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A Model for the Role of Metal Ions in the Enzyme-Catalyzed Hydrolysis of Polyphosphates*

Barry S. Cooperman†

ABSTRACT: Several new compounds containing pyrophosphate linkages and other oxygen and nitrogen ligands have been synthesized and purified. It was thought that the presence of these other ligand groups might induce metal ions to bind to the penultimate monoanionic phosphate position while leaving the terminal dianionic phosphate position unbound. This mode of binding would result in a metal ion–ligand complex resembling an unsymmetrical diester of pyrophosphoric acid. As previous work had indicated that such diesters hydrolyze extremely rapidly, it was hoped that the rates of pyrophosphate hydrolysis of the newly synthesized compounds would be greatly accelerated by metal ions, thus providing an attractive model for the binding of metal ions to substrates on the surface of polyphosphatases. The effects of a large number of metal ions on the hydrolysis rates of these compounds were studied and in no case was significantly large catalysis found, even though ^{31}P nuclear magnetic resonance studies revealed that the desired mode of binding could be at least partially obtained. These findings were taken as strong evidence against the validity of the proposed model.

Enzymes catalyzing polyphosphate hydrolysis have quite generally been shown to require divalent metal ions, but the precise catalytic role of the metal ions has not been established. Metal ion effects on nonenzymatic polyphosphate hydrolysis have been extensively studied (Schneider and Brintzinger, 1964; Miller and Westheimer, 1966; Tetas and Lowenstein, 1963; Hofstetter and Martell, 1959) and the catalysis observed is not large, the acceleration in the great majority of cases being less than a factor of 10. It is true that in the presence of solid metal salts, especially hydroxides, very fast hydrolyses have been observed (Torralba, 1960; Bamann, 1939; Bamann *et al.*, 1954), but these results have not been amenable to mechanistic interpretation.

That metal ions by themselves do not greatly accelerate polyphosphate hydrolysis does not necessarily prevent metal ions bound to enzymes from acting as efficient catalysts. However, it is evident that a mode of action not readily

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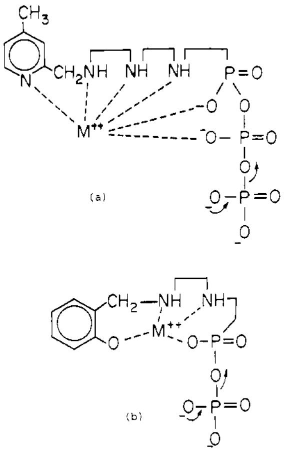
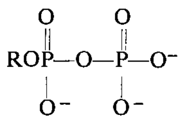


FIGURE 1: Desired binding mode: (a) of hexacoordinate metal ions to TAPPP; (b) of tetracoordinate metal ions to PhDAPP.

available to the metal ions in the nonenzymatic reaction would be required.

The rate of hydrolysis of an unsymmetrical diester of pyrophosphoric acid has been recently measured (Miller, 1969), and at neutral pH has been found to be about 10^6 times faster than that of the corresponding monoester. Since the natural substrates of the enzymes of interest can all be considered to be monoesters of pyrophosphoric acid (I),



$$\mathrm{RO\overset{\overset{O}{\|}}{\underset{\underset{O^-}{|}}{P}}{-}O{-}\overset{\overset{O}{\|}}{\underset{\underset{O^-}{|}}{P}}{-}O^-}$$

I

for PPi, R = H (pH 7–9)
for ATP, R = adenosyl 5′-OP(=O)O

an attractively simple model for the role of metal ion in the enzymatic hydrolysis is the conversion of pyrophosphate monoesters into unsymmetrical diesters. This could be accomplished if metal ion bound the penultimate, monoanionic phosphate group and neutralized its charge, while leaving the terminal phosphate group unbound and dianionic. Although this mode of binding is known to be quite unfavorable for M^{2+}–ATP complexes in solution (Cohn and Hughes, 1962; Sternlicht *et al.*, 1965), it could perhaps be made favorable on the enzyme surface by suitable arrangement of metal ion and/or pyrophosphate binding sites.

