Heinert, D., and Martell, A. E. (1963a), *J. Amer. Chem. Soc. 85*, 183.
Heinert, D., and Martell, A. E. (1963b), *J. Amer. Chem. Soc. 85*, 188.
Herries, D. G., Mathias, A. P., and Rabin, B. R. (1962), *Biochem. J. 85*, 127.
Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.
Hirs, C. H. W., and Kycia, J. H. (1965), *Arch. Biochem. Biophys. 111*, 223.
Ivanov, V. I., and Karpeisky, M. Ya. (1969), *Advan. Enzymol. 32*, 21.
Kartha, G., Bello, J., and Harker, D. (1967), *Nature (London) 213*, 862.
Konigsberg, W. (1967), *Methods Enzymol. 11*, 461.
Lowry, O. H., Rosebrough, N. J., Farr, A. J., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Marcus, F., and Hubert, E. (1968), *J. Biol. Chem. 243*, 4923.
Marfey, P. S., Nowak, H., Uziel, M., and Yphantis, D. A. (1965), *J. Biol. Chem. 240*, 3264.
Meadows, D. H., Roberts, C. G. and Jardetsky, O. (1969), *J. Mol. Biol. 45*, 491.
Means, G. E., and Feeney, R. E. (1971), *J. Biol. Chem. 246*, 5532.
Metzler, D. E. (1957), *J. Amer. Chem. Soc. 79*, 485.
Murdock, A. L., Grist, K. L., and Hirs, C. H. W. (1966), *Arch. Biochem. Biophys. 114*, 375.
Nolan, G., Novoa, W. B., Krebs, E. G., and Fisher, E. H. (1964), *Biochemistry 3*, 542.
Peterson, E. A., and Sober, H. A. (1954), *J. Amer. Chem. Soc. 76*, 169.
Piszkiewicz, D., Landen, M., and Smith, E. L. (1970), *J. Biol. Chem. 245*, 2622.
Rippa, M., Spanio, L., and Pontremoli, S. (1967), *Arch. Biochem. Biophys. 118*, 48.
Schnackerz, K. D., and Noltmann, E. A. (1971), *Biochemistry 10*, 4837.
Schroeder, W. A. (1967), *Methods Enzymol. 11*, 351.
Sela, M., and Anfinsen, C. B. (1957), *Biochim. Biophys. Acta 24*, 229.
Shapiro, S., Enser, M., Pugh, E., and Horecker, B. L. (1968), *Arch. Biochem. Biophys. 128*, 554.
Simpson, R. T., and Vallee, B. L. (1968), *Biochemistry 7*, 4336.
Smyth, D. G., Stein, W. H., and Moore, S. (1963), *J. Biol. Chem. 238*, 227.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Stock, A., Ortanderl, F., and Pfleiderer, G. (1966), *Biochem. Z. 344*, 353.
Strausbauch, P. H., and Fischer, E. H. (1970), *Biochemistry 9*, 233.
Ulmer, D. D., and Vallee, B. L. (1965), *Advan. Enzymol. 27*, 37.
Vallee, B. L., and Riordan, J. F. (1969), *Annu. Rev. Biochem. 38*, 733.
Vallee, B. L., and Williams, R. J. P. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 498.
Wilson, E. M., and Meister, A. (1966), *Biochemistry 5*, 1166.
Wyckoff, H. W., Hardman, K. D., Allewell, N. M., Inagami, T., Johnson, L. N., and Richards, F. M. (1967), *J. Biol. Chem. 242*, 3984.

Models for Metalloenzymes. Zinc Ion Catalyzed Phosphorylation of 1,10-Phenanthroline-2-carbinol by Adenosine Triphosphate†

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ABSTRACT: Zinc ion catalyzes the phosphorylation of 1,10-phenanthroline-2-carbinol by ATP. The reaction is absolutely dependent on the metal ion and proceeds through the formation of a reactive ternary complex composed of 1,10-phenanthroline-2-carbinol, ATP, and zinc ion. In addition to serving as a template for the reaction of two dissimilar ligands, the metal ion serves the additional catalytic functions of enhancing the nucleophilicity of the carbinol group by lowering its pK_a and of neutralizing the negative charge of the γ-phosphate to permit attack of an anionic nucleophile. The mechanistic function of the metal ion in this simple system corresponds to that postulated for the transition metal ion in terminal deoxynucleotidyl transferase from calf thymus.

Most enzymes which catalyze phosphoryl-transfer reactions require divalent metal ions (Mildvan, 1970). One approach to understanding the role of metal ions in these enzyme systems is to determine how metal ions can facilitate nonenzymic phosphoryl-transfer reactions.

An attractive hypothesis for the function of metal ions in pyrophosphate-bond cleavages stems from the highly reactive nature of unsymmetrical pyrophosphate diester dianions. For example, Miller (1969) has demonstrated that P_1,P_1-diethyl pyrophosphate dianion hydrolyzes with a half-life at 35° of 10 min most likely *via* a "metaphosphate" mechanism (Ia in eq 1). The suggested catalytic function of metal ions based on these studies would involve the metal ion binding to the penultimate, but not the terminal phosphate, and generating a species comparable in reactivity and structure by virtue of its neutralized charge on the penultimate phosphate (Ib in eq 1). Cooperman (1969) has made a direct

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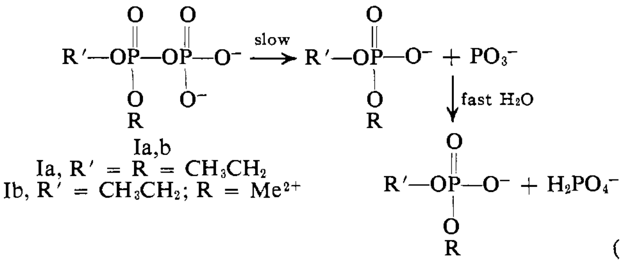
test of this hypothesis by synthesizing complex multidentate ligands containing pyrophosphates in order to produce the coordination of the metal ion in the manner proposed. However, he found no significant metal ion catalysis in pyrophosphate cleavage with the compounds he studied.



