above yielding starting material (18 mg) and 2,3,6,7-tetramethoxyphenanthrene (560 mg).

(b) In Dichloromethane–TFA (4:1). The electrolysis was carried out exactly as in acetonitrile yielding starting material (19 mg) and 2,3,6,7-tetramethoxyphenanthrene (557 mg).

Anodic Oxidation of 3,3′-Dimethoxybibenzyl (5). (a) In Acetonitrile. The compound (2 mmol, 484 mg) was oxidized as described above. Current corresponding to 3 F/mol was passed through the cell. The current was not reversed but the resulting dark-brown solution was worked up directly yielding only starting material (484 mg) and tars.

(b) In Dichloromethane–TFA (4:1). The electrolysis was carried out as in acetonitrile. The resulting solution was intensely green. Reversal of the current rendered the solution dark-brown after the passage of an amount of current corresponding to 0.8 F/mol. Work-up in the usual manner yielded starting material (16 mg) and 2,7-dimethoxy-9,10-dihydrophenanthrene (449 mg).

Anodic Oxidation of Bis(3-methoxyphenyl)methane (8). (a) In Acetonitrile with Tetrabutylammonium Fluoroborate as Supporting Electrolyte. The compound (2 mmol, 456 mg) was oxidized as described above. Current corresponding to 3 F/mol was passed through the cell. The current was not reversed but the resulting dark-brown solution was worked up directly yielding starting material (290 mg), 3,7-dimethoxyfluorene (60 mg), and polymers (50 mg).

(b) In Acetonitrile with Lithium Perchlorate (0.1 *M*) as Supporting Electrolyte. Current corresponding to 3 F/mol was passed through the cell. During the electrolysis a black precipitate was formed in the solution and on the anode surface. The precipitate dissolved in acetonitrile with an intense blue color and in nitromethane with an intense green color. Both solutions gave strong esr signals. The black precipitate was reduced with zinc powder in the electrolysis solution and after filtration work-up of the filtrate was continued in the usual manner yielding starting material (120 mg), 3,7-dimethoxyfluorene (180 mg), and polymers (95 mg).

(c) In Dichloromethane–TFA (4:1). The compound (2 mmol, 456 mg) was oxidized as described above. Current corresponding to 3 F/mol was passed through the cell. Reversal of the current rendered the solution colorless after the passage of an amount of current corresponding to 0.7 F/mol. Work-up in the usual manner yielded starting material (90 mg) and 3,7-dimethoxyfluorene (316 mg).

Anodic Oxidation of 3,3′,4-Trimethoxybibenzyl (11). (a) The compound (816 mg, 3 mmol) was dissolved in CH_2Cl_2–TFA (4:1, 150 ml) containing n-Bu_4NBF_4 (0.1 *M*) and under N_2 subjected to constant current (current density 3×10^{-5} A/cm²) electrolysis in a divided cell at −25°. When an amount of current corresponding to 2 F/mol had been passed through the cell, the electrolysis was interrupted and zinc powder (2 g) and water (50 ml) were added to the anolyte. After 2 min of stirring the color of the anolyte had changed from blue to yellow and the mixture was filtered. The organic phase was washed with a saturated bicarbonate solution (50 ml) and water (50 ml), dried over sodium sulfate, and evaporated. The resulting oil was chromatographed on 100 g of silica gel (benzene–ether 5:1) yielding starting material (208 mg) and a new compound (375 mg): mp 98–99°; nmr ($CDCl_3$) δ 2.67 (s, 8 H), 3.67 (s, 6 H), 3.83 (s, 6 H), 3.88 (s, 6 H), 6.50–7.33 (m, 12 H); *m/e* 542 (M^+). On the basis of these data structure **12** was assigned to the new compound. *Anal.* Calcd for $C_{34}H_{38}O_6$: C, 75.2; H, 7.0. Found: C, 75.4; H, 6.8.

(b) A similar experiment as under (a) was carried out with a current density of 1×10^{-1} A/cm². An amount of current corresponding to 6 F/mol was passed through the cell. Chromatography on silica gel yielded starting material (315 mg), **12** (96.5 mg), and 2,3,7-trimethoxyphenanthrene (11 mg) identified by comparison with an authentic sample.

Anodic Oxidation of 2,3,7-Trimethoxy-9,10-dihydrophenanthrene (13) in Dichloromethane–TFA (4:1). The compound (2 mmol, 540 mg) was oxidized as described above. Current corresponding to 2 F/mol was passed through the cell. The resulting green solution was rendered colorless by addition of zinc powder. Work-up in the usual manner yielded 2,3,7-trimethoxyphenanthrene (299 mg) and 2,3,7-trimethoxy-9,10-dihydrophenanthrene (13 mg).

