, in acetonitrile with varying amounts of water, and with various electrophiles present (*7*). This substrate (**2**) has the advantages of being a model for phosphate anions in biochemistry, reacting rapidly enough for convenient studies, and favoring reaction at phosphorus because of the high barrier to nucleophilic aromatic substitution which is required for cleavage of C–O bonds (8). The rates of cleavage of **2** by hydroxide (*9*) and by chymotrypsin (*10*) have been published, and derivatives have found utility as phosphorylating agents (*11*, *12*).

In this research, we have been able to observe effects which are relevant to understanding the role of electrophiles in enzymes which catalyze reactions at phosphorus,



especially those enzymes which contain Zn^{2+} at the active site. The list of such enzymes is frequently changed due to the difficulties and pitfalls in ascertaining that any enzyme is truly a zinc metalloenzyme. However, it appears to include alkaline phosphatases, nucleic acid polymerases, and phosphodiesterases. A recent review has summarized the information on Zn^{2+} in enzymes and chemical systems (*13*). Relatively little is known about the fundamental chemistry of Zn^{2+} and phosphates.

EXPERIMENTAL

Catechol cyclic phosphoric acid (**1**) was synthesized from catechol (Aldrich, 99+ %) and P_2O_5 (Mallinckrodt, analytical grade) (*9*, *14*). The phosphate was redistilled three times before its use in kinetic studies. It was stored in a desiccator in the dark because of reports that it may be light sensitive (*15*).

Materials. Imidazole (Pfaltz and Bauer or Aldrich, 99 %) was recrystallized three times from benzene (mp 89.5–90.0°C, uncorrected). *n*-Butylamine (Aldrich) was distilled through a Hempel column and stored over 4A molecular sieve. Hydrochloric acid (Harleco), perchloric acid (Baker, analyzed at 71.3 %), $(CH_3)_4NBr$ and $NaClO_4 \cdot H_2O$ (Fisher), $BaCl_2$, $MgCl_2$, NaCl, and KCl (Mallinckrodt, analytical), $Mg(ClO_4)_2$ (Mallinckrodt), and $Zn(ClO_4)_2$ (Amend, hydrated reagent) were used without further purification. Water was distilled and freed of CO_2 by boiling and cooling under N_2. Tetrahydrofuran (Mallinckrodt, stabilized analytical) and acetonitrile (Matheson, spectral) were stored over 4A molecular sieve.

Rate measurements. Stock solutions of imidazole/imidazolium buffer were prepared by weighing out the proper amount of amine, adding approximately three-fourths of the water required, adjusting to the proper pH with acid, diluting to volume, and then measuring the final pH with a Radiometer Model 26 pH meter. Stock solutions of imidazole and *n*-butylamine in acetonitrile–water were mixed by calculating the proper amounts of amine and perchloric acid necessary to give the desired ratio of protonated to unprotonated amine; pH values of these solutions were obtained after diluting with water. Catechol cyclic phosphate was warmed on a small hot plate to melt it, so that it could be weighed out and a stock solution prepared for each set of rate measurements; normally 0.1132 g of phosphate was dissolved in 2 ml of THF. Stock solutions were discarded after 2 hr.

The rate of hydrolysis of **2** was measured spectrophotometrically at 290 or 293 nm using a Hitachi 139 uv-vis spectrophotometer equipped with a thermostated sample compartment maintained at 30.0°C by means of a circulating water bath. The change in transmittance with time was printed out at appropriate intervals by a Digetec digital printer interfaced to the Hitachi spectrophotometer. Buffered media, prepared as described above, were pipetted into a stoppered cell and equilibrated in the Hitachi sample compartment for 15 min. A syringe was used to deliver 10 μl of a 0.329 *M* solution of **2**; the final concentration was 10^{-3} *M*. The cell was shaken vigorously and replaced in the sample compartment, and readings were taken. Generally, the pseudo-first-order rate constants were calculated based on 20 transmittance–time pairs taken from the first one to two half-lives of the reaction. Data were analyzed to give estimates for the rate constant, infinity point, and intercept. Experience with the reactions reported here demonstrated problems with measured infinity points. Therefore, the estimated values were refined iteratively using Taylor's expansion as applied to the integrated first-order rate equation. For the results reported here, iterations were performed until the values of two successive estimates of the unknowns were within 0.5% (*16*). Second-order rate constants were calculated from a given series of pseudo-first-order rate constants according to the equation $k_{obs} = k[X] + C$ by a digital computer program using the method of least squares.

