Models for Metalloenzymes. The Zinc(II)-Catalyzed Transesterification of *N*-(β-Hydroxyethyl)ethylenediamine by *p*-Nitrophenyl Picolinate

David S. Sigman* and Catherine T. Jorgensen

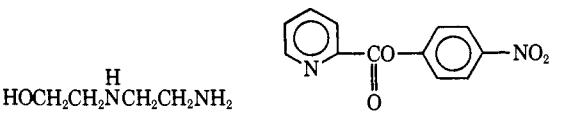
Contribution from the Department of Biological Chemistry, University of California at Los Angeles, School of Medicine, Los Angeles, California 90024. *Received March 20, 1971*

Abstract: Zinc ion catalyzes a facile transesterification reaction between *N*-(β-hydroxyethyl)ethylenediamine and *p*-nitrophenyl picolinate. The principal and unique feature of this reaction is the formation of a reactive ternary complex composed of zinc ion, *N*-(β-hydroxyethyl)ethylenediamine, and *p*-nitrophenyl picolinate. In this ternary complex the zinc ion serves a dual catalytic role. It perturbs the pK_a of the hydroxyethyl group of *N*-(β-hydroxyethyl)ethylenediamine to provide a high concentration of an effective nucleophile and it acts as a template for the intracomplex nucleophilic attack of the ionized hydroxyethyl group on the carbonyl of *p*-nitrophenyl picolinate. The reactive ternary complex is therefore analogous to metalloenzyme–substrate complexes in which the metal ion serves as a bridge to orient a substrate for nucleophilic attack either by an amino acid residue of the enzyme or by another substrate.

Many enzymes require metal ions for catalytic activity.[1-3] Although some metal ion requiring enzymes involve the reaction of only a single substrate (*e.g.*, oxaloacetic acid decarboxylase),[4] many metalloenzymes and metal ion activated enzymes catalyze nucleophilic displacement reactions between two substrates where one of these substrates is sometimes water. Examples of metal ion dependent enzymes of this type are phosphoryl transferring enzymes such as creatine kinase, pyruvate kinase, and adenylate kinase, as well as hydrolases such as alkaline phosphatase and carboxypeptidase.

One possible function of metal ions in these enzymes is to serve as a template for the reaction between two coordinated substrates.[5] Additional mechanistic roles of the metal ion may be to promote the nucleophilicity or electrophilicity of the substrates through coordination to the appropriate functional group.[3,6] Since few examples of metal ions serving as a template between coordinated ligands are known, we sought to examine such a reaction in order to isolate those factors necessary for facile reactions within enzyme–metal–substrate complexes. The type of nucleophilic displacement reaction chosen was an acyl-transfer reaction.

In the present article, we wish to report that zinc ion catalyzes the esterification of *N*-(β-hydroxyethyl)ethylenediamine (**1**) by *p*-nitrophenyl picolinate (**2**) to yield the picolinoyl ester of *N*-(β-hydroxyethyl)ethylenediamine (**3**). The central feature of this reaction is the formation of a reactive ternary complex composed of zinc ion, *N*-(β-hydroxyethyl)ethylenediamine, and *p*-nitrophenyl picolinate. One further interesting aspect of this reaction is that it is one of the few unambiguous examples of the activation of a nucleophile for reaction as a consequence of its direct coordination or interaction with a metal ion.



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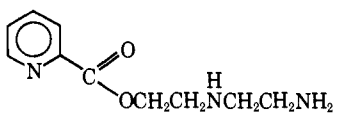

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These two relatively novel features of the present reaction have been studied in other systems. For example, Breslow and Chipman[7] have presented a clear example of an acyl-transfer reaction within a mixed complex composed of a metal ion, a chelating ester, and a chelating agent with an uncoordinated nucleophile. In particular, they studied the acetylation of the zinc complex of 2-pyridine aldoxime by 8-acetoxyquinoline-5-sulfonate and by *p*-nitrophenyl acetate. They found that although the rate of acylation of the uncomplexed anion of 2-pyridine aldoxime by *p*-nitrophenyl acetate was tenfold that of 8-acetoxyquinoline-5-sulfonate, the rates of acetylation of the zinc complex of the aldoxime by the two acetate esters were equal. This equality of rates, therefore, represented an enhanced reactivity of 8-acetoxyquinoline-5-sulfonate for the zinc complex of 2-pyridine aldoxime and probably arose from the formation of a mixed complex composed of the metal, the quinoline, and the aldoxime in which acyl transfer was facilitated by the favorable geometry of the complex.

In a series of papers, Buckingham, *et al.*,[8-10b] have demonstrated that the reactivity of a nucleophile can be enhanced by coordination to a metal ion. For ex-

(1) B. L. Vallee, *Proteins*, **5**, 1 (1970).
(2) B. L. Vallee and J. E. Coleman, *Comp. Biochem.*, **12**, 165 (1964).
(3) A. Mildvan, *Enzymes*, **2**, 446 (1970).
(4) G. W. Kosicki and F. H. Westheimer, *Biochemistry*, **7**, 4303 (1968).
(5) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, p 33.
(6) D. H. Busch, *Science*, **171**, 241 (1971).
(7) R. Breslow and D. Chipman, *J. Amer. Chem. Soc.*, **87**, 4195 (1965).
(8) D. A. Buckingham, D. M. Foster, and A. M. Sargeson, *ibid.*, **91**, 4102 (1969).
(9) D. A. Buckingham, D. M. Foster, and A. M. Sargeson, *ibid.*, **91**, 3451 (1969).
(10) (a) D. A. Buckingham, D. M. Foster, and A. M. Sargeson, *ibid.*, **92**, 6151 (1970); (b) D. A. Buckingham, C. B. Davis, and A. M. Sargeson, *ibid.*, **92**, 6159 (1970).