Catalysis of the β-Elimination of *O*-Phosphothreonine by Pyridoxal and Metal Ions[1]

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Abstract: The β-elimination reaction of *O*-phosphothreonine was investigated in aqueous media at 45.0 ± 0.1° in the presence of pyridoxal and various metal ions. Of the six metal ions employed only copper(II) and oxovanadium(IV) ions have strong catalytic effects. In the pyridoxal–copper(II) catalysis the rate increased as pH was raised from neutrality with a rate plateau being observed in the region of pH 8–10. For the pyridoxal–vanadyl(IV) catalysis the rate maximum was reached in the region of $-\log [H^+] \simeq 5.5$. On the basis of equilibrium studies of the pyridoxal–metal–threonine and related systems by means of electronic spectroscopy, the observed catalysis was ascribed to the formation of metal complexes of the amino acid–pyridoxal Schiff base having a 1:1 molar ratio of ligand to metal ion. The addition of organic bases (*i.e.*, piperidine and morpholine) resulted in further rate enhancement. This catalysis was attributed to a rate-determining step involving combination of the base with a proton abstracted from the α position of the amino acid, and the concomitant liberation of the phosphate group by carbon–oxygen fission. The catalytic activity observed in these systems seem to be derived from the electronic effect of metal ions in the Schiff base complexes in a direction which promotes hydrogen abstraction. A reaction mechanism for metal ion–pyridoxal catalysis of elimination of phosphate groups substituted in the β position of amino acids is proposed.

Among various nonenzymatic reactions of α-amino acids, β-elimination reactions were observed to occur with appropriate β-substituted amino acids in the presence of pyridoxal and metal ions. The substrates include serine,[2a] threonine,[2b] β-chloroalanine,[3]

(1) This work was supported in part by the National Institute of Arthritis Metabolic and Digestive Diseases, U. S. Public Health Service, under Research Grant No. AM 11694.

(2) (a) D. E. Metzler and E. E. Snell, *J. Biol. Chem.*, **198**, 353 (1952); (b) D. E. Metzler, J. B. Longenecker, and E. E. Snell, *J. Amer. Chem. Soc.*, **76**, 639 (1954).

(3) R. I. Gregerman and H. N. Christensen, *J. Biol. Chem.*, **220**, 765 (1956).

cysteine,[2a] cystathionine, lanthionine,[4] *O*-phosphoserine, *O*-phosphothreonine, *O*-carbamylserine, azaserine,[5] *O*-sulfoserine, and *O*-sulfothreonine.[6] Although metal ions do not seem to be involved directly in the vitamin B_6 enzyme system,[7] the nonenzymatic reactions promoted by metal ions have many similarities in their mechanistic behavior to the corresponding enzymatic ones. It is, therefore, of interest to study the reaction mechanisms of these metal ion catalyzed reactions to assist in the understanding of the corresponding enzymatic reactions, and as a further step in the designing of more sophisticated model systems.

The dephosphorylation of *O*-phosphothreonine has been previously reported by Longenecker and Snell.[5] In order to provide more mechanistic information on this reaction, this research is concerned with kinetic and equilibrium measurements of *O*-phosphothreonine under β-elimination conditions in the presence of various metal ions.

Experimental Section

Materials. Pyridoxal hydrochloride and pyridoxamine dihydrochloride were obtained from Mann Research Laboratories, Inc. *O*-Phosphothreonine was obtained from Sigma Chemical Co.. and also prepared by the phosphorylation of threonine with phosphorus oxychloride. Alternatively, pyrophosphoric acid (a mixture of 85% orthophosphoric acid and phosphorus pentoxide) may be employed. The crude *O*-phosphothreonine thus produced was refined by cation exchange (Dowex 50W-X8) column chromatography and further purified by recrystallization from water. 2-Aminoethyl phosphate and *O*-phospho-α-methylserine were prepared in a similar manner by the phosphorylation of the corresponding alcohols with pyrophosphoric acid and were recrystallized from aqueous ethanol. α-Ketobutyric acid was obtained by the thermal decarboxylation of methyloxalacetic anhydride.[8] All of these organic materials were subjected to elemental analysis, and gave percentages of carbon, hydrogen, and nitrogen that are very close to the theoretical values. For phosphorus analysis, the sample was completely decomposed with nitric acid and the inorganic phosphate produced was determined colorimetrically by Allen's method.[9]

Stock solutions of metal ions were prepared from their nitrate salts except for the oxovanadium(IV) ion, which was obtained as the sulfate salt. The metal ion solutions were standardized by conventional chelatometric titrations.