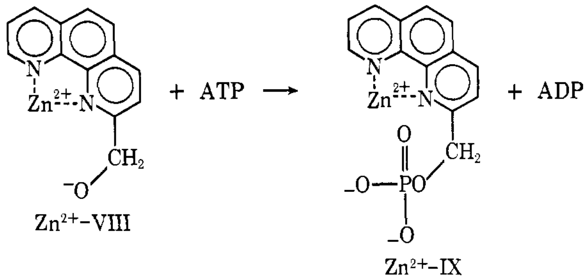
$$\mathrm{R'{-}O\overset{\overset{O}{\|}}{\underset{\underset{R}{|}}{\underset{O}{P}}}{-}O\overset{\overset{O}{\|}}{\underset{O^-}{\underset{|}{P}}}{-}O^-} \xrightarrow{\text{slow}} \mathrm{R'{-}O\overset{\overset{O}{\|}}{\underset{\underset{R}{|}}{\underset{O}{P}}}{-}O^-} + \mathrm{PO_3^-} \xrightarrow{\text{fast } \mathrm{H_2O}} \mathrm{R'{-}O\overset{\overset{O}{\|}}{\underset{\underset{R}{|}}{\underset{O}{P}}}{-}O^-} + \mathrm{H_2PO_4^-} \qquad (1)$$

Ia,b

Ia, $R' = R = CH_3CH_2$

Ib, $R' = CH_3CH_2$; $R = Me^{2+}$

To provide alternative nonenzymic systems to study the role of metal ions in phosphoryl-transfer reactions, we sought to examine reactions in which the metal ion would principally facilitate nucleophilic attack on the terminal phosphate rather than promote the generation of "metaphosphate" (PO_3^-). In the present communication, we wish to report that a metal ion can effectively promote a phosphoryl transfer if the phosphate donor and acceptor can simultaneously coordinate to the metal ion to generate a reactive ternary complex with a favorable geometry for a nucleophilic displacement reaction. Specifically, we have found that zinc ion catalyzes the phosphorylation of 1,10-phenanthroline-2-carbinol by ATP to yield 1,10-phenanthroline-2-carbinol phosphate and ADP (eq 2) and that this reaction proceeds by the formation of a ternary complex composed of Zn^{2+}, ATP, and 1,10-phenanthroline-2-carbinol in a 1:1:1 ratio.



(2)

Recently Lloyd and Cooperman (1971) have demonstrated that phosphorylimidazole rapidly phosphorylates the anionic oxygen of Zn^{2+}–pyridine-2-carbaldoxime also *via* the formation of a reactive ternary complex. The metal ion catalyzed phosphorylation of inorganic phosphate by triphosphates has been known for some time and may proceed through a reactive ternary complex but the stoichiometry of the active species is uncertain (Lowenstein, 1958; Miller and Westheimer, 1966b). In addition, the nonenzymatic activation of acetate by ATP in the presence of Be^{2+} to yield a derivative which reacts with hydroxylamine to produce acethydroxamate has been reported (Lowenstein and Schatz, 1961). This reaction may proceed through a reactive ternary complex but the exact nature of the primary product which is converted to acethydroxamate has not been determined.

Experimental Section

Materials

1,10-Phenanthroline-2-carbinol was synthesized by the scheme indicated in Figure 1. The synthesis of 1,10-phenanthroline-2-carboxylic acid by the route indicated has been previously described (Corey *et al.*, 1965).

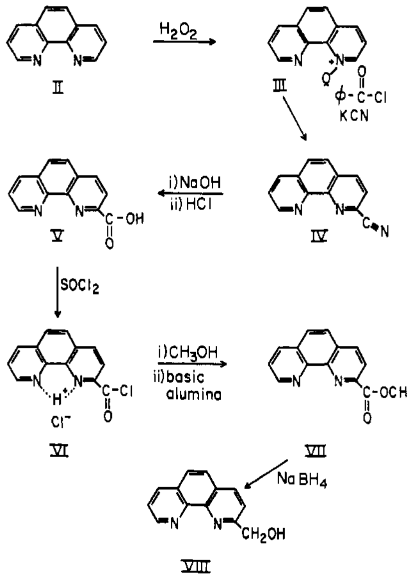


FIGURE 1: Synthetic route to 1,10-phenanthroline-2-carbinol.

The acid chloride (VI) was prepared by suspending 1.2 g of V (5.31 mmoles) in 50 ml of thionyl chloride and refluxing until V had dissolved to give a pale orange solution (2.5 hr). After the reaction mixture was cooled to room temperature, 100 ml of benzene was added to precipitate out the product. The thionyl chloride and benzene were then removed on the rotatory evaporator. To remove any remaining thionyl chloride, the residue was suspended in 100 ml of benzene and then evaporated to dryness. This process was repeated twice more with 50 ml of benzene. The resulting pale yellow powder was dried in a stream of dry nitrogen to yield 1.2 g of the hydrochloride salt, VI.

To prepare VII, 1.2 g of the hydrochloride salt, VI, without any further purification, was dissolved in 100 ml of methanol and then passed slowly through 24 g of methanol-washed basic alumina (AG-10, 100–200 mesh). The resin was then washed twice with 50-ml aliquots of methanol to remove any residual methyl ester from the alumina. The free base from VII in the methanol effluent was then concentrated to approximately 20 ml on the rotatory evaporator. The addition of 100 ml of water caused the ester to crystallize as white plates: yield 83%, mp 112–114°.