The aim of the work reported here was to test this model by duplicating the proposed function of the enzyme in an organic molecule of comparatively simple structure. To this end, the molecules PhDAPP,[1] PhDAPPP, TAPP, and TAPPP were designed and synthesized (Figures 2 and 3). It was hoped that these multidentate ligands would form complexes with divalent metal ions having the structures shown in Figure 1 for PhDAPP and TAPPP,[2] and then hydrolyze rapidly *via* a rapid metaphosphate elimination.

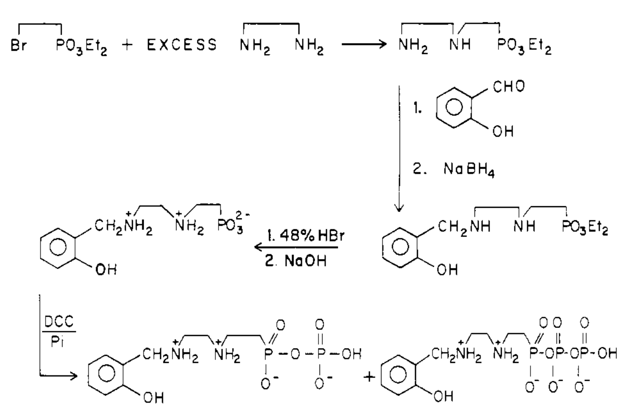


FIGURE 2: Scheme for PhDAPP and PhDAPPP syntheses.

We have studied the rates of hydrolysis of these compounds at two or more pH values in the presence of a large number of divalent, and some trivalent, metal ions. In no case was a significantly enhanced rate observed. In an effort to interpret these results we have also studied other physicochemical properties of the metal ion complexes of these compounds. The results of ^{31}P nuclear magnetic resonance studies are of particular importance, since they indicate that the desired mode of binding was at least partially obtained in some cases.

These results constitute strong evidence against the model under consideration.

Experimental Section

Materials and Equipment. PhDAPP, PhDAPPP, TAPP, and TAPPP were synthesized according to the schemes shown in Figures 2 and 3. They were all isolated by ion-exchange (Dowex 50, or a combination of Dowex 50 and 1) chromatography as well-resolved peaks, and each gave a single spot on paper chromatography. For all four compounds, the infrared, ultraviolet, ^1H nuclear magnetic resonance, and ^{31}P nuclear magnetic resonance spectra, as well as the stoichiometry of acid-labile phosphate, were fully consistent with the assigned structures. In addition, satisfactory C, H, N, and P elemental analyses were obtained for the crystalline solids PhDAPP and TAPP, as well as for the monolithium salt of PhDAPPP. The overall isolated yields, based on diethyl bromoethyl phosphonate, were PhDAPP, 3.0%; PhDAPPP, 0.9%; TAPP, 1.2%; and TAPPP, 1.0%.[3]

Metal ion salts of certified high purity were used. *N*-Ethylmorpholine (Union Carbide, technical grade) was redistilled before use (bp 136–138°). Fisher 0.10 N $AgNO_3$ was used for chloride ion titrations.

Ultraviolet spectra were taken on a Cary recording spectrophotometer and optical density measurements were made on

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[1] The abbreviations used for the multidentate compounds studied emphasize the nature of the ligand groups in the molecules. Thus, PhDAPP is an abbreviation for phenoxydiaminophosphonyl phosphate, while TAPPP is short for tetraaminophosphonyl pyrophosphate. The systematic names for these compounds are as follows: PhDAPP, [7-(2-hydroxyphenyl)-3,6-diazaheptyl]phosphonyl phosphate; PhDAPPP, [7-(2-hydroxyphenyl)-3,6-diazaheptyl)]phosphonyl pyrophosphate; TAPP, [10-(4-methyl-2-pyridyl)-3,6,9-triazadecyl]phosphonyl phosphate; TAPPP, [10-(4-methyl-2-pyridyl)-3,6,9-triazadecyl]phosphonyl pyrophosphate.