Product analysis. After hydrolysis of **2**, water was evaporated; the residue was dissolved in acetonitrile, dried with anhydrous sodium sulfate, and recrystallized three times from acetonitrile–benzene. The recrystallized *o*-hydroxyphenyl phosphate (mp (136–139°C) (*9*) (**3**) was dissolved at an equivalent concentration to that of **2** in a kinetic run; uv spectra of the known product were compared on a Cary 14 spectrophotometer to the spectra obtained in rate determinations. These spectra supported hydrolysis as the predominant reaction.

RESULTS

Accuracy and Reproducibility

For most kinetic results reported here the accuracy and reproducibility were such that the standard deviations of the calculated rate constants were within 5 to 10%. However, catechol cyclic phosphate is not easy to handle and we suspect that problems of polymerization, hydrolysis, oxidation, and light sensitivity caused the large standard deviations that were frequently observed. A further complication is that at low pH the change in absorbance is small. We have removed rate constants for which the standard deviation was greater than one-third of the observed rate constant. It is clear that the inaccuracy of the rate constants is considerably greater than the standard deviation.

Salt Effects

Table 1 presents the results of ionic strength measurements using $(CH_3)_4NBr$ at pH 7.55 and $NaClO_4$ at pH 8.3. There is no significant dependence on $(CH_3)_4NBr$ but $NaClO_4$ causes slight catalysis.

Amines in Water

The dependence on buffer concentration at ratios of imidazole to imidazolium of 4/1, 3/1, 2/1, and 1/1 is presented in Table 2. There is no dependence at the 4/1 ratio (the slight negative slope is due to the slight negative salt effect at this pH), a very small dependence at 3/1, and a large nonlinear dependence at 2/1 and 1/1. To insure that the dependence at 2/1 and 1/1 was not due to salt effects, the experiment at 1/1 imidazole/imidazolium was repeated at a constant ionic strength of 1 *M* maintained with $NaClO_4$ (Table 3). It seems clear that the rate acceleration is due to a specific effect of imidazolium ion.

TABLE 1

SALT EFFECTS IN AQUEOUS SOLUTION, 30.0°C

$[(CH_3)_4NBr]$ (*M*) at pH 7.55[a]	$10^5\ k(\mathrm{sec}^{-1})$	$[NaClO_4]$ (*M*) at pH 8.30[a]	$10^5\ k(\mathrm{sec}^{-1})$
0.050	4.4 ± 0.1	0.100	14.9 ± 1.7
0.100	4.8 ± 0.4	0.400	11.9 ± 1.1
0.150	4.4 ± 0.3	0.800	15.9 ± 3.5
0.200	4.4 ± 1.2	1.00	19.6 ± 1.1
0.250	4.0 ± 0.2	1.20	22.6 ± 1.3
0.300	4.2 ± 0.3		
0.700	5.0 ± 0.2		
0.800	3.3 ± 0.1		

[a] pH maintained by imidazole buffer.

TABLE 2

DEPENDENCE ON BUFFER CONCENTRATION AT VARYING RATIOS OF IMIDAZOLE/IMIDAZOLIUM

Total [imidazole] (*M*)	$10^5\ k(\mathrm{sec}^{-1})$[a]	Total [imidazole] (*M*)	$10^5\ k(\mathrm{sec}^{-1})$[a]
4/1, pH 7.55, with Cl^- as the counter ion		3/1, pH 7.43, with ClO_4^- as the counter ion	
0.050	4.82 ± 0.43	0.100	10.7 ± 0.86
0.100	3.95 ± 0.07	0.200	9.21 ± 0.81
0.300	3.68 ± 0.09	0.600	14.4 ± 0.80
0.500	3.05 ± 0.14	0.800	11.3 ± 0.85
0.700	3.07 ± 0.09	1.00	13.4 ± 6.44
		1.20	16.4 ± 3.51
2/1, pH 7.25, with ClO_4^- as the counter ion		1/1, pH 6.95, with ClO_4^- as the counter ion	
0.100	7.92 ± 0.21	0.200	5.00 ± 0.46
0.200	5.44 ± 0.26	0.600	13.6 ± 0.88
0.600	19.9 ± 1.70	0.800	18.5 ± 1.4
0.800	16.4 ± 1.25	1.00	34.6 ± 3.6
1.00	27.0 ± 2.22	2.00	158.0 ± 7.3
1.20	57.0 ± 7.00		

[a] ± Values are standard deviations.