Paper chromatography (ascending technique) was run on sheets of Toyo No. 50 or 51A filter paper with the use of the following solvent systems: (1) 1-butanol 40, acetic acid 10, and water 20 parts by volume; and (2) 1-propanol 70, concentrated aqueous ammonia 20, and water 20 parts by volume. The chromatographic spots were developed with the 0.1% ninhydrin solution for amino compounds and with molybdate solution[10] for phosphorus-containing compounds. In the latter the spots were exposed to gaseous hydrogen sulfide subsequent to treatment with molybdate.

Kinetic Measurements. Electrodes for pH measurements were usually a set of Beckman glass electrode No. 39099 and Metrohm reference electrode EA-402, or a combination of Beckman glass electrode No. 43509 and reference electrode No. 39402. Another combination, glass electrode HG-6005 and reference electrode HC-605 of TOA Electronics, Ltd., Tokyo, was adopted occasionally. The pH value of an experimental solution was maintained constant during each run within an accuracy of ± 0.02 with the use of a TOA pH stat HS-1B which was fitted with an autoburet and incorporated with a Beckman pH meter SS-2. In cases when TOA electrodes were used, a combination of TOA pH stat HS-2A and TOA pH meter HM-5A was adopted.

The initial substrate concentration was adjusted to either 1.00 or 2.00×10^{-3} *M* and the concentrations of pyridoxal and metal ions were set at 5.00×10^{-4} *M* in all cases. The ionic strength was maintained at either 0.10 or 1.0 with potassium nitrate, depending upon the concentrations of other components in an experimental solution. The reaction rate at $45.0 \pm 0.1°$ was determined by measuring the amount of inorganic phosphate liberated in the course of reaction. The reaction was initiated by adding 5.0 ml of the metal salt solution to 50 ml of a metal-free reaction mixture. Aliquot samples were withdrawn from the reaction mixture at appropriate time intervals, cooled, and treated with a cold mixture of 60% perchloric acid (2 ml) and water (10 ml). All sample solutions were then kept in a refrigerator prior to analysis for inorganic phosphate by Allen's method[9] or by a slightly modified method.[11] In the latter method, 2.5 ml of 15 vol % sulfuric acid was employed (to prevent precipitation of the supporting electrolyte) in place of the 2.0 ml of perchloric acid used in Allen's method. In both procedures, the absorbance was measured at 720 nm on a Shimadzu-Bausch & Lomb Spectronic 20 colorimeter.

The pH was calibrated before and after each kinetic run by the use of standard buffers prepared in accordance with the recipe, JIS-Z8802-1964, and the pH variation was controlled within ± 0.03 pH unit. The pH values thus obtained were then converted to hydrogen ion concentrations. The logarithm of the apparent activity coefficient for the hydrogen ion (ΔpH) was evaluated as the difference between the pH meter reading and the negative logarithm of the stoichiometric concentration, as established by titration of perchloric or hydrochloric acid of known concentration with standard sodium hydroxide.

Equilibrium Measurements. Equilibrium studies were made by measurement of the spectral changes produced upon Schiff base formation. In these binary pyridoxal–amino acid systems the total concentration of pyridoxal was set at 1.00×10^{-4} *M* and the total concentrations of amino acids were varied in a range from 0.010 to 0.10 *M*, while the ionic strength of aqueous systems was maintained at 1.0 with potassium chloride. Equilibria existing in the pyridoxal–metal–amino acid systems were also investigated spectrophotometrically by preparing the aqueous solutions having ionic strength 0.10 with potassium chloride. In these ternary systems the total concentrations of pyridoxal and amino compounds were set at 2.00×10^{-4} and 1.00×10^{-2} *M*, respectively, and the concentrations of metal ions were adjusted to 5.00×10^{-4} *M* for threonine and to 1.00×10^{-4} *M* for 2-aminoethyl phosphate. Although equilibrium was reached in a short period of time for the binary systems, the rate of equilibration was rather slow in the ternary systems. Therefore, these sample solutions were allowed to stand overnight to assure the attainment of equilibrium. The addition of amino acids in excess of the metal ion concentration employed also served to facilitate equilibration.

Electronic spectra were measured on a Hitachi Model EPS-2 recording spectrophotometer by the use of a pair of matched silica cells of 1.00-cm path length equipped with a jacket through which water was circulated to maintain the temperature at $25.0 \pm 0.1°$. The hydrogen ion concentration was measured with a Beckman pH meter SS-2 fitted with a Metrohm combination electrode EA-125. Equilibrium constants were evaluated with the computational procedure described by Nagano and Metzler.[12,13] The calculations were programmed by means of the Fortran language for use with a FACOM 230-60 electronic computer of the Computation Center of Kyushu University.