[2] Using Catalin space-filling models, it was found that the proposed structures could be built. However, structures involving binding to the terminal phosphate were also possible.

[3] For further details, see Cooperman (1967).

TABLE I: Hydrolysis Rate Constants, k (min^{-1} × 10^4), pH 7.8,[a] and T = 75.4 ± 0.2°.

	Ligand[b]		
Metal Ion	PhDAPP	TAPP	TAPPP
None	(0.89)[c]	1.0	5.3
Cu^{2+}	2.5	1.4	12
Ni^{2+}	0.65	0.14	12
Zn^{2+}	0.15	0.18	10
$Co^{2+} \rightarrow Co^{3+}$[d]	0.26	0.29	48
Mn^{2+}	2.0[e]	0.76	9.9
Pd^{2+}	9.7	1.4	4.2
Hg^{2+}	(0.92)[c]	1.2	4.0
Mg^{2+}	(0.59)[c]	1.1	6.7
Mg^{2+} (5:1)	(0.45)[c]	0.60	
Ca^{2+}	(0.99)[c]	1.0	12
Ca^{2+} (10:1)	(1.4)[c,f]	1.3	
Fe^{3+}	0.46		

[a] *N*-Ethylmorpholine buffer. [b] Metal ion:ligand is 1.0:1.0 unless otherwise indicated. [c] Decomposition of phenolic moiety taking place. [d] Spectral evidence was obtained for Co^{2+} oxidation to Co^{3+} during runs. [e] Spectral evidence was obtained for Mn^{2+} oxidation during runs. [f] Precipitate present.

a Zeiss PMQ-II spectrophotometer. ^1H nuclear magnetic resonance spectra were taken at room temperature on a Varian Model A-60 analytical nuclear magnetic resonance spectrometer, using tetramethylsilane as an external standard. ^{31}P nuclear magnetic resonance spectra were taken at room temperature on a Varian high-resolution spectrometer, Model V-4300B, operating at 24.3 Mc, on sample volumes of 1.0–1.5 ml and at concentrations of 0.2–0.5 M. The pH was adjusted with concentrated HCl or tetramethylammonium hydroxide solutions. H_3PO_4 (85%) was used as an external standard, and spectra were calibrated using side bands of the H_3PO_4 peak.

Kinetic Procedures. Stock substrate solutions were brought to basic pH and stored at 0°. Under these conditions, no inorganic phosphate release could be detected after several months. Concentration was determined by testing aliquots for acid-hydrolyzable phosphate (Doose, 1959) and by optical density measurement, using the known extinction coefficients (Cooperman, 1967). All metal ion solutions were prepared from the respective chlorides. Concentrations of $HgCl_2$ and K_2PdCl_4 were calculated from the weight of crystalline solids used. All other concentrations were determined by potentiometric titration of chloride ion with standard $AgNO_3$.

The pH of the buffers used for the kinetic studies were measured at the temperatures used in the kinetic runs following a procedure described by Tanford (1960).

Reaction mixtures were between 1.5 × 10^{-4} and 3.1 × 10^{-4} M in substrate, 0.014 M in buffer, and were brought to an ionic strength of 0.05 with KCl.

Reactions were carried out in a brass-jacketed Teflon container, fitted with a take-off tube leading to a stopcock. A positive nitrogen pressure was maintained during kinetic runs.