1,10-Phenanthroline-2-carbinol is prepared by adding 0.76 g of VII dissolved in 100 ml of methanol to 2.42 g of sodium borohydride over a 7-min period. The reaction mixture is then refluxed for 2 hr. After the reaction mixture is allowed to cool down, 100 ml of distilled water is added, and the total volume is reduced to 100 ml on the rotatory evaporator. The resulting solution is then extracted five times with 50-ml aliquots of chloroform which were combined and in turn extracted twice with 20-ml aliquots of water. The chloroform solution was then dried over sodium sulfate, treated with charcoal and then concentrated to 10 ml. Upon addition of petroleum ether (bp 30–60°), crystals formed which could be recrystallized from acetonitrile: yield 53%; mp 139–143° dec. *Anal.* Calcd for $C_{13}H_{10}N_2O$: C, 74.28; H, 4.76. Found: C, 74.15; H, 4.96.

ATP, GTP, CTP, UTP, ADP, AMP, and *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes) were pur-

TABLE I: Rates of the Phosphorylation Reaction as a Function of the Composition of the Reaction Mixtures.[a]

Expt	[VIII]	[Zn^{2+}]	[II]	[Ca^{2+}]	% of IX at 5 days[b]
1	2.0	2.0	0	0	26 ± 7
2	2.0	0	0	0	<1
3	2.0	1.0	0	0	1.5 ± 0.5
4	2.0	2.0	2.1	0	3 ± 1
5	2.0	2.0	0	2.0	42 ± 2
6	2.0	1.0	0	2.0	3 ± 1

[a] pH 7.2, 0.03 M Hepes–0.1 M NaCl, 37°. 2.1×10^{-3} M ATP present in all reaction mixtures. Concentrations of other components are indicated as M $\times 10^3$. With the exception of expt 6, the rates of ADP production in all experiments corresponded to the rates of production of IX. ADP production in expt 6 is greater than IX due to the calcium-catalyzed hydrolysis of ATP. [b] The per cent of IX is obtained by multiplying the fraction of IX by 100. See the Experimental Section for method used to calculate fraction of IX formed.

chased from Calbiochem and reagent grade anhydrous zinc chloride from J. T. Baker. All these chemicals were used without any further purification. Doubly distilled water was used for the preparation of all reaction mixtures.

Methods

All reaction mixtures were analyzed by descending paper chromatography using Whatman No. 3MM paper and isopropyl alcohol–water–trichloroacetic acid–ammonia (75:25:5:0.2, vvwv) as the developing solvent. This solvent system clearly resolves all four reactants and products of interest in the reaction, 1,10-phenanthroline-2-carbinol (VIII), the monophosphate ester of 1,10-phenanthroline-2-carbinol (IX), ADP, and ATP. The two phenanthroline derivatives can be detected as blue-fluorescent spots under a mercury lamp while the adenosine derivatives are visible as dark ultraviolet-absorbing spots on the white background of the paper. Compound IX, ADP, and ATP move 0.83, 0.264, and 0.094 times as rapidly as VIII in the paper chromatography system indicated above. Phosphorus containing spots were specifically stained by the method of Hanes and Isherwood (1949).

To measure the rates of the phosphorylation reactions, the following procedure was generally employed. Reaction mixtures with an initial volume of 3.0 ml were prepared containing the Zn^{2+}, VIII, and the pyrophosphate ester under study in the concentration range of $1–4 \times 10^{-3}$ M. pH was maintained with Hepes buffers (0.03 M) and the ionic strength with 0.1 M NaCl. Part of the reaction mixture was withdrawn and frozen at zero time while the rest of the reaction mixture was incubated at 37°. At various time intervals (of the order of days), 100-μl aliquots of the reaction mixture that had been incubated at 37° were spotted and chromatographed. The various ultraviolet-absorbing and fluorescent spots of interest were cut out and eluted with 3 ml of 10^{-3} M HCl. The absorption spectrum of the resulting solutions were then obtained with a Cary 14 recording spectrophotometer.

An extinction coefficient of 14.1×10^3 M^{-1} cm^{-1} at 257 mμ was used to calculate the concentrations of ATP and ADP. Since the spectra of VIII and IX in 0.01 M HCl were very similar, extinction coefficients of 3.05×10^4 and 3.29×10^4 M^{-1} cm^{-1} at 275 and 279 mμ, respectively, determined for VIII in 0.01 M HCl, were used to calculate the concentrations of VIII and IX. The fraction of VIII or IX in a reaction mixture at a given time was measured by dividing the absorbance of VIII or IX by the sum of the absorbances of the spots corresponding to VIII and IX. A comparable procedure was used in measuring the conversion of ATP to ADP. Usually greater than 80% of the initial concentrations of both ATP and VIII were recovered as ATP plus ADP and VIII plus IX, respectively. Very little IX (less than 1%) was detected in chromatograms of the reaction mixtures that had been frozen or had been analyzed at zero time. Therefore IX is not generated during the course of the 20-hr paper chromatography. However, some ADP comprising between 5 and 10% of the total ATP recovered following chromatography, was sometimes observed in reaction mixtures that had been frozen or those that had been analyzed at zero time. Consequently, the rate of formation of IX from VIII was a more reliable method to assay the phosphorylation reaction. For all experiments reported in Table I except expt 6, the rate of ADP production was equal within experimental limits to the rate of IX production. For expt 6, the rate of ADP production was greater due to the calcium-catalyzed hydrolysis of ATP.