ample, they have shown that in the cobalt(III)-promoted hydrolysis and amidolysis of glycine esters, a prominent reaction pathway involves the reaction of a nucleophile coordinated to the metal ion.[8,9] These same workers have further demonstrated that the cobalt(III)-promoted hydrolysis of glycinamides[10a] and a lysis reaction at a saturated carbon also involve nucleophilic attack of a metal ion bound hydroxide ion.[10b]

Experimental Section

Materials. Doubly distilled water was passed through a deionizer prior to use. 2,6-Lutidine (Aldrich Chemical Co.) was redistilled three times prior to use, bp 142–142.5°. *N*-(β-Hydroxyethyl)ethylenediamine (**1**) (Eastman Chemical Co.) was distilled at 84–90° (0.3 mm) and redistilled at 112–114° (2.6 mm). Diethylenetriamine (Eastman Chemical Co.) and ethylenediamine (Eastman Chemical Co.) were redistilled, bp 52–54 (0.3 mm) and 116°, respectively. Acetonitrile was redistilled twice from phosphorus pentoxide and once from K_2CO_3, bp 81°. Anhydrous zinc chloride (Baker Reagent Grade), EDTA disodium salt (Baker Reagent Grade), *p*-nitrophenyl acetate (Aldrich), and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid[11] (Calbiochem) were used without further purification.

p-Nitrophenyl picolinate (**2**) was prepared by stirring 4.08 g of picolinic acid, 4.61 g of *p*-nitrophenol, and 6.87 g of dicyclohexylcarbodiimide in 100 ml of pyridine overnight. After 20 hr the reaction mixture was cooled in an ice bath, the dicyclohexylurea was filtered off, and the pyridine was removed on a rotatory evaporator. The residue was crystallized after treatment with charcoal from absolute methanol and white fluffy needles were obtained. These needles were recrystallized from methanol and dried. The product melted with decomposition, 144–146°. The infrared spectrum was consistent with **2** (1730, 1520, 1350, 1250, 1245, 1220 cm^{-1}). The uv spectrum of the ester in 0.1 *N* NaOH was the sum of the spectra of the expected hydrolysis products, picolinic acid and *p*-nitrophenol. *Anal.* Calcd for $C_{12}H_8N_2O_4$: C, 59.02; H, 3.30; N, 11.47. Found: C, 58.74; H, 3.54; N, 11.15.

Methods. Kinetics were generally run on a Zeiss PMQ II spectrophotometer coupled to a Photovolt Varicord Model 43 Linear/Log Recorder and equipped with a thermostated cell compartment. The reaction was initiated by addition of ester in an acetonitrile solution and the rate of nitrophenol production was followed either by the increase of absorbance at 400 or 320 mμ depending on the pH of the reacting solution. Pseudo-first-order rate constants were calculated by plotting log $[(OD_\infty) - (OD_0)]/[(OD_\infty) - (OD)_t]$ *vs.* time and determining the slope by the method of least squares. All reaction mixtures contained between 0.5 and 1.7% acetonitrile; the rates were not affected by acetonitrile concentration up to 5% acetonitrile. Total buffer concentration was 0.03 *M* (either 2,6-lutidine or Hepes[11]) and ionic strength was maintained with 0.1 *M* NaCl. No significant buffer effects were observed with either 2,6-lutidine or Hepes.

A Radiometer automatic titration assembly was used to determine pH's, pK_a's, and stability constants. pK_a's and stability constants were determined by titrating the hydrochlorides of the various amines in the presence and absence of zinc ion. All titrations were performed under nitrogen at 25 ± 0.1° in the presence of 0.1 *M* NaCl. Usually 3–20 × 10^{-3} *M* chelating agent was titrated with about 0.25 *N* NaOH. The base was standardized with $KH(IO_3)_2$. The pK_a's were obtained by determining the pH at which half the ionizable protons were titrated. The stability constants were determined by the method of Gustafson and Martell.[12] In this procedure equimolar amounts of chelating agent and metal ion are employed to maximize the concentration of 1:1 complexes.

Hydroxamate tests were performed according to Hestrin.[13] Ethyl picolinate was used as the standard in the hydroxamate test. Descending paper chromatography was generally done on Whatman 3M paper. The most generally used solvent system was freshly prepared *n*-butyl alcohol–acetic acid–water (40:6:15). Compounds possessing aromatic rings were detected as dark spots on a light background when the paper was examined with a high-pressure mercury lamp. A ninhydrin spray (95 ml of collidine–5 ml of ethanol–0.1 g of ninhydrin) was used to detect compounds which possessed *N*-(β-hydroxyethyl)ethylenediamine. After the paper was sprayed with ninhydrin it was placed in an 80° oven for color development. A Cary Model 14 recording spectrophotometer was used for scans of the ultraviolet and visible regions of the spectrum. Infrared spectra of the compounds were obtained in either KBr pellets or chloroform solution with a Perkin-Elmer Model 237 B recording spectrophotometer.

(11) Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

(12) R. Gustafson and A. Martell, *J. Amer. Chem. Soc.*, **81**, 525 (1959).

(13) S. Hestrin, *J. Biol. Chem.*, **180**, 249 (1949).

Results

Specificity of Reaction for Ester and for Chelating Agent. Table I indicates that the rate of nitrophenol production from *p*-nitrophenyl picolinate is significantly greater in the presence of both Zn^{2+} and **1** than the sum of rates when either component is present separately. Table I further demonstrates that of the five amines studied, only *N*-(β-hydroxyethyl)ethylenediamine (**1**) and zinc ion together show a rate of nitrophenol release significantly greater than that observed with zinc alone.