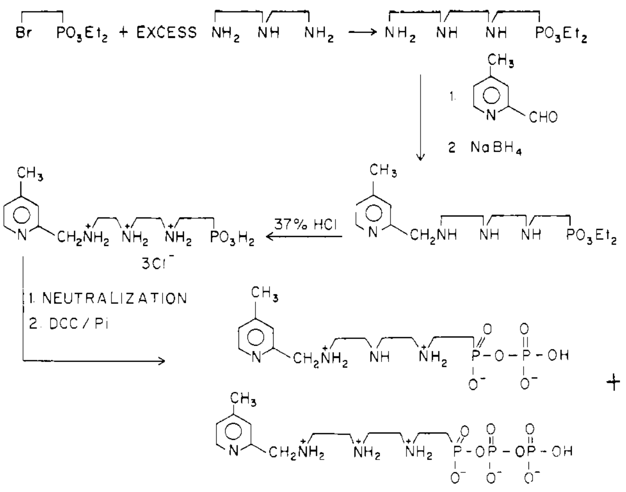


FIGURE 3: Scheme for TAPP and TAPPP syntheses.

Aliquots were taken by opening the stopcock, quenched by rapid cooling to 0°, and analyzed for P_i using the Marsh method (Marsh, 1959) with some minor modifications. In almost all cases the final pH was within 0.2 pH unit of the initial pH (both measured at room temperature). In a few runs the variation was as high as 0.3 pH unit.

The PP_i:P_i ratio in reaction aliquots was determined as follows. Aliquots were treated with charcoal (prepared following Lipkin *et al.*, 1954) to remove all (>95%) ultraviolet-absorbing material. The mixture was filtered and the filtrate was analyzed for inorganic phosphate and for total phosphate after acid hydrolysis. PP_i was then set equal to half the difference between the latter and former quantities.

Results

Kinetics. First-order rate constants for the hydrolysis of the terminal phosphate group of the compounds PhDAPP, TAPP, and TAPPP, in the presence and absence of metal ions, were obtained using the usual graphical methods and are summarized in Tables I and II. The values listed are considered accurate to ±15%. In general, five points were taken per run and each run was performed twice. For PhDAPP and TAPP hydrolyses were followed to 25–60% completion. For TAPPP, in order to minimize contribution to the buildup of P_i from side reactions, hydrolyses were followed only to 15–20% of completion. In some cases TAPPP also hydrolyzed to give TAP and PP_i to a significant extent. The PP_i:P_i ratio was used as a quantitative measure of this side reaction and the rate constants listed in Tables I and II are corrected for it.

Kinetic studies on PhDAPP at pH 7.8 and 75° were complicated by the fact that the molecule decomposes at a rate faster than that of P_i release. This decomposition is thought to proceed *via* a quinone methide intermediate, on the basis of chromatographic and spectrophotometric evidence as well as by analogy to well-studied decompositions in molecules of similar structure (Turner, 1964). Interestingly, those metal ions which bind the phenolic oxygen (as shown by their effects of the phenolic ultraviolet spectrum), *i.e.*, Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, and Pd^{2+}, were found to prevent decomposition.

Several preliminary kinetic runs were performed on

TABLE II: Hydrolysis Rate Constants at pH 5,[a] k (min^{-1} × 10^3).

Metal Ion	Ligand[b]		
	PhDAPP[c]	TAPP[c]	TAPPP[d]
None	1.3	1.9	0.46
Cu^{2+}	0.38	0.78	0.25
Ni^{2+}	0.31	0.13	0.21
Ni^{2+} (1.3:1)		0.13	
Zn^{2+}	0.69	0.16	0.18
Zn^{2+} (1.3:1)		0.16	
$Co^{2+} \rightarrow Co^{3+}$[e]	0.79	0.19[f]	2.7
Mn^{2+}	1.0	1.7	0.52
Mn^{2+} (3:1)	0.85	1.1	
Pd^{2+}	0.96	0.81	0.23
Hg^{2+}	1.2	1.2	0.31
Mg^{2+} (3:1)	1.2[g]		0.63
Mg^{2+} (10:1)	0.98	1.5	
Ca^{2+} (3:1)	1.2	2.0	0.60
Ca^{2+} (10:1)	1.1	1.8[g]	
Fe^{3+}	0.24		

[a] Potassium acetate buffer. [b] Metal ion:ligand is 1.0:1.0 unless otherwise indicated. [c] T = 75.4 ± 0.2°, pH 5.2. [d] T = 60.2 ± 0.2°, pH 5.1. [e] Spectral evidence was obtained for Co^{2+} oxidation to Co^{3+} during runs. [f] Potassium phthalate buffer (pH 5.2) was used; the hydrolysis rate constant in the absence of metal ion in this buffer was 1.7 × 10^{-3} min^{-1}. [g] Precipitate present.