Metal Ion Catalysis in Water

We looked at metal ion effects (metals as perchlorate salts) in 1 *M* total imidazole at a ratio of imidazole/imidazolium of 1/1. From Table 4, we estimate relative rate enhancements for sodium:magnesium:zinc = 1:4:33. The rate due to the imidazole buffer

TABLE 3

DEPENDENCE ON 1/1 IMIDAZOLE/IMIDAZOLIUM PERCHLORATE AT 1 *M* CONSTANT IONIC STRENGTH[a]

Total [imidazole] (*M*)	$10^5 k(\text{sec}^{-1})$
0.100	1.05 ± 0.16
0.400	7.02 ± 0.25
0.800	15.6 ± 1.0
1.00	22.7 ± 1.6
1.40	57.2 ± 6.9
1.80	104.0 ± 11.0
2.00	158.0 ± 7.00

[a] Ionic strength maintained by added $NaClO_4$.

TABLE 4

METAL ION CATALYSIS IN WATER (IMIDAZOLE BUFFER, pH = 6.95[a])

Concentration (*M*)	$10^4 k(\text{sec}^{-1})$
Sodium perchlorate	
0.000	3.51 ± 0.23
0.01	4.44 ± 0.27
0.05	6.38 ± 0.79
0.10	7.32 ± 1.32
Magnesium perchlorate	
0.010	6.88 ± 0.37
0.080	8.41 ± 0.64
0.100	11.2 ± 0.32
0.250	20.9 ± 3.66
Zinc perchlorate	
0.000	3.46 ± 0.36
0.010	7.78 ± 1.56
0.100	21.6 ± 3.75
0.200	83.4 ± 1.61
0.250	119.0 ± 0.02

[a] Total concentration of imidazole = 1 *M*, ratio of imidazole/imidazolium perchlorate = 1/1.

alone at this pH is about 3.5×10^{-4} sec^{-1} compared to 120×10^{-4} sec^{-1} if 0.25 *M* zinc is added, a 34-fold increase.

Zinc perchlorate was soluble at very low concentrations and at high concentrations over the range of 0.01 to 0.35 *M*, but at intermediate concentrations a precipitate forms, possibly due to a zinc–phosphate complex.

Studies in Acetonitrile–Water

Effect of solvent. In a 1/1 imidazole/imidazolium buffer, the rate of reaction of **2** in acetonitrile containing 1 *M* water is about half of the rate in water (see Table 2); at 0.80 *M* total buffer, $k = (10.8 \pm 0.4) \times 10^{-5}$ sec^{-1} and at 1.00 *M*, $k = (17.5 \pm 0.6) \times 10^{-5}$ sec^{-1}.

Metal ion effects. All metal ion experiments in acetonitrile–water used the metals as perchlorate salts for reasons of adequate solubility. With *n*-butylamine (Table 5), the relative rate of reaction with and without 1.0 *M* zinc (the standard state) is $10^3/1$. Since

TABLE 5

DEPENDENCE ON $Zn(ClO_4)_2 \cdot 6H_2O$ IN ACETONITRILE + 1 *M* WATER[a]

$[Zn(ClO_4)_2 \cdot 6H_2O]$ (*M*)	$10^3\ k$(sec^{-1})
0	0.10
0.010	1.20 ± 0.32
0.020	2.03 ± 0.07
0.040	5.14 ± 0.09
0.050	6.77 ± 0.34
0.100	13.9 ± 1.27

[a] 1 *M* total concentration of *n*-butylamine, ratio of amine/ammonium perchlorate = 1/1.

TABLE 6

METAL ION CATALYSIS IN ACETONITRILE + 1 *M* WATER, IMIDAZOLE BUFFER[a]

Metal salt	[Metal salt] (*M*)	$10^3\ k$(sec^{-1})
None	0.00	0.175 ± 0.006
$Zn(ClO_4)_2 \cdot 6H_2O$	0.05	1.13 ± 0.12
$Zn(ClO_4)_2 \cdot 6H_2O$	0.15	3.39 ± 0.28
$Zn(ClO_4)_2 \cdot 6H_2O$	0.20	5.99 ± 0.92
$NaClO_4 \cdot H_2O$	0.15	3.91 ± 0.49
$NaClO_4 \cdot H_2O$	0.20	3.83 ± 0.19
$NaClO_4 \cdot H_2O$	0.25	3.07 ± 0.09
$Mg(ClO_4)_2$	0.05	2.04 ± 0.09
$Mg(ClO_4)_2$	0.10	3.04 ± 0.08
$Mg(ClO_4)_2$	0.15	3.27 ± 0.74
$Mg(ClO_4)_2$	0.20	2.86 ± 0.85

[a] 1 *M* total concentration of imidazole, ratio of imidazole/imidazolium perchlorate = 1/1.

the zinc salt was the hexahydrate, adding zinc also added water which must be added to the 1 *M* water to give the total water concentration. However, as noted in the above paragraph and in Table 7, water does not have a large effect on the rate. The zinc salt was not soluble in concentrations above 0.1 *M*, presumably because an *n*-butylamine complex is required to keep zinc hydroxide from precipitating.