Table I. Rate Constants for Nitrophenol Release from *p*-Nitrophenyl Picolinate as a Function of Amine Structure[a]

Amine	[Amine], *M*	[Zn^{2+}], *M*	k_{obsd}, min^{-1} × 10^2
	0	0	0.149
	0	1.52×10^{-3}	4.86
N-(β-Hydroxyethyl)-ethylenediamine (**1**)	1.56×10^{-3}	0	1.65
N-(β-Hydroxyethyl)-ethylenediamine (**1**)	1.56×10^{-3}	1.52×10^{-3}	26.8
Ethylenediamine	1.56×10^{-3}	0	3.03
Ethylenediamine	1.56×10^{-3}	1.52×10^{-3}	5.17
1,5-Diaminopentane	1.54×10^{-3}	0	1.03
1,5-Diaminopentane	1.54×10^{-3}	1.52×10^{-3}	5.20
	0	1.00×10^{-3}	3.29
Diethylenetriamine	1.00×10^{-3}	0	1.37
Diethylenetriamine	1.00×10^{-3}	1.00×10^{-3}	2.42
	0	1.25×10^{-3}	3.14[b]
Aminoethanol	1.31×10^{-3}	0	0.64[b]
Aminoethanol	1.31×10^{-3}	1.25×10^{-3}	3.78[b]

[a] 0.03 *M* lutidine buffer, 0.1 *M* NaCl, pH 7.15; $T = 25.0 \pm 0.1°$. [b] 0.03 *M* Hepes buffer, 0.1 *M* NaCl, pH 7.10; $T = 25.0 \pm 0.1°$. Concentration of *p*-nitrophenyl picolinate varied from 7.8×10^{-5} *M* to 11.86×10^{-5} *M*.

The Zn^{2+}–**1** complex, present only in solutions containing both zinc ion and **1**, is effective in accelerating the rate of nitrophenol production from *p*-nitrophenyl picolinate but not from *p*-nitrophenyl isonicotinate and *p*-nitrophenyl acetate. The data reported in Table II show that the rate of nitrophenol production from the latter two esters in the presence of both Zn^{2+} and **1** is in fact less than the rate obtained in the presence of **1** at comparable concentrations. The results of Table II suggest that *p*-nitrophenyl picolinate must react in an entirely different manner with the Zn^{2+}–**1** complex than the *p*-nitrophenyl esters of isonicotinic and acetic acids.

Product Characterization. The unique reactivity demonstrated by *N*-(β-hydroxyethyl)ethylenediamine strongly suggests that the picolinoyl ester of **1** (**3**) was generated during the course of the zinc ion catalyzed reaction with *p*-nitrophenyl picolinate (**2**). Kinetic measurements obtained by following the rate of nitrophenol release in the presence of an excess of the Zn–**1** complex relative to **2**, however, cannot reveal if the

Table II. Rate Constants for *p*-Nitrophenol Release as a Function of Ester Structure in the Presence of Zinc Ion and *N*-(β-Hydroxyethyl)ethylenediamine[a]

p-Nitrophenyl ester	$[Zn^{2+}]$, *M*	[1], *M*	k_{obsd} min^{-1} $\times 10^2$
Picolinate	0	0	0.208
Picolinate	1.34×10^{-3}	0	7.79
Picolinate	0	1.34×10^{-3}	2.11
Picolinate	9.89×10^{-4}	9.89×10^{-4}	52.3
Isonicotinate	0	0	0.365
Isonicotinate	2.00×10^{-3}	0	0.431
Isonicotinate	0	2.00×10^{-3}	1.27
Isonicotinate	1.50×10^{-3}	1.50×10^{-3}	1.04
Acetate	0	0	0.038
Acetate	0	2.00×10^{-3}	0.369
Acetate	2.00×10^{-3}	0	0.041
Acetate	1.50×10^{-3}	1.50×10^{-3}	0.179

[a] 0.03 *M* lutidine, pH 7.33, 0.1 *M* NaCl; esters ranged in concentration from 7.16 to 8.58×10^{-5} *M*.

ester is a stable product or if the ester is an unstable intermediate which in turn is subjected to rapid metal ion catalyzed hydrolysis. Only if the reaction could have been studied under conditions where the ester was in excess relative to the Zn^{2+}–**1** complex would it have been possible to resolve this problem by kinetic methods. The reason kinetic studies were restricted to the concentration ranges indicated in Table I is that the stability constant for the formation of the Zn^{2+}–**1** complex in the neutral pH range is not very great (Table III). At low total concentrations of **1** and Zn^{2+} (about 10^{-4}) very little rate acceleration is observed because the concentration of the Zn–**1** complex is small under these conditions.

Table III. Stability Constants of Zinc Ion–Amine Complexes

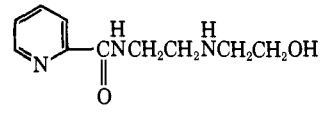
Chelating agent	pK_{a1}	pK_{a2}	pK_{a3}	Stability constant
N-(β-Hydroxyethyl)-ethylenediamine	6.71	9.68		1.69×10^5
Ethylenediamine	7.32	10.06		3.52×10^5
Diethylenetriamine	4.34[a]	9.13[a]	9.94[a]	4.92×10^8

[a] L. Sillen and A. Martell, "Stability Constants of Metal-Ion Complexes," *Chem. Soc., Spec. Publ.*, No. 17 (1964).