PhDAPPP using a modified procedure from that described in the Experimental Section. Four points were taken per run, and the runs were done only once. Rates were calculated assuming a simple first-order process and were not corrected for PP_i information. The results are presented in Table III.

TABLE III: Rate Constants for PhDAPPP Hydrolysis at 65.0°, k (min^{-1} × 10^4).

M^{2+}	M^{2+}: PhDAPPP	pH 7.7[a]	pH 6.5[a]	pH 5.7[b]
None		(1.3)[c]	2.7	4.2
Cu^{2+}	1.0		4.3	5.2
Cu^{2+}	1.2	2.9		
Ni^{2+}	1.2	19		
Ni^{2+}	1.3		18	13
Zn^{2+}	1.0		8.9	6.7
Zn^{2+}	1.2	7.8		
Mn^{2+}	1.2	5.1		
Cd^{2+}	1.0	5.2	5.5	6.1
Pd^{2+}	1.0	2.3	4.9	6.2

[a] N-Ethylmorpholine buffer, 0.035 M. [b] Potassium acid phthalate buffer, 0.035 M. [c] Decomposition assumed to be taking place.

TABLE IV: PP_i:P_i in TAPPP Hydrolysis.

Metal Ion[a]	pH 7.8,[b] 75.4°	pH 5.1,[c] 60.2°
None	0.51	0.21
Cu^{2+}	0.17	0.26
Ni^{2+}	0.24	0.15
Zn^{2+}	0.18	0.13
Mn^{2+}	2.1	0.37
Pd^{2+}	1.4[d]	0.23
Hg^{2+}	0.40	0.30
Mg^{2+}	0.69	
Mg^{2+} (3:1)		0.10
Ca^{2+}	0.59	
Ca^{2+} (3:1)		0.18

[a] Metal ion:TAPPP is 1.0:1.0 unless otherwise indicated. [b] N-Ethylmorpholine buffer. [c] Potassium acetate buffer. [d] This value represents an upper limit due to incomplete removal of ultraviolet-absorbing material on treatment with charcoal.

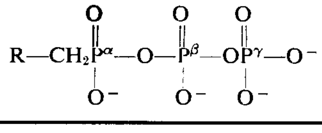
No studies were conducted on the possible decomposition of PhDAPPP but, by analogy with the studies on PhDAPP, this presumably does take place.

The PP_i:P_i Ratio. The PP_i content of TAPPP reaction mixtures was measured in order to determine if the observed slow rates of metal ion catalyzed P_i production could be masking a prior rapid hydrolysis of TAPPP to TAP and PP_i. The results presented in Table IV show this not to be the case. However, the amounts of PP_i found at pH 7.8, both in the presence and absence of metal ion, are considerably higher than those found with ATP under similar conditions (Tetas and Lowenstein, 1963), and it is possible that the amine

TABLE V: Effect of Paramagnetic Metal Ions on ^{31}P Nuclear Magnetic Resonance Spectra.