A complete analysis of the reaction kinetics was not undertaken since the chromatographic method of analysis was time consuming and the overall reaction rate was slow. An additional difficulty which impeded kinetic analysis, in particular studying the effect of pH on the reaction rate, was that reaction mixtures possessed traces of insoluble material even at pH 7.2. For the experiments reported in Table I, the rate of conversion of VIII to IX was studied in reaction mixtures that contained small amounts of precipitate and in the clear supernatant of reaction mixtures that were centrifuged immediately after they were prepared. Since the rates of production of IX from VIII were very similar in the two cases, the reaction must proceed largely if not totally in solution. The recovery of the adenine nucleotides and 1,10-phenanthroline derivatives from the centrifuged reaction following paper chromatographic analysis were greater than 80% in the experiments reported in Table I, so that less than 20% of the total concentration of ATP and VIII added initially could have been present in the precipitate.

Chromatography on Dowex 1 (Cl^-) provided an alternate way to analyze aliquots of the reaction mixture. Stepwise elution with water, 0.01 N HCl, 0.01 N HCl with 0.01 M NaCl, and 0.01 N HCl with 0.1 M NaCl removed VIII, IX, ADP, and ATP, respectively. Although variable recoveries of the phenanthroline derivatives prevented this column procedure from being a good method to follow the kinetics, the technique was nevertheless useful as a means of isolating enough IX to be able to characterize it with respect to its phosphate content according to the method of Bartlett (1959).

Results

The zinc ion catalyzed phosphorylation of 1,10-phenanthroline-2-carbinol (VIII) by ATP can readily be observed when all the components of the reaction mixture are present at 2×10^{-3} M at pH 7.2 in 0.03 M Hepes buffer containing 0.1 M NaCl. Under these conditions 26 ± 7% of the 1,10-phenanthroline-2-carbinol (VIII) is converted to its monophosphate ester (IX) in 120 hr (5 days) at 37° (expt 1, Table

I). This rate corresponds to an observed first-order rate constant for the production of IX of 7.0 × 10^{-7} ± 2.3 × 10^{-7} sec^{-1}. A comparable rate is obtained if KCl is substituted for 0.1 M NaCl. The rate of production of the chromatographic spot identified as IX, is paralleled by the rate of production of ADP. One mole of ADP is formed for each mole of IX produced and the rate of ADP production is the same as that for IX within experimental error. In agreement with this observation is that zinc ion does not catalyze the hydrolysis of IX to inorganic phosphate and VIII under the experimental conditions in which the phosphorylation reaction is observed. Therefore there is no net catalyzed hydrolysis of ATP to ADP and inorganic phosphate. The stability of the Zn^{2+}–IX complex to hydrolysis is consistent with the inability of zinc ion to catalyze the hydrolysis of 2-pyridylmethyl phosphate (Murakami and Takagi, 1969).

The rate of production of ADP in the presence of 2 × 10^{-3} M ATP, VIII, and Zn^{2+} is comparable, within the large experimental error, to that observed with 2 × 10^{-3} M Zn^{2+}, and ATP but in the absence of VIII. In reaction mixtures containing 2 × 10^{-3} M ATP and VIII but lacking zinc ion, no chromatographic spot, could be found corresponding to the phosphate ester of 1,10-phenanthroline-2-carbinol (expt 2, Table I). The phosphorylation reaction therefore appears to be absolutely dependent on the presence of the metal ion.

Product Analysis. The identification of the new derivative which appeared as a function of time as the phosphate ester of 1,10-phenanthroline-2-carbinol is based on the following considerations. First, the spectroscopic and chromatographic behavior of the new derivative is consistent with its possessing a 1,10-phenanthroline nucleus as well as a phosphate group. The new compound is certainly closely related to 1,10-phenanthroline-2-carbinol (VIII) since its fluorescence on the paper and its absorption spectrum when it is eluted from the paper with 10^{-2} M HCl or isolated by column chromatography is indistinguishable from VIII. Since compounds with a greater negative charge possess significantly lower R_F values in the paper chromatography system used, the new 1,10-phenanthroline derivative's lower R_F value relative to VIII is consistent with its possessing a phosphate group.

Direct evidence for the presence of phosphate in the new derivative comes from staining the paper chromatograms of the reaction mixture by the method of Hanes and Isherwood (1949). With this procedure, the new phenanthroline derivative, tentatively identified as IX, was clearly shown to possess a phosphoryl group, supporting its suggested structure. As expected, ADP and ATP give positive and VIII negative tests by this procedure. As further proof that the new derivative possessed a phosphate group, a reaction mixture composed of 2 × 10^{-3} M Zn^{2+}, ATP, and VIII, which had incubated for 17 days and possessed considerable amounts of IX and ADP, was treated with alkaline phosphatase and then chromatographed. After the enzymic digestion, the only phenanthroline derivative present was VIII and adenosine was the only adenine derivative present. When VIII and IX were isolated by elution from the paper following chromatographic analysis of a reaction mixture and then digested with alkaline phosphatase and rechromatographed, all the suspected phosphate ester was converted to VIII.

Final proof that the product of the reaction was the monophosphate of 1,10-phenanthroline-2-carbinol involved isolation of the new derivative by column chromatography and the determination of its phosphate content by the method of Bartlett (1959). Corrections for trace amounts of phosphate in the samples of IX isolated by column chromatography were done by the method of Lowry and Lopez (1946). The concentration of IX was determined by assuming the extinction coefficient of IX was the same as that of VIII in 0.01 N HCl. This assumption was reasonable, since as indicated above, the absorption spectra of both compounds were very similar with respect to both the positions and the relative intensities of the various absorption maxima, minima, and shoulders. When quantitative phosphate analyses were performed on 0.038, 0.076, and 0.114 μmole of IX, 0.037, 0.067, and 0.106 μmole of inorganic phosphate were obtained, respectively. Therefore, IX contained 0.92 ± 0.035 mole of phosphate per mole of 1,10-phenanthroline-2-carbinol.