One obvious direct approach to determine if any reaction takes place subsequent to nitrophenol production involves measuring the number of titratable protons that were generated when Zn^{2+} and **1** were allowed to react with *p*-nitrophenyl picolinate (**2**) in the neutral pH range followed by a comparison of that number with the moles of nitrophenol produced as determined spectrophotometrically. If these values were the same, after taking into account the pK_a of nitrophenol, a stable ester (or amide) product would be indicated. However, if picolinic acid was generated, twice the amount of hydrogen ions would have been generated per mole of nitrophenol produced. This type of experiment proved unreliable since **1** is an effective buffer at neutral pH's and the chelating agent was generally present in a significant excess over the ester substrate. Superimposed upon this problem was that zinc ion slowly poisoned the electrodes and thereby generated spurious pH alterations.

The picolinoyl ester of **1** was demonstrated to be the major product in the following manner. Three different 50-ml reaction mixtures containing 3.2×10^{-4} *M* *p*-nitrophenyl picolinate in 8×10^{-3} *M* Hepes buffer pH 7.12 were prepared. Solution A contained 2×10^{-3} *M* **1**, solution B contained 2×10^{-3} *M* Zn^{2+}, and solution C contained 2×10^{-3} *M* **1** and 2×10^{-3} *M* Zn^{2+}. The rate of nitrophenol production was followed spectrophotometrically for each of the three solutions (A, B, C). After each of the three solutions had reacted for 8 half-lives, corresponding to their own characteristic rates of nitrophenol production, 3 ml of 0.1 *M* EDTA, pH 6.8, was added to each reaction mixture. Then 0.1 mmol of Zn^{2+} was added to solution A and 0.1 mmol of **1** was added to solution B. No further addition was made to solution C. All three solutions were then lyophilized. The resulting residues were then dissolved in 2.5 ml of water and subjected to descending paper chromatography using *n*-butyl alcohol–acetic acid–water as the developing solvent. Picolinic acid was used as an internal standard.

Solution B had only a single uv absorbing spot other than nitrophenol. It had the same R_f value (0.52) as the picolinic acid standard, confirming that the products of the zinc-catalyzed hydrolysis of *p*-nitrophenyl picolinate are picolinic acid and nitrophenol. Solution A, in addition to nitrophenol, demonstrated a faint picolinic acid spot and a more intense spot which moved 1.1 times the rate of the picolinic acid spot of solution B. The latter spot was only faintly positive to ninhydrin and is most likely the secondary amide **4**. This identification is further supported by the finding that when **1** and **2** react in acetonitrile in the absence of zinc ion a product is obtained with an ir spectrum consistent with **4** and identical chromatographic behavior as the



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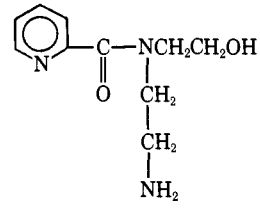
major uv absorbing spot of solution A.

Solution C yielded two distinct uv absorbing spots in addition to nitrophenol. The faster spot was the minor product and migrated with the same R_f as the picolinic acid of solution B. As expected, this spot was ninhydrin negative. The second and more intense uv absorbing spot moved only 0.86 times as fast as picolinic acid. It yielded a bright bluish-purple spot characteristic of primary amines when sprayed with ninhydrin. This strongly uv absorbing and ninhydrin positive spot was tentatively identified as the expected picolinoyl ester of *N*-(β-hydroxyethyl)-ethylenediamine (**3**).

The only other product it might be is the tertiary amide **5**. This is an unlikely product since diethylene-



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triamine and zinc are ineffective in accelerating the rate of nitrophenol production from *p*-nitrophenyl picolinate. In addition, the reaction products of solution C showed a positive hydroxamate test, characteristic of an ester, whereas the products of solutions A and B did not.

However, to exclude **5** as a possible product, a 300-ml reaction mixture identical in all other respects with solution C was prepared and products were isolated in the manner previously described. After lyophilization, the dried residue was stirred for 48 hr at room temperature with 60 ml of chloroform. The infrared spectrum of the chloroform extract possessed a strong 1712-cm^{-1} absorption maximum characteristic of an ester. Paper chromatographic analysis of the chloroform extract showed it contained the ultraviolet and ninhydrin positive spot presumed to be the ester. In summary, the paper chromatographic and infrared evidence, in combination with the unique specificity of the reaction for **1**, all support the conclusion that the picolinoyl ester of **1** (**3**) is the primary and stable product of the zinc-catalyzed reaction between *N*-(β-hydroxyethyl)ethylenediamine and *p*-nitrophenyl picolinate.

Stoichiometry of the Reactive Complex. The data summarized in Table I suggest that the pseudo-first-order rate constant (k_{obsd}) determined in the presene of zinc ion and **1** is composed of four important rate constants under our experimental conditions at any given pH. Therefore

$$k_{obsd} = k_0 + k_M[Zn] + k_A[\mathbf{1}] + k_c[Zn^{2+}\text{–}\mathbf{1}] \quad (1)$$

where, at any given pH, k_0 is the rate constant in buffer; k_M represents the rate constants which depend on zinc ion alone; k_A includes all the rate constants which depend on **1** alone; and k_c is the second-order rate constant which describes the reaction between the kinetically important complex of zinc ion and **1**.