Esr spectra on the pyridoxal–threonine–copper(II) system were measured in aqueous solution at room temperature with a JEOL-ME-3X spectrometer equipped with a 100-KHz field modulation unit.

Identification of Products. The experimental solutions used for kinetic runs were recovered and maintained at 45° for 5 hr at pH 10. The keto acid produced was isolated as its 2,4-dinitrophenylhydrazone in a manner similar to that described by Metzler and

(4) F. Binkley, *J. Amer. Chem. Soc.*, 77, 501 (1955); F. Binkley and M. Boyd, *J. Biol. Chem.*, 217, 67 (1955).
(5) J. B. Longenecker and E. E. Snell, *J. Biol. Chem.*, 225, 409 (1957).
(6) J. H. Thomas, K. S. Dodgson, and N. Tudball, *Biochem. J.*, 110, 687 (1968).
(7) T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," W. A. Benjamin, New York, N. Y., 1966, Chapter 8.
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(9) R. J. L. Allen, *Biochem. J.*, 34, 858 (1940).
(10) E. Karl-Kroupa, *Anal. Chem.*, 28, 1091 (1956).
(11) M. Nakamura, *Nogei Kagaku Zasshi*, 24, 1 (1950).
(12) K. Nagano and D. E. Metzler, *J. Amer. Chem. Soc.*, 89, 2891 (1967).
(13) The authors are grateful to Dr. Nagano of the Faculty of Pharmaceutical Science, University of Tokyo, for providing detailed information about the computer program employed.

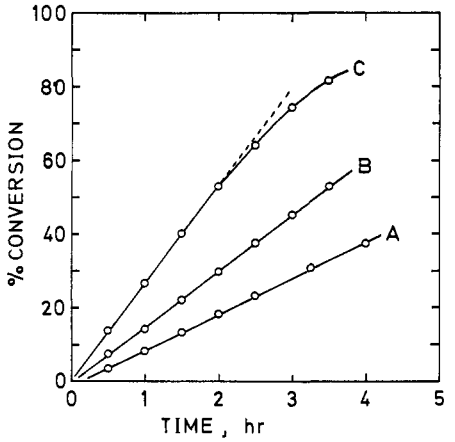


Figure 1. Zero-order plots for the pyridoxal-catalyzed β-elimination of *O*-phosphothreonine in the presence of copper(II) at 45.0 ± 0.1° and μ = 1.0 (KNO_3): *O*-phosphothreonine 2.00 × 10^{-3} *M*, pyridoxal 5.00 × 10^{-4} *M*, copper(II) 5.00 × 10^{-4} *M*; A, $-\log [H^+]$ = 8.30; B, $-\log [H^+]$ = 9.25 and 0.05 *M* morpholine present; C, $-\log [H^+]$ = 9.86 and 0.05 *M* piperidine present.

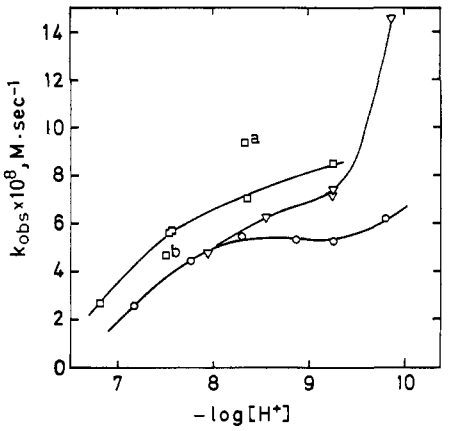


Figure 2. pH–rate (zero order) profile for the pyridoxal-catalyzed β-elimination of *O*-phosphothreonine in the presence of copper(II) at 45.0 ± 0.1° and μ = 1.0 (KNO_3): *O*-phosphothreonine 2.00 × 10^{-3} *M*, pyridoxal 5.00 × 10^{-4} *M*, copper(II) 5.00 × 10^{-4} *M*; (○) without organic bases; (▽) 0.05 *M* piperidine present; (□) 0.05 *M* morpholine present (a, 0.10 *M*; b, 0.02 *M*).

Snell[14] and was recrystallized from methanol. The keto acid formed was identified as α-ketobutyric acid by comparing its infrared and visible spectra with those of an authentic sample. Aqueous solutions prepared by mixing *O*-phosphothreonine, pyridoxal, and copper(II) ion at the concentrations of 1.0 × 10^{-2} *M* were also warmed at 45° at pH ~10 to the completion of reaction, and the resulting mixture was examined by paper chromatography. Neither pyridoxamine nor threonine was detected, clearly indicating that neither hydrolysis nor transamination proceeded to any meaningful extent under these experimental conditions.