TABLE 7

DEPENDENCE ON WATER CONTENT IN ACETONITRILE

$[H_2O]$ (*M*)	10^3 k(sec^{-1})
A. 1 *M* total concentration on *n*-butylamine, ratio of amine/ammonium perchlorate = 1/1, 0.02 *M* $Zn(ClO_4)_2 \cdot 6H_2O$	
1.00	2.03 ± 0.07
2.00	2.99 ± 0.10
3.00	4.28 ± 0.12
4.00	4.96 ± 0.07
5.00	5.79 ± 0.22
7.00	7.24 ± 0.51
10.0	9.35 ± 0.57
B. 1 *M* total concentration of imidazole, ratio of imidazole/imidazolium perchlorate = 1/1, 0.05 *M* $Zn(ClO_4)_2 \cdot 6H_2O$	
1.00	1.1 ± 0.1
2.00	3.2 ± 1.0
3.00	4.3 ± 0.6
4.00	1.9 ± 0.6
5.00	3.0 ± 0.3
7.00	1.6 ± 0.1
10.0	2.7 ± 0.5

With an *imidazole buffer* we also find catalysis by Zn^{2+} (Table 6) but Na^+ and Mg^{2+} have complex effects. It appears that there is a catalytic effect of these metal ions by comparison with the rate with no metal ion present, but increased concentrations decrease the rate. We conclude that one must consider not only the interaction of metal ion with substrate but also with imidazole and water. Therefore, the water (Table 7) and imidazole (Table 8) concentrations were varied with zinc ion present.

Dependence on water concentration. The dependence of rate on the concentration of water was measured in *n*-butylamine (0.02 *M* $Zn(ClO_4)_2 \cdot 6H_2O$ and in imidazole (0.05 *M* zinc salt) buffers (Table 7). In both cases, the total concentration of amine plus protonated amine was 1 *M* and the ratio of unprotonated to protonated amine was 1/1 with perchlorate as the counter ion. Although the imidazole data show considerable scatter due to the problems of accuracy which are inherent in this system, it appears that the effect on rate due to increased water concentration is small in both cases.

TABLE 8

DEPENDENCE ON IMIDAZOLE IN ACETONITRILE + 1 *M* TOTAL WATER[a]

Total [imidazole] (*M*)	$10^3\,k(\mathrm{sec}^{-1})$
A. 0.10 *M* $Zn(ClO_4)_2 \cdot 6H_2O$ + 0.40 *M* water	
0.400	No change in absorbance after 20 000 sec
0.800	1.77 ± 0.44
1.00	5.97 ± 0.24
1.20	5.84 ± 0.32
1.40	6.17 ± 0.34
1.80	7.53 ± 0.53
B. 0.05 *M* $Zn(ClO_4)_2 \cdot 6H_2O$ + 0.70 *M* water	
0.200	No change in absorbance after 10 700 sec
0.400	0.29 ± 0.08
0.800	2.97 ± 0.27
1.00	4.36 ± 0.16
1.40	5.49 ± 0.70
1.80	5.86 ± 0.37

[a] Ratio of imidazole/imidazolium = 1/1, maintained with ClO_4^-.

Dependence on imidazole/imidazolium concentration. The dependence of rate on 1/1 imidazole was measured in acetonitrile with 0.1 *M* and 0.05 *M* $Zn(ClO_4)_2 \cdot 6H_2O$ with enough added water to bring the total amount of water to 1 *M*. The data in Table 8 and Fig. 1 show that concentrations of imidazole equal to approximately three times the concentration of zinc salt are required for a measurable rate.

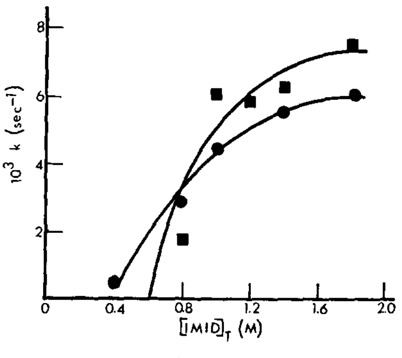


FIG. 1. Dependence of imidazole in acetonitrile + 1 *M* water (ratio of imidazole/imidazolium perchlorate = 1/1). ■, 0.10 *M* $Zn(ClO_4)_2 \cdot 6H_2O$ + 0.40 *M* water; ●, 0.05 *M* $Zn(ClO_4)_2 \cdot 6H_2O$ + 0.70 *M* water.