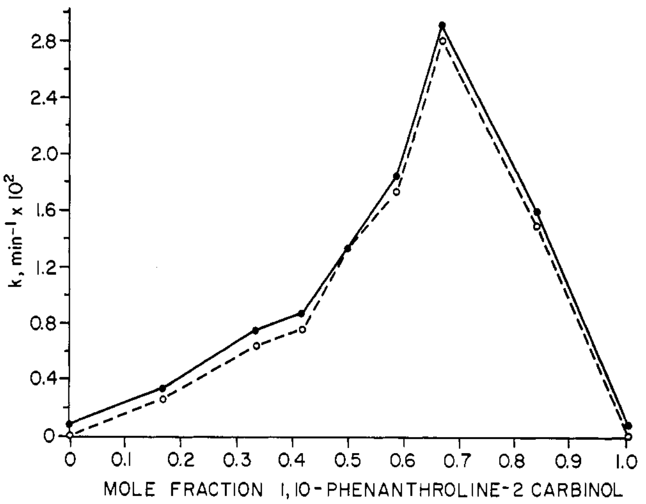


FIGURE 2: The method of continuous variations applied to the reaction of Zn^{2+}–1,10-phenanthroline-2-carbinol complexes with *p*-nitrophenyl acetate. The sum of the concentrations of zinc ion and 1,10-phenanthroline-2-carbinol is 1.60 × 10^{-3} M and the *p*-nitrophenyl acetate concentration is 8.40 × 10^{-5} M. The kinetics were run at 25° in pH 7.20, 0.03 M Hepes buffer in the presence of 0.1 M NaCl. The solid line is k_{obsd} (eq 3) while the dashed line is k_c' (eq 4) which has been corrected for the small contributions due to buffer and free Zn^{2+} and VIII.

Kinetic Properties of the Zn^{2+}–VIII Complexes. Although the kinetic data for the phosphorylation reaction could be analyzed in terms of first-order reaction kinetics, indicating the obligatory formation of a ternary complex composed of Zn, VIII, and ATP, the accuracy and precision of the kinetic data were insufficient to exclude rigorously a bimolecular reaction between the Zn^{2+}–VIII complex and ATP. Further proof was needed, in terms of the overall specificity of the reaction, to demonstrate the existence of a reactive ternary complex. This was provided by comparing the reactivities of 1:1 and 1:2 Zn^{2+}–VIII complexes with *p*-nitrophenyl acetate to their reactivities with ATP. Whereas the 1:2 complex reacts more rapidly than the 1:1 complex with *p*-nitrophenyl acetate, the former complex barely reacts at all with ATP. As will be discussed further, the 1:2 complex cannot react with ATP because no reactive ternary complex can be formed.

Figure 2 summarizes the results of a kinetic version of the method of continuous variations (Job plot) (Martell and Chaberek, 1959) which was designed to investigate the effect of Zn^{2+} and VIII on the rate of *p*-nitrophenol production from *p*-nitrophenyl acetate at 25° in a pH 7.20, 0.03 M Hepes buffer containing 0.1 M NaCl, the same buffer generally used in studying the phosphorylation reaction. The sum of the concentrations of Zn^{2+} and VIII were kept constant but their mole fractions were varied. The first-order rate constant describing the production of nitrophenol, k_{obsd}, is defined by eq 3, where k_0 is the first-order rate constant for hydrolysis in a given buffer, $k_{Zn^{2+}}$ and k_{VIII} are the second-order rate constants describing the effect of Zn^{2+} and VIII on the release of nitrophenol when present alone, and k_c is the second-order rate constant reflecting the rate of nitrophenol production from *p*-nitrophenyl acetate by complexes of Zn^{2+} and VIII. The pseudo-first-order rate constant for nitrophenol production, k_c', can be simply determined by subtracting the negligible contribution due to the Zn^{2+} and VIII-dependent reactions from the observed first-order rate constant (eq 4). Figure 2 shows that although VIII is nucleophilic both in 1:1 and 1:2 Zn^{2+}–VIII complexes, it is a more effective nucleophile toward *p*-nitrophenyl acetate in 1:2 Zn^{2+}–VIII complexes than in 1:1 complexes even when the rate constant, k_c, is calculated in terms of the concentration of chelated VIII. One possible reason for the enhanced reactivity of VIII in the 1:2 complex is that the catalytic efficiency of zinc is enhanced because of its coordination to another 1,10-phenanthroline nucleus.

$$k_{obsd} = k_0 + k_{Zn^{2+}}(Zn^{2+}{}_f) + k_{VIII}(VIII) + k_c(Zn^{2+}\text{–}VIII) \quad (3)$$

$$k_c' = k_c(Zn^{2+}\text{–}VIII) = k_{obsd} - k_0 - k_{Zn^{2+}}(Zn^{2+}) - k_{VIII}(VIII) \quad (4)$$

This suggestion was tested directly by examining the effect of 1,10-phenanthroline on the rate of the reaction between *p*-nitrophenyl acetate and the 1:1 Zn^{2+}–VIII complex. The observed first-order rate constant for *p*-nitrophenol production from *p*-nitrophenyl acetate in the presence of 1×10^{-3} M VIII, 1.02×10^{-3} M Zn^{2+}, and 1.04×10^{-3} M 1,10-phenanthroline is 18.3×10^{-3} min^{-1}; in the absence of 1,10-phenanthroline and at the same concentration of VIII and Zn^{2+}, the rate constant is 9.5×10^{-3} min^{-1}. Since the rate constant for *p*-nitrophenol production in the presence of 1.02×10^{-3} M Zn^{2+} and 1.04×10^{-3} M 1,10-phenanthroline is 0.6×10^{-3} min^{-1}, the ternary complex composed of 1,10-phenanthroline, VIII, and Zn^{2+} must be more nucleophilic than the binary 1:1 complex composed only of Zn^{2+} and VIII. The demonstration that 1,10-phenanthroline can enhance the catalytic efficiency of the zinc ion supports the suggested explanation for the greater reactivity of VIII in the 1:2 Zn^{2+}–VIII complex relative to that in the 1:1 Zn^{2+}–VIII complex toward *p*-nitrophenyl acetate. The ability of nonreactive ligands to enhance the catalytic efficiency of metal ions has been previously demonstrated by Rund and Claus (1967) for the manganese ion catalyzed decarboxylation of dimethyloxaloacetic acid.