In order to determine the stoichiometry of the kinetically important complex of zinc ion and **1**, it was first necessary to determine the stability constant for the complex composed of Zn^{2+} and **1** so that appropriate corrections for the reactions involving the metal ion alone and the chelating agent alone could be made. Since kinetic experiments were generally performed under conditions in which the concentrations of zinc ion and **1** were equal, the method of Gustafson and Martell[12] was used to determine the stability constants. Table III summarizes the values obtained for zinc ion and three of the chelating agents used in this study. One consequence of the results reported in Table III is that the concentration of Zn^{2+}–**1** is a very sharp function of pH in the pH range 6.5–7.5.

The stoichiometry of the kinetically active complex of zinc ion and **1** was determined with a kinetic version of a Job plot.[14] A series of kinetic measurements was performed in which the sum of the zinc ion and *N*-(β-hydroxyethyl)ethylenediamine (**1**) concentrations was kept constant but the mole fractions were varied. The observed rate constants were then plotted as a function of mole fraction of **1**. The mole fraction of **1** which corresponds to the maximum of the observed rate constants indicates the stoichiometry of the kinetically active species. In Figure 1 are plotted the raw first-order rate constants obtained in this set of experiments. Also plotted in Figure 1 are corrected pseudo-first-order rate constants (k_c') describing only the reaction of [Zn^{2+}–**1**] with *p*-nitrophenyl picolinate (**2**). These constants were obtained by subtracting the contributions of free zinc ion and **1** from the observed first-order rate, according to eq 2, where the kinetic constants are

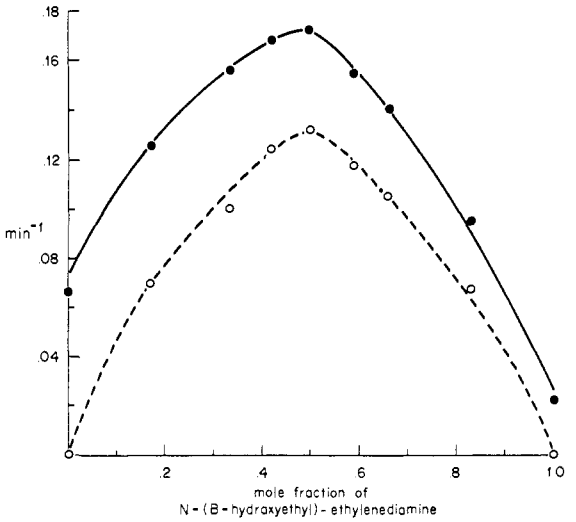


Figure 1. The change in k_c' as a function of the mole fraction of *N*-(β-hydroxyethyl)ethylenediamine: pH 7.10; 0.03 *M* Hepes; 0.1 *M* NaCl. Total concentration of Zn^{2+} plus **1** is 2.06×10^{-3} *M*. In all runs, the *p*-nitrophenylpicolinate concentration is 7.78×10^{-5}: solid line, uncorrected rate constants; dashed line, rate constants corrected for free zinc ion and free **4** concentrations.

$$k_c' = k_c[Zn^{2+}\text{–}\mathbf{1}] = k_{obsd} - (k_0 + k_M[Zn^{2+}] + k_A[\mathbf{1}]) \quad (2)$$

defined in the same manner as in eq 1. The concentrations of free zinc ion and **1** were determined by using the stability constants indicated in Table III, and k_M and k_A were determined by studying the rates of nitrophenol production in the presence of zinc ion and **1** separately. The results reported in Figure 1 indicate that the stoichiometry of the kinetically active complex is 1:1.

pH Dependence. The observed first-order rate constant describing the rate of nitrophenol production from *p*-nitrophenyl picolinate in the presence of constant total concentrations of **1** and zinc ion increases sharply with increases in pH (Table IV). This pH dependence can be attributed to (1) an increase in the concentration of the Zn–**1** complex at higher pH's and (2) an increase in k_c, the second-order rate constant, as a function of pH. The former reason for the pH dependence is a direct result of the stability constant and the pK_a's reported in Table III. The pH dependence of k_c is reflected in Figure 2 where the logarithm of k_c is plotted as a function of pH. The slope of the line in Figure 2 computed from a linear regression analysis is 0.85. A slope of 1.0 is expected if the 1:1 complex of Zn^{2+} and **1** reacts *via* a specific base catalyzed reaction.[15,16]

(14) A. Martell and S. Chaberek, "Organic Sequestering Agents," Wiley, New York, N.Y., 1959, p 508.

(15) T. C. Bruice and S. Benkovic, "Bioorganic Mechanisms," Vol. 1, W. A. Benjamin, New York, N. Y., 1966.

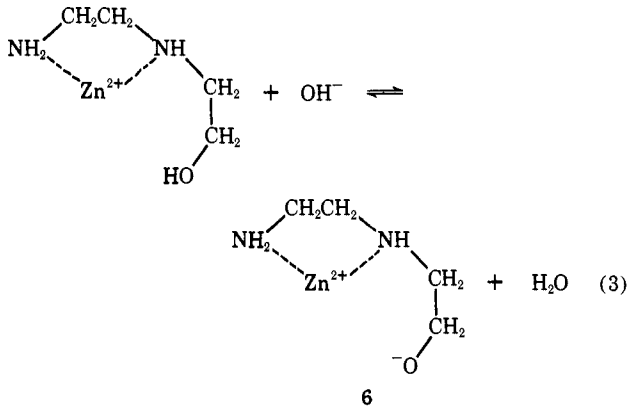
(16) T. C. Bruice and D. Piszkiewicz, *J. Amer. Chem. Soc.*, **89**, 3568 (1967).