Ligand	Metal Ion	Metal Ion: Ligand	pH	α[a]	β	γ
TAPPP	Cu^{2+}	0.02	8.6	sh	br	ext
TAPPP	Mn^{2+}	0.03	9.1	br	ext	ext
TAPP	Cu^{2+}	0.008	6.8	sh	ext	
TAPP	Cu^{2+}	0.008	9.3	sh	br	
TAPP	Cu^{2+}	0.04	9	sh	ext	
PhDAPP	Cu^{2+}	0.09	6.8	ext	ext	
PhDAPP	Cu^{2+}	0.09	9.5	sh	sh	
PhDAPP	Mn^{2+}	0.004	7.5	ext	ext	
PhDAPP	Mn^{2+}	0.004	9.3	br	br	

[a] The labeling of the phosphorus nuclei is



$$R{-}CH_2P^{\alpha}(=O)(O^-){-}O{-}P^{\beta}(=O)(O^-){-}OP^{\gamma}(=O)(O^-){-}O^-$$

TABLE VI: Effect of Diamagnetic Metal Ions on ^{31}P Nuclear Magnetic Resonance Spectra (δ in parts per million from 85% H_3PO_4).

Ligand	M^{2+}	pH	α	β	γ	$\Delta\alpha$	$\Delta\beta$	$\Delta\gamma$
TAPP	None	4.6	−10.7	+9.9		0.0	0.0	
		5.8	−11.9	+6.6		0.0	0.0	
		6.7	−11.4	+6.3		0.0	0.0	
		8.8	−11.2	+6.1		0.0	0.0	
TAPP	Zn^{2+}	4.7	−13.9	+7.6		−3.2	−2.3	
		5.7	−15.4	+7.1		−3.5	+0.5	
		6.7	−18.3	+6.0		−6.9	+0.3	
		8.9	−19.7	+5.7		−8.5	−0.4	
TAPP	Ca^{2+}	4.7	−12.3	+7.3		−1.6	−2.6	
		5.7	−12.4	+6.9		−0.5	+0.3	
		6.6	−12.3	+6.5		−0.9	+0.2	
		8.9	−13.9	+6.1		−2.7	0.0	
TAPPP	None	8.7	−13.0	+21.4	+6.3	0.0	0.0	
TAPPP	Zn^{2+}	8.5	−19.4	+19.9	+6.0	−6.4	−1.5	−0.3

positions in TAPPP are involved in some sort of nucleophilic or general base catalysis of hydrolysis leading to PP$_i$ formation.

PP$_i$ determinations were also made for reaction mixtures containing Zn^{2+}–TAPP at pH 5.2 and 7.8, and Ni^{2+}–TAPP and Zn^{2+}–PhDAPP at pH 7.8, to examine the possible importance of nucleophilic P$_i$ attack on the phosphonyl phosphate complexes. In no case was a significant amount of PP$_i$ found.

Nuclear Magnetic Resonance Spectra—Metal Ion Effects. The effects of the paramagnetic metal ions Mn^{2+} and Cu^{2+} on the ^{31}P nuclear magnetic resonance spectra of PhDAPP, TAPP, and TAPPP are presented in Table V. The symbols used have the following meaning: sh means that the peak remained sharp in the presence of metal ion, br means that the peak was broadened in the presence of metal ion, and ext means that the peak was extinguished in the presence of metal ion. These symbols are only significant for the conditions specified, since peak broadening increases with increasing (metal ion)/(ligand) and, at fixed (metal ion)/(ligand), with decreasing pH.

The effects of the diamagnetic metal ions Zn^{2+} and Ca^{2+} on the ^{31}P nuclear magnetic resonance spectra of TAPP and TAPPP are presented in Table VI. Values of Δ represent the difference in chemical shift between the complexed and uncomplexed ligand. Values of $|\Delta| \leq 0.5$ ppm are not necessarily significant.

^{1}H nuclear magnetic resonance spectra of TAPPP were taken in the presence and absence of Mn^{2+} and Cu^{2+}, at pD 9.1. In both cases, all peaks present in the original spectrum were broadened in the presence of metal ion.

Discussion

The data presented in Tables I–III provide a clear demonstration that none of the metal ions tested greatly accelerate the rates of hydrolysis of the compounds examined in this study. If any generalization can be made, it is that metal ions are somewhat poorer catalysts for these compounds than for other polyphosphates. Indeed, the modest rate decreases for TAPP and PhDAPP hydrolysis in the presence of Zn^{2+}, Ni^{2+}, and Co^{2+} → Co^{3+}, of the order of 5-fold at pH 7.8 and up to 14-fold at pH 5.1, are in marked contrast to the modest rate increases that these metal ions give for ADP hydrolysis (3- to 5-fold at pH 9, 0.6- to 6-fold at pH 5) (Tetas and Lowenstein, 1963).