Results

Kinetic Measurements. The pyridoxal-catalyzed dephosphorylation of *O*-phosphothreonine was investigated in aqueous media at 45° in the presence of various metal ions. All the kinetic runs were carried out in homogeneous systems except for those in the presence of the thorium(IV) ion, for which measurements were made with suspensions. The $-\log [H^+]$ values at which kinetic measurements for metal ions other than copper(II) and vanadyl(IV) were performed are as follows: aluminum(III) 7.37, 8.64; iron(III) 7.04, 9.10; nickel(II) 9.67; thorium(IV) 6.3. With these metal ions the amount of inorganic phosphate liberated was too small to permit accurate rate estimations in reasonably accessible reaction times. The copper(II)–pyridoxal and the vanadyl(IV)–pyridoxal systems demonstrated profound rate enhancements under the same general reaction conditions. On the other hand, copper(II) and vanadyl(IV) ions did not promote dephosphorylation of two closely related substrates, the pyridoxal Schiff bases of *O*-phospho-α-methylserine and 2-aminoethyl phosphate, under the same experimental conditions.

Copper(II) Catalysis. When the initial substrate concentration was set at 2.00 × 10^{-3} *M*, *i.e.*, four times the catalyst concentration, the reaction followed zero-order kinetics with respect to the unreacted substrate concentration up to about 60% conversion (Figure 1). The presence of organic bases resulted in the zero-order reaction kinetics in a similar manner, though at high conversions to reaction product the rate showed negative deviation from the zero-order plot. When the initial substrate concentration was reduced to 1.00 × 10^{-3} *M* the adherence to zero-order kinetics was retained only at relatively low conversions of the substrate. The zero-order rate constants thus obtained at high initial substrate concentrations are plotted against $-\log [H^+]$ as shown in Figure 2. The rate increases first as pH is raised from neutrality and then reaches a plateau region above $-\log [H^+] \sim 8$.

The presence of 0.050 *M* imidazole resulted in a slight retardation of the reaction rate. On the other hand, morpholine and piperidine at the same concentration promoted the dephosphorylation reaction, as can be seen from the pH dependence of the corresponding rate constant plots shown in Figure 2. In view of the p*K* values of these catalytic bases (morpholine 8.36,[15a] piperidine 11.22[15b]), it seems that both the free and protonated forms have catalytic activity. The profile of the more basic catalyst piperidine indicates a very marked increase at high pH corresponding to an increase in equilibrium concentration of the free base. Upon doubling the morpholine concentration the overall rate was further increased, while its reduction to 0.020 *M* resulted in a decrease in the rate of catalysis, but the concentration effect is apparently not linear. Because of the seeming complexity of the solution equilibria the specific rate constants of individual species in solution were not evaluated and the subsequent discussion is made in terms of the overall rate constants.

Vanadyl(IV) Catalysis. In vanadyl(IV) catalysis the reaction rate followed neither first-order nor zero-order kinetics with respect to the unreacted substrate concentration. For convenience, the apparent first-order rate constants were estimated from the initial stage of the reaction. As illustrated in Figure 3, the pH–rate profile seems to be a typical bell-shaped curve with a maximum rate at $-\log [H^+] \simeq 5.5$. In spite of the apparent differences in reaction type, rate constants, and pH–rate profile, the reaction mechanisms for vanadyl(IV) and copper(II) catalysis seem to resemble each other as described below.

Equilibrium Measurements. Schiff Base Formation. The formation of Schiff bases from pyridoxal and amino

(14) D. E. Metzler and E. E. Snell, *J. Amer. Chem. Soc.*, 74, 979 (1952).

(15) (a) H. K. Hall, Jr., *ibid.*, 78, 2570 (1956); (b) S. Searles, M. Tamres, F. Block, and L. A. Quarterman, *ibid.*, 78, 4917 (1956).