DISCUSSION

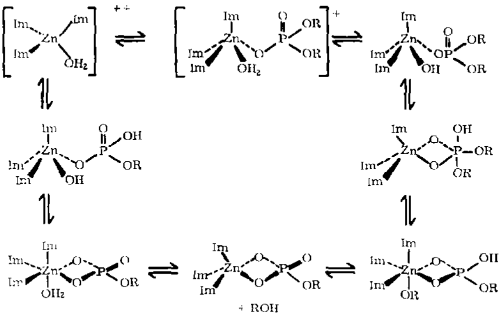
Many enzymes which catalyze reactions at phosphorus appear to utilize positively charged groups such as Ca^{2+}, Zn^{2+}, imidazolium ion, guanidinium ion, and the ammonium ion of lysine. An important question concerns the magnitude of catalysis by these electrophilic cations. Although **2** turned out to be a troublesome substrate because of the problems mentioned in the Introduction, the results in this paper demonstrate the catalytic potency of positive ions with an anionic phosphate substrate; sodium, imidazolium, zinc, and magnesium ions have been shown to be catalytic.

Nucleophilic catalysis by imidazole does not seem to be competitive with attack by water (Table 2). Product analysis (Experimental section) and the results indicate that a water molecule is the predominant nucleophile both with and without electrophilic catalysis. If the previously published rate of attack by HO^- on **2** is extrapolated to pH 7, we get k (at 25°C) $= 9 \times 10^{-8}$ sec^{-1}, considerably less than any of the rates we have found (*9*).

The kinetically indistinguishable alternative to H_2O attack on **2** is HO^- attack on **1**. Since the pK_a of **1** should be approximately 1.0 (*17*), at pH 7 the rate constant for $v = k'[HO^-]$ [**1**] would be approximately (4×10^{-5}) $([\mathbf{2}]/[\mathbf{1}]) = 4 \times 10^{-11}$ sec^{-1}. This is below the diffusion-controlled limit for nucleophilic attack of HO^- on a neutral molecule (in contrast to proton transfer from a cation to HO^-).

We suggest that the initial rise in rate on increase in water concentration (Table 7) is due to the need to have a water in the coordination sphere of Zn^{2+}; this water molecule probably acts as a nucleophile (*13*, *18*). Large increases in concentration of water appear to cause a decrease in rate of catalysis by zinc. We suggest that this is due to competition by water and substrate for a coordination site on the zinc.

The results in Table 8 demonstrate that *the catalytic potency of zinc is strongly affected by imidazole*! Figure 1 indicates that two to three imidazoles must coordinate to Zn^{2+} before it becomes catalytic. Since imidazole residues of histidines are involved in binding Zn^{2+} in metalloenzymes and the coordination number found in Zn^{2+}-enzymes by crystallography is 3 (*13*), this seems to be a very significant observation. We have found a similar result with another phosphorus substrate (*5*). The saturation of rate (Fig. 1) on adding excess imidazole is probably due to competition between substrate and imidazole for coordination to Zn^{2+}.



SCHEME 1. Catalysis of hydrolysis of **2** (Im = imidazole).

Tentatively, our results and the literature (*13*) suggest the hypothesis that catalysis by Zn^{2+} in metalloenzymes may involve equilibria between tetra-, penta-, and possibly hexacoordinate zinc as the substrate moves along the reaction coordinate. This hypothesis helps explain the biological importance of zinc ions in metalloenzymes: Not only does the zinc have the advantage of an intense field due to its small ionic radius, but the filled shell of *d* electrons minimizes the barriers to changes in coordination number and geometry. Scheme 1 is an example of this hypothesis as applied to phosphate diesters. A similar scheme could be written to describe the mechanism of action of carboxypeptidase, carbonic anhydrase (*19*), and other Zn^{2+}-metalloenzymes (*19*). Scheme 1 is meant primarily to exemplify the possible value of changes in coordination number and geometry and to suggest that bond-making and bond-breaking could be facilitated if they occur within the coordination sphere around zinc; this also may help explain the high turnover numbers of Zn^{2+}-metalloenzymes (*13, 19*).

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