Failure to observe the desired catalytic effects can be attributed to one of two causes. Either the design of the compounds is deficient in that metal ions remain bound to the terminal phosphate position, thus providing no test of the metaphosphate elimination mechanism, or metal ions are bound in the hoped-for manner, but this mode of binding does not lead to rapid hydrolysis.

The effects of Cu^{2+} and Mn^{2+} on the TAPPP ^{1}H nuclear magnetic resonance spectrum and the observation that some metal ions prevent PhDAPP decomposition both suggest that the nonphosphate portions of these molecules are binding metal ions. This suggestion was further supported by the marked effects of metal ions on the ultraviolet spectrum and pH titration curve of PhDAPP, as well as by the very large values measured for the association constants of Cu–TAPP and Cu–TAPP.[4] Thus in this respect the compounds are behaving as planned.

The ^{31}P nuclear magnetic resonance studies provide direct information on metal ion binding to the phosphate ligands. Using peak broadening as a measure of binding, the results presented in Table V indicate that for the TAPP, TAPPP, and PhDAPPP complexes of Cu^{2+} and Mn^{2+} the terminal phosphate position is always bound and thus these complexes do not test the proposed mechanism.

The results for the complex Zn^{2+}–TAPP (Table VI) are more interesting. The very large chemical shift in the α peak is a clear indication of strong binding to the α position, while the lack of a significant shift in the β peak is evidence that the β position is at best weakly bound. The arguments in support of weak binding to the β position are threefold. First, in Ca^{2+}–TAPP the β peak is also hardly shifted, yet given the strong preference of Ca^{2+} for oxygen over nitrogen ligands, it would be highly unusual if the tetraamino ligand system of

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[4] Both found to be about 10^{20} (Cooperman, 1967).

TAPP could draw Ca^{2+} off the terminal dianionic phosphate position. Lack of a shift is therefore not definitive evidence against binding. Second, in Zn^{2+}–TAPPP, a shift of 1.5 ppm in the β peak is a clear indication of binding. If Zn^{2+} binds to the monoanionic β position of TAPPP, it would be expected to bind to the dianionic β position of TAPP. Third, the Ca^{2+}, Mg^{2+}, and Zn^{2+} complexes of ATP, as well as the Mg^{2+} complex of ADP, all show significant shifts in the peaks corresponding to the terminal phosphate positions (Cohn and Hughes, 1962). Lack of such a shift constitutes evidence against strong binding. Thus, the binding of Zn^{2+} to the phosphonyl phosphate portion of TAPP is a close approximation to what was originally hoped for. The observed decelerating effect of Zn^{2+} on TAPP hydrolysis must then be taken as strong evidence against the model proposed in the introduction for the catalytic role of metal ions in enzymatic polyphosphate hydrolysis.

The question as to what is the correct role of metal ions is left open by this work, but if the metaphosphate mechanism is incorrect, then a mechanism invoking metal ion assistance of nucleophilic attack at the terminal phosphate becomes well worth examining. It has already been demonstrated that several metal ions give rise to phosphorylation of inorganic phosphate by ATP, a reaction which is undetectable in their absence (Lowenstein, 1958; Lowenstein and Schatz, 1961). It has also been claimed that metal ions lead to phosphorylation of acetate ion by ATP (Lowenstein and Schatz, 1961), although this result has been seriously challenged (Lawlor, 1967). What is lacking now are demonstrations that conditions can be found or that model systems can be synthesized in which the rate of these transphosphorylation reactions can be vastly increased, to a point where they provide a reasonable mechanism for the role of metal ions in the catalytic action of polyphosphatases. Efforts in this direction are currently under way.

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