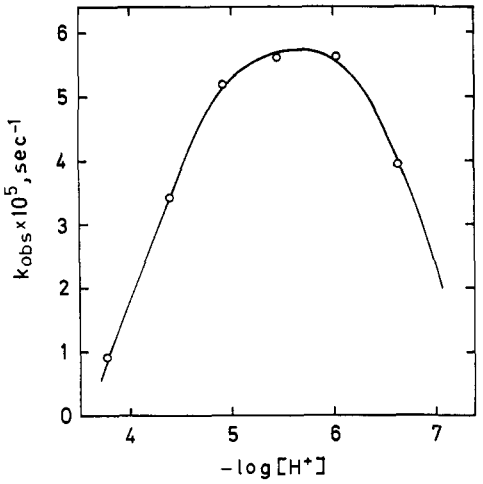


Figure 3. pH–rate (first order) profile for the pyridoxal-catalyzed β-elimination of *O*-phosphothreonine in the presence of vanadyl(IV) at 45.0 ± 0.1° and μ = 1.0 (KNO_3): *O*-phosphothreonine 2.00 × 10^{-3} *M*, pyridoxal 5.00 × 10^{-4} *M*, vanadyl(IV) 5.00 × 10^{-4} *M*.

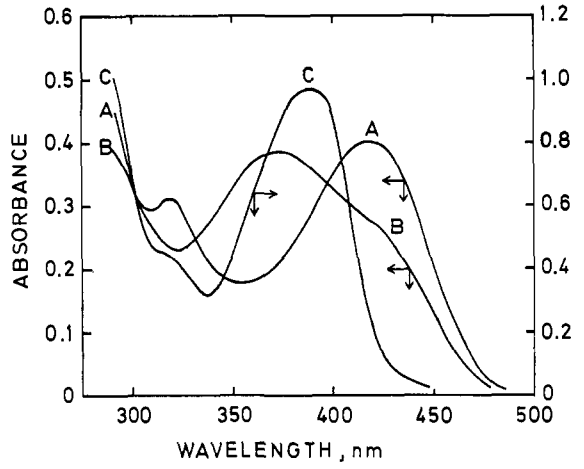
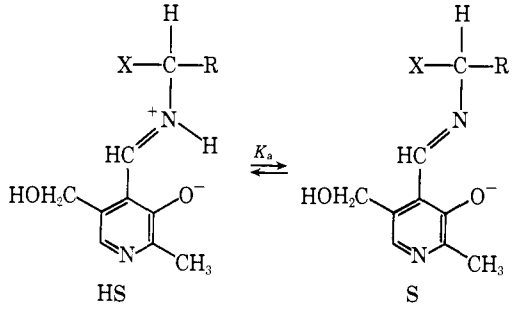


Figure 4. Electronic spectra for pyridoxal–threonine and pyridoxal–threonine–copper(II) systems at 25.0 ± 0.1°; μ = 1.0 for A and B, 0.10 for C with KCl: (A) pyridoxal 1.00 × 10^{-4} *M*, threonine 1.00 × 10^{-1} *M*, $-\log[H^+]$ = 8.07; (B) pyridoxal 1.00 × 10^{-4} *M*, threonine 1.00 × 10^{-1} *M*, $-\log[H^+]$ = 9.81; (C) pyridoxal 2.00 × 10^{-4} *M*, threonine 1.00 × 10^{-2} *M*, copper(II) 5.00 × 10^{-4} *M*, $-\log[H^+]$ = 4.22.

acids was detected spectrophotometrically in the presence of excess amino acids as has been reported previously by Metzler.[16] For all the amino acids examined, two ionic species of the corresponding Schiff bases were observed over the pH range 7–10, the protonated species (HS) absorbing at ~420 nm and the deprotonated form (S) at ~370 nm. One example is shown in Figure 4 for the pyridoxal-threonine system. The formation constant (log K_s) and the acid dissociation constant (pK_a) of the corresponding aldimine defined by eq 1 and 2, respectively, were calculated according to the computational procedure described by Nagano and Metzler[12]



$$K_s = [S]/[P][A] \quad (1)$$

$$K_a = [H][S]/[HS] \quad (2)$$

where P, A, and S stand for the highest deprotonated forms of pyridoxal, amino compound, and Schiff base, respectively. The results are summarized in Table I.

Table I. Formation Constants and Acid Dissociation Constants of Schiff Bases Derived from Pyridoxal and Amino Compounds at 25.0 ± 0.1° and μ = 1.0 (KCl)

	Amino compd			
	O-Phosphothreonine	Threonine	2-Aminoethyl phosphate	Alanine[a]
log K_s	0.70	1.48	1.81	1.61
pK_a	10.48	9.89	9.96	9.91

[a] Reference 12 (at 50°).

These values are comparable in magnitude to the literature values for similar systems.[12]

Formation of Metal–Schiff Base Complexes. When a metal salt solution was added to a solution of a Schiff base, a new broad absorption band appeared near 380 nm, as has also been reported in the literature,[17] with λ_{max} depending somewhat on the amino compound and metal ion employed. For a particular combination of metal ion and ligand, however, the value λ_{max} did not change substantially throughout the whole pH range studied. The absorbance at λ_{max} which is plotted against $-\log[H^+]$ in Figures 5 and 6 for the pyridoxal–threonine–metal and the pyridoxal–2–aminoethyl phosphate–metal systems, respectively, may be taken as a direct measure of the extent of Schiff base–metal complex formation in these solutions.