If the reaction of the 1:1 Zn^{2+}–VIII complex with ATP proceeds *via* complex formation rather than by way of a bimolecular process, no reaction of ATP with the 1:2 Zn^{2+}–VIII complex should take place even though the 1:2 complex is more than twice as nucleophilic as the 1:1 complex toward *p*-nitrophenyl acetate. Reaction between ATP and the 1:2 Zn^{2+}–VIII complex should be prohibited because the reactive ternary complex would not be able to form because all the available coordination positions around the zinc ion would be occupied by the two 1,10-phenanthroline-2-carbinol molecules. Since very little IX was generated in expt 3 of Table I where the predominant Zn^{2+}–VIII complex is the 1:2 complex, the obligatory formation of a ternary complex composed of ATP, Zn^{2+}, and VIII is indicated and the reaction of the 1:1 Zn^{2+}–VIII complex probably does not proceed to any significant extent *via* a bimolecular mechanism.

Further support for this conclusion can be obtained by incubating ATP with Zn^{2+}, VIII, and 1,10-phenanthroline (II) as indicated in expt 4 of Table I. If the reaction of Zn^{2+}–VIII with ATP was bimolecular in nature, II would not significantly inhibit the formation of IX. In fact, the effect of 1,10-phenanthroline on the rate of the reaction of Zn^{2+}–VIII with *p*-nitrophenyl acetate would suggest a rate acceleration with ATP should be observed if a bimolecular reaction took place. However, if the reaction proceeds *via* the formation of a ternary complex composed of VIII, Zn^{2+}, and ATP, significant inhibition would be expected because II can compete with ATP for available coordination positions on the Zn^{2+}–VIII complex. Since the rate of formation of IX is sharply reduced in the presence of 1,10-phenanthroline, the phosphorylation of the Zn^{2+} complex most likely proceeds *via* a ternary complex. The displacement by 1,10-phenanthroline of ATP rather than VIII from the ternary complex is more likely to occur since the stability constant for the formation of ATP–Zn^{2+} is 1.62×10^5 M^{-1} (Sigel *et al.*, 1967) while the stability constant for the formation of the Zn^{2+}–II complex is 3×10^6 M^{-1} (Sillen and Martell, 1964). Studying the effect of the addition of 2.1×10^{-5} M ATP on the difference spectrum of the 1:1:1 Zn^{2+}:VIII:II complex (each component present at 2×10^{-5} M) confirmed this expectation. At 300 mμ where the difference absorbancy for the ternary complex can be analyzed in terms of equal contributions from VIII and II, the added nucleotide decreased the absorbancy by only 8%. Therefore most of the zinc ion was coordinated simultaneously to 1,10-phenanthroline (II) and 1,10-phenanthroline-2-carbinol (VIII) although the presence of small amounts of ternary complexes of Zn^{2+}, 1,10-phenanthroline, and ATP and of Zn^{2+}, 1,10-phenanthroline-2-carbinol, and ATP cannot be excluded.

Although the formation of a ternary complex of Zn^{2+}, VIII, and ATP is strongly indicated by the results of expt 3 and 4 in Table I, an alternative explanation might be that IX is formed through a bimolecular reaction between the Zn^{2+}–VIII complex and Zn^{2+}–ATP complex. To investigate this possibility expt 5 and 6 of Table I were performed. Calcium ion, which is not readily chelated by 1,10-phenanthroline derivatives but is effectively coordinated by ATP, was added to ensure that charge neutralization of the phosphates of ATP would be achieved even when the concentration of free Zn^{2+} was very low due to high concentrations of phenanthroline derivatives. The very slow rate of production of IX in expt 6 of Table I, when the 1:2 Zn^{2+}–VIII complex is present, underscores the importance of a reactive ternary complex for facile reaction to take place. The results of expt 3 and 4 in Table I therefore cannot readily be interpreted in terms of a bimolecular reaction between the Zn^{2+}–VIII complex and the Zn^{2+}–ATP complex. The rapid rate of formation of IX observed in expt 5 of Table I, compared to that in expt 1 of Table I, suggests that ATP, as well as its calcium complex, can form a ternary complex with the 1:1 Zn^{2+}–VIII complex. The enhanced rate observed in expt 5 is probably due to the greater charge neutralization possible when the phosphates of ATP coordinate to the Zn^{2+} and Ca^{2+} simultaneously.

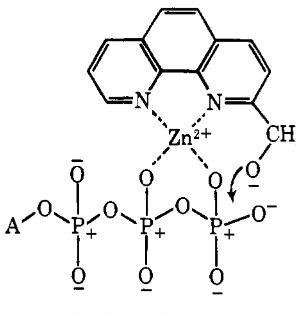
Specificity for Nucleotide. The phosphorylation of the Zn^{2+}–VIII complex to yield IX is also accomplished to a measurable extent by ADP, UTP, GTP, and CTP when Zn^{2+}, VIII, and any of the above nucleotides are present at concentrations of 2×10^{-3} M. In each case the only readily observable product is IX and either AMP, UDP, GDP, or CDP. Although a careful kinetic analysis was not attempted, all these nucleotides reacted with Zn^{2+}–VIII at a rate roughly one-fifth to one-third as rapidly as ATP at comparable concentrations.