Table IV. pH Dependence of Transesterification Reaction with *p*-Nitrophenyl Picolinate (2)[a]

pH	[1], *M*	[Zn^{2+}], *M*	[2], *M*	k_{obsd}, min^{-1}	k_c', min^{-1}	k_c, M^{-1} min^{-1}
7.50	2.02×10^{-3}	2.02×10^{-3}	9.80×10^{-5}	1.06	0.965	956
7.20	2.02×10^{-3}	2.02×10^{-3}	9.80×10^{-5}	0.612	0.549	753
7.02	2.04×10^{-3}	2.03×10^{-3}	9.80×10^{-5}	0.283	0.230	414

[a] 0.03 *M* Hepes buffer with 0.1 *M* NaCl used to maintain pH; ester concentration was 9.80×10^{-5} *M*.

These rate data suggest that the equilibrium indicated in eq 3 is important prior to the formation of the



(3)

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reactive ternary complex and that **6** is the kinetically reactive complex of Zn^{2+} and **1** in the complete reaction system containing **1**, **2**, and zinc ion.

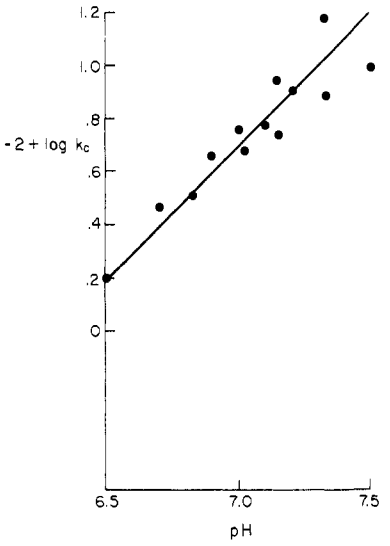


Figure 2. The effect of pH on log k_c. Lutidine and Hepes buffers were employed; 0.1 *M* NaCl was present in all buffers; $T = 25°$.

Ester Specificity. *o*-Nitrophenyl picolinate can replace *p*-nitrophenyl picolinate as the ester substrate in the reaction with Zn^{2+} and **1**. Table V demonstrates that the observed rate of *o*-nitrophenol release is greater in the presence of Zn^{2+} and **1** together than the sum of the rates observed when either Zn^{2+} or **1** is present separately.

Ethyl picolinate does not serve as an ester substrate. In fact, **1** slightly retards the zinc ion catalyzed hydrolysis of ethyl picolinate to ethanol and picolinic acid. Diethylenetriamine inhibits the zinc ion catalyzed hydrolysis of ethyl picolinate more effectively than **1**.

Table V. Rate Constants for *o*-Nitrophenol Release from *o*-Nitrophenyl Picolinate (7) in the Presence of Zinc Ion and *N*-(-β-Hydroxyethyl)ethylenediamine[a]

[Zn^{2+}], *M*	[1], *M*	k_{obsd}, $min^{-1} \times 10^2$
0	0	0.455
0	1.01×10^{-3}	1.76
0	2.04×10^{-3}	2.53
1.01×10^{-3}	0	2.95
2.02×10^{-3}	0	5.68
1.01×10^{-3}	1.01×10^{-3}	20.6
2.02×10^{-3}	2.04×10^{-3}	48.7

[a] 0.03 *M* Hepes buffer (pH 7.20) with 0.1 *M* NaCl; ester concentration was 1.14×10^{-4} *M*.

Reaction Inhibitors. The data presented in Table II strongly imply that the Zn–**1** complex reacts specifically with *p*-nitrophenyl picolinate (**2**) because of the formation of a reactive ternary complex composed of Zn^{2+}, **1**, and **2**. Implicit in this suggestion is that chelating agents must exist which can inhibit the reaction with **2** by competing for the available ligand positions in the Zn–**1** complex. Chelating agents which can inhibit the reaction are 1,10-phenanthroline, picolinic acid, and ethyl picolinate. The latter compound inhibits the rate of nitrophenol production from **2** by 50% at a concentration of 4.04×10^{-2} *M* when the concentrations of Zn^{2+}, **1**, and **2** are 1.04×10^{-3}, 1.03×10^{-3}, and 1.03×10^{-4} *M*, respectively. Since the first stability constant for the coordination of Zn^{2+} by pyridine is 25 M^{-1} (4×10^{-2} *M* as a dissociation constant) the concentration of ethyl picolinate which causes 50% inhibition probably roughly reflects the affinity of *p*-nitrophenyl picolinate (**2**) for Zn–**1** under our reaction conditions.

An attempt was made to determine if added *p*-nitrophenol could inhibit the rate of nitrophenol production from *p*-nitrophenyl picolinate (**2**) in the presence of Zn^{2+} and **1**. No inhibition could be demonstrated up to 2×10^{-4} *M* nitrophenol at pH 7.3, consistent with the conclusion that **3** is the stable product of the reaction.

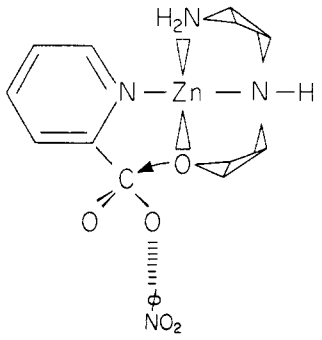
Discussion

Zinc ion catalyzes a facile transesterification reaction between *p*-nitrophenyl picolinate (**2**) and *N*-(β-hydroxyethyl)ethylenediamine (**1**). All the kinetic and specificity data support the suggestion that the reaction proceeds through the formation of a ternary complex composed of Zn^{2+}, **1**, and **2** that has well-defined geometric restrictions. Although the presently available data do not permit the precise determination of the structure of this ternary complex, *N*-(β-hydroxyethyl)ethylenediamine most likely coordinates to the metal ion primarily through the nitrogens of the ethylenediamine moiety[17] while *p*-nitrophenyl picolinate

(17) B. Das Sarma, G. L. Tennenhouse, and J. C. Bailar, Jr., *J. Amer. Chem. Soc.*, **90**, 1362 (1968).

must coordinate to the metal ion through the heterocyclic nitrogen of the pyridine.