The copper(II) and vanadyl(IV) complexes of the pyridoxylidenethreonine Schiff base were formed in acid solution at pH values as low as 3. While the vanadyl complex appeared to exist only in the acid region (pH <7), the copper complex was stable throughout the pH range investigated. The lack of stability of the vanadyl complexes in the alkaline region is considered to be due to dissociation and hydrolysis of the metal ion, as occurs with many other vandyl complex systems. With nickel(II), formation of the Schiff base complex was observed to occur at higher pH values ($-\log[H^+] \geq 5$), indicating that the nickel(II) complex is less stable than the copper(II) and oxovanadium(IV) complexes.

When threonine was replaced by 2-aminoethyl phosphate, the 380-nm band was not observed with oxovanadium(IV) present at any pH within the pH range studied, indicating lack of stability of the Schiff base complex relative to other vanadyl species formed. On the other hand, with copper(II) and nickel(II) Schiff base formation was detected spectrophotometrically about 2 pH units higher than those observed for the pyridoxylidenethreonine systems. Thus, it is seen that the carboxylate group in threonine provides

(16) D. E. Metzler, *J. Amer. Chem. Soc.*, 79, 485 (1957).

(17) L. Davis, F. Roddy, and D. E. Metzler, *ibid.*, 83, 127 (1961).

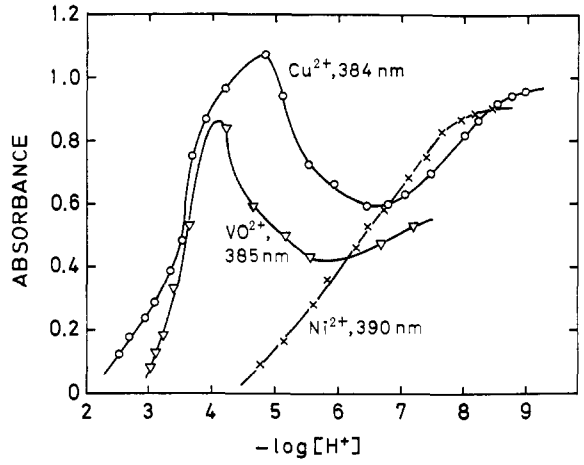


Figure 5. pH dependency of absorbance at λ_{max} for the pyridoxal–threonine–metal systems at 25.0 ± 0.1° and μ = 0.10 (KCl): pyridoxal 2.00 × 10^{-4} M, threonine 1.00 × 10^{-2} M, metal ion 5.00 × 10^{-4} M.

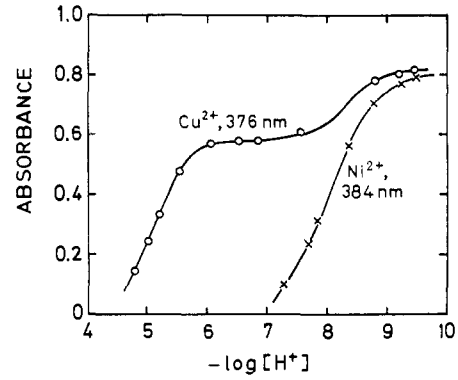
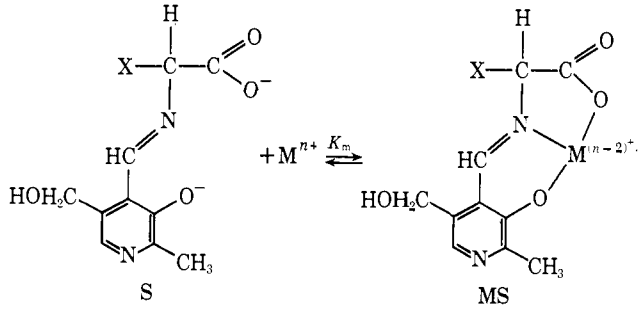


Figure 6. pH dependency of absorbance at λ_{max} for the pyridoxal–2-aminoethyl phosphate–metal systems at 25.0 ± 0.1° and μ = 0.10 (KCl): pyridoxal 2.00 × 10^{-4} M, 2-aminoethyl phosphate 1.00 × 10^{-2} M, metal ion 1.00 × 10^{-4} M.

an additional donor group and the Schiff base derived from pyridoxal and threonine (or *O*-phosphothreonine) functions as a terdentate ligand. The carboxylate group therefore seems to be essential for stable chelate formation at relatively low pH and, for the oxovanadium(IV) complexes, it is essential for complex formation at any pH.