Discussion

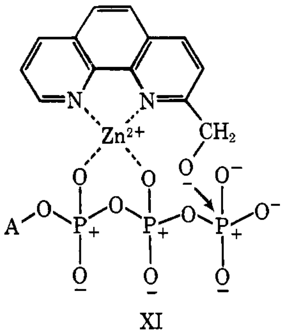
Zinc ion has three probable catalytic functions in the phosphorylation of 1,10-phenanthroline-2-carbinol by ATP. The first is to serve as a template upon which both 1,10-phenanthroline-2-carbinol and ATP are able to coordinate simultaneously. The geometry of the resulting ternary complex must permit and likely facilitate nucleophilic attack by the carbinol on the γ-phosphate of ATP. The second catalytic function of the zinc ion is to perturb the pK_a of the carbinol group of VIII so that an effective nucleophile is generated at neutral pH. Finally, the interaction of the zinc ion with the phosphates of ATP probably significantly reduces the electrostatic barrier for the nucleophilic attack of the anionic alkoxide on the negatively charged γ-phosphate.

Three other simple systems have been studied in which zinc ion promotes a nucleophilic displacement reaction by formation of a ternary complex in which two dissimilar ligands coordinate to metal ion. Chipman and Breslow (1965) have shown that zinc ion catalyzes the acetylation of pyridine-2-carbaldoxime by 8-acetoxyquinoline-5-sulfonic acid. Using the same metal ion associated nucleophile, Lloyd and Cooperman (1971) have shown that phosphorylimidazole, phosphoryl-*N*-methylimidazole, and phosphoramidate efficiently phosphorylate the Zn^{2+}–pyridine-2-carbaldoxime complex *via* formation of a reactive ternary complex. Sigman and Jorgensen (1972) have recently demonstrated that zinc ion effectively catalyzes the esterification of *N*-(β-hydroxyethyl)-ethylenediamine by *p*-nitrophenyl picolinate.

Two possible schematic representations of the active ternary complex in the phosphorylation would be either X or XI. Either of these alternatives are consistent with data presented in this study and the known coordination properties of ATP and 1,10-phenanthroline derivatives. Since several investigators (Cohn and Hughes, 1962; Hammes and Miller, 1967; Rimai and Hyde, 1970) have reported that the β- and γ-phosphate groups of ATP are the primary ligands in the coordination of the nucleotide to the zinc ion and the affinity of ATP to Zn^{2+} is the same as that of ATP to the 1:1 Zn^{2+}–2,2-bipyridine complex (Sigel *et al.*, 1967), X is probably more stable thermodynamically than XI. Although an additional site of interaction between ATP and Zn^{2+} is most likely N-7 of adenine, the ability of CTP and UTP to substitute for ATP in the present reaction system, indicates that the latter interaction cannot be absolutely essential for the formation of the reactive ternary complex.



X, A = adenosine



XI

Examination of molecular models of X reveals that the carbinol group is suitably located for nucleophilic attack on either the β- or γ-phosphate. Since the only derivative of 1,10-phenanthroline-2-carbinol that can be detected is its monophosphate and very little AMP is formed, attack on the β-phosphate must be excluded. The greater double-bond character of the two P–O bonds of the central phosphorus in trimetaphosphate (Pauling, 1960), and the likelihood that a comparable situation exists in ATP, may be responsible for prohibiting nucleophilic attack at the β-phosphorus in X. An equally possible explanation is that the reaction proceeds through the less stable but more reactive ternary complex XI. The possibility that reactions involving the cleavage of pyrophosphate bonds are funneled through very reactive yet thermodynamically unstable structures, has been proposed by previous workers. For example, Miller and Westheimer (1966a) have shown that the hydrolysis of γ-phenylpropyl diphosphate in the pH region 3–6 most likely proceeds through a thermodynamically unfavorable but highly reactive prototropic form. Tetas and Lowenstein (1963) have suggested a possible explanation for the pH dependence of the Cu^{2+} and Zn^{2+} catalysis for the hydrolysis of ATP is that the most reactive forms of the metal–ATP complex involve coordination through the α- and β-phosphoryl group and not the more stable β–γ-coordinated structures. In summary, although evidence can be presented to support both X and XI as the reactive species, the relative importance of X and XI cannot be assessed conclusively.

Two other important components of the zinc ion catalysis have been incorporated in both X and XI. One of these is that the reactive nucleophilic form of the carbinol is probably its ionized or alkoxide form. This feature is suggested, but not proven, by examination of the pH dependence of the reaction of the Zn–VIII complex with the *p*-nitrophenyl and *o*-nitrophenyl esters of picolinic acid. The pH–rate profile describing the nitrophenol release from these esters can be interpreted in terms of the carbinol group of the Zn–VIII complex having a pK_a of 7.5 and the ionized form being the reactive nucleophilic species. Two kinetically equivalent mechanisms, which would yield identical or similar pH dependences, would be general base catalysis of the nucleophilic attack of the un-ionized carbinol group by (a) free hydroxide ion or (b) metal ion bound hydroxide which is generated from a metal ion bound water molecule with a pK_a of 7.5. The latter alternative is unlikely in view of the exceptional reactivity of the 1:2 Zn^{2+}–VIII complex while the former alternative, although it cannot be rigorously excluded, is unlikely since it would require very large third-order rate constants for both picolinate esters. Since the rate constants for nucleophilic attack of the Zn^{2+}–VIII complex on *p*-nitrophenyl acetate do not show a dependence on buffer concentration, the possibility that the rate depends on a basic component of the buffer can be excluded. The most likely of the possible mechanisms to explain the pH dependence of the picolinate esters is therefore the zinc ion induced perturbation of the pK_a of the carbinol in the 1:1 Zn^{2+}–VIII complex. The large apparent pK_a perturbation of the carbinol group in the Zn^{2+}–VIII complex is comparable to that obtained for the hydroxyethyl group in the *N*-(β-hydroxyethyl)ethylenediamine–zinc complex (Sigman and Jorgensen, 1972). Because of the similarities in the pK_a perturbations for these two systems, the pK_a alteration in the Zn^{2+}–VIII complex most likely does not arise from electronic effects generated by the metal ion in the aromatic 1,10-phenanthroline nucleus but rather results from the close proximity of the hydroxyl group of the carbinol group to the metal ion. The latter alternative is the only reasonable one to explain the pK_a alteration in the N-(β-hydroxyethyl)ethylenediamine–Zn^{2+} system.