The strongest support for the formation of a reactive ternary complex is the observation that the *p*-nitrophenyl esters of isonicotinic and acetic acids do not react readily with the Zn^{2+}–**1** complex (Table II). Since both these esters have intrinsic reactivities roughly comparable to *p*-nitrophenyl picolinate, the reaction of *p*-nitrophenyl picolinate with the Zn^{2+}–**1** complex cannot be a simple bimolecular reaction. The formation of a kinetically important ternary complex is the most likely explanation of the unusual reactivity of *p*-nitrophenyl picolinate since only this ester can form a ternary complex in which the hydroxyethyl group of **1** is favorably oriented for intracomplex nucleophilic attack on the carbonyl group. The ester carbonyl in the ternary complex *p*-nitrophenyl isonicotinate might form with the Zn–**1** complex would be remote from the hydroxyethyl group. *p*-Nitrophenyl acetate is unlikely to form any ternary complex with the Zn–**1** complex since it has no functional group that can serve as an effective ligand to the metal ion. One reasonable structure, among the many possible structures for the ternary complex composed of **1**, **2**, and Zn^{2+}, may be **8**.

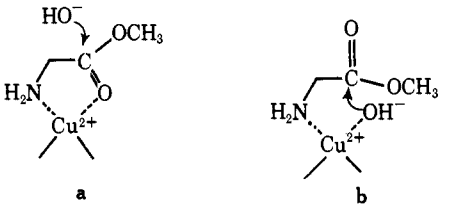


8

The inhibition of the reaction between the Zn^{2+}–**1** complex and **2** by 1,10-phenanthroline, picolinic acid, and ethyl picolinate provides further support for the formation of a kinetically important ternary complex. The kinetic effect of these bidentate ligands is most likely exerted through their competition with **2** for the available coordination positions on the Zn^{2+}–**1** complex. As a consequence, the concentration of the mixed complex composed of Zn^{2+}, **1**, and **2** is decreased in the presence of the bidentate chelating agents and the extent of intracomplex nucleophilic attack of the hydroxyethyl group of **1** on the carbonyl of **2** is correspondingly reduced.

A further interesting feature of the present system is the specificity for *N*-(β-hydroxyethyl)ethylenediamine. Table I indicates that aminoethanol, 1,5-diaminopentane, ethylenediamine, and diethylenetriamine are ineffective in promoting the release of nitrophenol from *p*-nitrophenyl picolinate in the presence of zinc ion. Metal ion catalyzed aminolysis and transesterification reactions in which the amino or hydroxyl group is external to the immediate coordination sphere of the zinc ion are clearly excluded by these data as an explanation for the unique reactivity of **1**. Of particular interest is the negligible reactivity of the diethylenetriamine–zinc ion complex toward *p*-nitrophenyl picolinate. Generally, amino groups are more effective nucleophiles than hydroxyl groups in acyl-transfer reactions.[15] The probable cause for the low reactivity of the diethylenetriamine complex is that the aminoethyl groups of diethylenetriamine, unlike the hydroxyethyl group of **1**, coordinate the zinc ion so tightly that their nucleophilicity is masked. Rigid geometric restrictions therefore must be satisfied in order for intracomplex acyl-transfer reactions to proceed. If a potential nucleophile is too strong a ligand and is not rigidly constrained from coordinating the metal ion, it is unlikely to participate in intracomplex reactions.

The observation of a rapid zinc-catalyzed transesterification reaction between *N*-(β-hydroxyethyl)-ethylenediamine and *p*-nitrophenyl picolinate permits some suggestions as to the mechanism of nucleophilic displacement reactions catalyzed by divalent metal ions in general. Analysis of reactions catalyzed by divalent metal ions such as Cu^{2+}, Ni^{2+}, and Zn^{2+} is usually complicated by the ligand lability in complexes of these metal ions. For example, the cupric ion catalyzed hydrolysis of glycine methyl ester can proceed by two mechanisms which are difficult to distinguish kinetically (mechanisms a and b).[18]



In mechanism a, the function of the cupric ion is to stabilize the developing negative charge on the carbonyl oxygen caused by attack of the hydroxide ion external to the metal ion coordination sphere. The function of the metal ion in mechanism b is to serve as a template for the reaction between the coordinated ester substrate and metal-bound OH^-. An additional component of the metal ion catalysis in mechanism b is its ability to increase the local concentration of hydroxide by perturbing the pK_a of the water molecule coordinated to it.

Although the same mechanistic ambiguity can exist for related inert Co(III) complexes, the relative importance of the two pathways represented by mechanisms a and b can be resolved through ^{18}O-tracer experiments[7,9] in these cases. The results of such studies indicate that both pathways operate to roughly the same extent in the hydrolysis of glycinamide[7] and the isopropyl ester of glycine.[9] The pathway represented by mechanism b appears particularly effective in the hydrolysis of glycinamide. Buckingham, *et al.*, have estimated that the hydrolysis of *cis*-$(Co(en)_2(OH)(glyNH_2)$ may be as much as 10^{11} times more rapid than the hydrolysis of uncoordinated glycinamide.[7]

The zinc ion catalyzed reaction between *N*-(β-hydroxyethyl)ethylenediamine and *p*-nitrophenyl picolinate demonstrates that pathways analogous to mechanism b are also important for labile complexes. However, since *p*-nitrophenyl and *o*-nitrophenyl picolinate react rapidly with the Zn–**1** complex while ethyl picolinate does not, the present experiments suggest

(18) R. Breslow, R. Fairweather, and J. Keana, *J. Amer. Chem. Soc.*, **89**, 2135 (1967).

that pathways analogous to mechanism b may only be important in labile complexes for esters composed of alcohols that are good leaving groups. Further experiments are required to resolve this point.