A final important catalytic function of the zinc ion in the phosphorylation reaction is indicated in both X and XI. In both structures the zinc ion is coordinated to the phosphates of ATP. A consequence of this coordination is that the net negative charge on the γ-phosphate will be reduced. This, in turn, will reduce the electrostatic barrier in the approach of the negative alkoxide to the negatively charged γ-phosphate. Since nucleophilic attack of anions on the monoesters of phosphoric acid is very slow (Kirby and Younas, 1970), the ability of the zinc ion to neutralize negative charge, as indicated in either X or XI, probably represents an important catalytic function of the metal ion. For example, in addition to its inability to form a ternary complex, one reason the 1:2 zinc ion–1,10-phenanthroline-2-carbinol complex does not react appreciably with ATP, even though it is an effective nucleophile with respect carboxylic acid esters, is that no charge neutralization of ATP by the zinc ion is possible. The coordination scheme with ATP indicated in XI, in addition to permitting charge neutralization by the metal ion, enables a stable chelated form of ADP to become the leaving group in the nucleophilic displacement reaction. In the absence of further work, it is not possible to state whether this is a valid reason for favoring XI over X.

The absolute dependence on zinc ion of the phosphorylation of 1,10-phenanthroline-2-carbinol by ATP indicates that the rate enhancement attributable to the metal ion must be very significant. By analogy, a metal ion serving a similar function in an enzymic phosphorylation reaction could make a very substantial contribution to the overall catalytic efficiency. A recent review of probable reaction mechanisms of a variety of metalloenzymes (Mildvan, 1970) has indicated that the metal ion in a number of enzymic reactions plays a comparable mechanistic role as the zinc ion in the phosphorylation of 1,10-phenanthroline-2-carbinol by ATP.

One enzyme which catalyzes the nucleophilic attack of an hydroxyl group on a pyrophosphate linkage and requires a transition metal ion (likely zinc) is terminal deoxynucleotidyl transferase from calf thymus (Chang and Bollum, 1970). The competitive inhibition by 1,10-phenanthroline with respect to the oligodeoxythymidylate primer has prompted the suggestion that the catalytic function of the metal ion is to interact with the 3′-hydroxyl of the growing end and activate it for nucleophilic attack on the α-phosphate of the incoming nucleotide triphosphate (Chang and Bollum, 1971). This postulated catalytic role of the metal ion is supported by the ability of zinc ion to catalyze the phosphorylation of 1,10-phenanthroline-2-carbinol by ATP.

References

Bartlett, G. (1959), *J. Biol. Chem. 234*, 466.

Chang, L., and Bollum, F. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 1041.

Chipman, D., and Breslow, R. (1965), *J. Amer. Chem. Soc. 87*, 4195.

Cohn, M., and Hughes, T. R., Jr. (1962), *J. Biol. Chem. 237*, 176.

Cooperman, B. S. (1969), *Biochemistry 8*, 5005.

Corey, E. J., Borror, A. L., and Foglia, T. (1965), *J. Org. Chem. 30*, 289.

Hammes, G. M., and Miller, D. L. (1967), *J. Chem. Phys. 46*, 1533.

Hanes, C. S., and Isherwood, F. A. (1949), *Nature (London) 164*, 1107.

Kirby, A. J., and Younas, M. (1970), *J. Chem. Soc. B*, 1165.

Lloyd, G. J., and Cooperman, B. S. (1971), *J. Amer. Chem. Soc. 93*, 4883.

Lowenstein, J. (1958), *Biochem. J. 70*, 222.

Lowenstein, J., and Schatz, M. (1961), *J. Biol. Chem. 236*, 305.

Lowry, O., and Lopez, J. A. (1947), *J. Biol. Chem. 162*, 421.

Martell, A., and Chaberek, B. (1959), Organic Sequestering Reagents, New York, N. Y., John Wiley, p 508.

Mildvan, A. (1970), *in* The Enzymes, Vol. II, Boyer, P. D., Ed., New York, N. Y., Academic Press, p 445.

Miller, D. L., and Ukena, T. (1969), *J. Amer. Chem. Soc. 91*, 3050.

Miller, D. L., and Westheimer, F. H. (1966a), *J. Amer. Chem. Soc. 88*, 1507.

Miller, D. L., and Westheimer, F. H. (1966b), *J. Amer. Chem. Soc. 88*, 1514.

Murakami, Y., and Takagi, M. (1969), *J. Amer. Chem. Soc. 91*, 5130.

Pauling, L. (1960), The Nature of the Chemical Bond, Ithaca, N. Y., Cornell University Press, p 323.

Rimai, L., and Heyde, M. E. (1970), *Biochem. Biophys. Res. Commun. 41*, 313.

Rund, J. V., and Claus, K. C. (1967), *J. Amer. Chem. Soc. 89*, 2256.

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

Sigman, D. S., and Jorgensen, C. (1972), *J. Amer. Chem. Soc. 94*, 1724.

Sillen, L., and Martell, A. (1964), Stability Constants of Metal Ion Complexes, London, Chemical Society.

Tetas, M., and Lowenstein, J. M. (1963), *Biochemistry 2*, 350.