The most important similarity between the zinc ion catalyzed transesterification between *N*-(β-hydroxyethyl)ethylenediamine and *p*-nitrophenyl picolinate and mechanism b is that both reactions involve the nucleophilic attack of one coordinated ligand upon another. Another significant similarity is that in both cases the concentration of the nucleophilic species is increased as a consequence of coordination or proximity to the metal ion. In the present case, the pK_a of the hydroxy group of *N*-(β-hydroxyethyl)-ethylenediamine in the Zn–**1** complex (see eq 3) can be estimated to be about 8.4 by plotting k_c *vs.* $k_c[H^+]$ from the same kinetic data presented in Figure 2. This probably represents a pK_a perturbation of at least 3–4 pK_a units for the hydroxyethyl group in the Zn–**1** complex **6** relative to the hydroxyethyl group of **1** when it is free in solution.

In summary, metal ions which form both labile and inert complexes can catalyze acyl-transfer reactions by mechanisms in which the dual catalytic function of the metal ion is to increase the concentration of an effective nucleophile at neutral pH values and to serve as a template for the reaction between two coordinated ligands. The present study shows that both these catalytic properties are important in reactions of coordinated ligands and suggests that both are likely to be important factors in the catalytic properties of metalloenzymes.

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Molecular Luminescence Studies of Flavins. I. The Excited States of Flavins[1]

Ming Sun,[2a] Thomas A. Moore,[2b] and Pill-Soon Song*

Contribution from the Department of Chemistry, Texas Tech University, Lubbock, Texas 79409. *Received April 28, 1971*

Abstract: The lowest excited singlet and triplet states of flavins have been investigated by means of luminescence polarization (photoselection) and P–P–P SCF–MO CI methods. The fluorescent and phosphorescent states of flavins of varying structures such as isoalloxazines (*e.g.*, riboflavin), alloxazines (*e.g.*, lumichrome), and lumazines (*e.g.*, 6,7,8-trimethyllumazine) have been assigned (π,π^*) symmetry. However, the (n,π^*) states have been found to play a significant role in the luminescence processes of flavins, particularly of alloxazines. Attempts have been made to characterize various electronic transitions of flavins in the visible and near-uv regions of the absorption spectra, and to reconcile diverse spectroscopic data reported in the literature.

Flavins are implicated in a variety of photobiological processes such as photodynamic action, phototropism, phototaxis, and photosynthesis.[3] In order to elucidate the role of flavins in these and related photochemical systems, a considerable amount of work on the photophysics and photochemistry of flavins *in vitro* has been published during the last decade and is summarized in recent review articles.[4,5] The aim of the present paper is to report a comprehensive luminescence study of flavins in an attempt to characterize and reconcile the diverse spectroscopic properties of these molecules. Three aspects of the excited states of flavins are described herein: namely, (a) assignments of various electronic transitions in the near-uv and visible regions, (b) characteristics of the fluorescent state, and (c) characteristics of the phosphorescent state. In the second part of the present work,[6] dimeric and charge-transfer complexes of flavins will be described.

Experimental Section

Materials. Riboflavin (6,7-dimethyl-(1′-D-ribityl)isoalloxazine, RF) was obtained as described elsewhere.[7] Riboflavin tetrabutyrate (RFTB) was purchased from Tokyo Tanabe Co., and was purified by thin-layer chromatography with chloroform–acetone (1,2 v/v) as the developing solvent. Lumiflavin (6,7-dimethyl-9-methylisoalloxazine, LF), 8-methylisoalloxazine (M1S), 3-methyl-6,7-dichlorolumiflavin, ClLF), 1,3-dimethyl-5,8-dibromolumichrome (1,3-dimethyl-5,8-dibromo-6,7-dimethylalloxazine, BrLC), and 1,3-dimethyllumichrome (MLC) were gifts from Dr. J. Koziol, and luminescent impurities were absent as checked on tlc. 3-Methyllumiflavin (MLF) was a gift from Dr. M. J. Gibian and was used without further purification. Alloxazine (benzo[g]pteridine-2,4-(1*H*,3*H*)-dione), pfs grade, was obtained from the Sigma Chemical Co. and was recrystallized or chromatographed on tlc prior to the preparation of samples. When the compound was found to be free of luminescent impurities, as judged from the excitation spectra which were independent of emission wavelengths monitored, no

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(2) (a) Robert A. Welch Foundation Predoctoral Fellow, 1968–1971; (b) National Science Foundation Predoctoral Trainee, 1968–1971.

(3) For a discussion of the photobiological role of flavins, see J. B. Thomas, "Primary Photoprocesses in Biology," Wiley, New York, N. Y., 1965.

(4) G. R. Penzer and G. K. Radda, *Quart. Rev., Chem. Soc.*, **21**, 43 (1967), and references therein.

(5) P. S. Song in "Flavins and Flavoproteins," H. Kamin, Ed., University Park Press, Baltimore, Md., 1971, pp 37–60.

(6) Part II: T. A. Moore and P. S. Song, submitted for publication.

(7) P. S. Song and D. E. Metzler, *Photochem. Photobiol.*, **6**, 691 (1967).