In order to determine the composition of a metal complex, the mole ratio method was applied to the pyridoxal–threonine–copper(II) system at $-\log [H^+]$ = 9.1. As indicated in Figure 7, a break in the absorbance–mole ratio plot at slightly above a 1:1 ratio of pyridoxal and threonine to copper(II) indicates that a 1:1 copper(II)–Schiff base complex is the principal species formed. The lower slope of the plot in Figure 7 above a molar ratio of unity is probably due to the background absorbance of the excess ligand, but formation of a small amount of a weak 2:1 complex is not ruled out.

The formation constant (log K_m) for a Schiff base complex defined by eq 3 was calculated under the assumptions that (1) the solution equilibria involved are formation of a fully deprotonated metal–Schiff base complex at a 1:1 molar ratio, and also 1:1 and 2:1 amino acid–metal complexes (eq 4 and 5), and (2)



$$K_m = [MS]/[M][S] \quad (3)$$

$$M + A \rightleftharpoons MA \quad (4)$$

$$MA + A \rightleftharpoons MA_2 \quad (5)$$

A = amino compound

species absorbing in the near-uv region are pyridoxal, Schiff base, and metal–Schiff base complex in various degrees of protonation. In order to determine more accurate formation constants, other equilibria of lesser importance, such as formation of mixed ligand complexes, should be taken into account, as reported by Leussing, *et al.*[18] Nevertheless, the Schiff base complexes having a 1:1 molar ratio of metal to ligand (eq 3) are the ones of primary importance to the catalytic studies under investigation, and are considered the predominant species involved in the complex-forming equilibria under the present experimental conditions. Accurate values of the formation constants were not obtained for the pyridoxylidenethreonine complexes of copper(II) and oxovanadium(IV). However, approximate values were estimated by comparing absorbance (at λ_{max})–pH profiles for these metal systems with the corresponding nickel(II) system, and by comparison with literature values.[17,19] The equilibrium data thus obtained are listed in Table II.

Table II. Formation Constants of Metal–Schiff Base Complexes at 25.0 ± 0.1° and μ = 0.10 (KCl)

	Amino compd			
	Threonine	2-Aminoethyl phosphate	Valine[c]	Glycine[d]
Cu^{2+}	>14[a]	~10[a]	14.5	
Ni^{2+}	9.84	6.98	10.8	10.3
VO^{2+}	>14[a]	*b*		

[a] Estimated, see text. [b] Formation of the complex was not observed spectroscopically. [c] Reference 17. [d] Reference 19.

Discussion

Catalytically Active Complexes and Role of Metal Ions. The β-elimination of *O*-phosphoserine has been observed in the pH range of 7–13 at 100° in the absence of pyridoxal and metal ions. The reaction rate was relatively slow and essentially independent of pH, with a first-order rate constant of approximately 2 × 10^{-2} hr^{-1}.[20] The elimination rate was somewhat raised when pyridoxal was present as a catalyst species. But the pyridoxal–metal ion systems were the only catalysts that gave considerable rate enhancement.[5]

(18) W. L. Felty, C. G. Ekstrom, and D. L. Leussing, *J. Amer. Chem. Soc.*, **92**, 3006 (1970).
(19) D. L. Leussing and N. Hug, *Anal. Chem.*, **38**, 1388 (1966).
(20) D. Samuel and B. L. Silver, *J. Chem. Soc.*, 289 (1963).

In the present kinetic study of the β-elimination of *O*-phosphothreonine, copper(II) and vanadyl(IV) ions were found to be active catalysts upon incorporation of pyridoxal, while nickel(II), iron(III), aluminum-(III), and thorium(IV) were found to be inactive under the experimental conditions employed.

A calculation based on the data in Table I shows that the fraction of Schiff base in the pyridoxal-amino compound systems never exceeded 10% of the total substrate. This low formation of the Schiff base could be partly responsible for the observed low rate of β-elimination in the absence of metal ions. With metal ions present, the concentrations of Schiff base (as the metal chelate) are much higher, as can be calculated from the data in Table II, which summarizes the equilibrium information obtained thus far for these systems. Since the ternary systems containing *O*-phosphothreonine did not reach equilibrium in the limited time available prior to extensive dephosphorylation, threonine was employed as a model for the substrate to obtain applicable equilibrium data.

Copper(II) forms stable complexes with the Schiff base derived from pyridoxal and threonine. As can be seen from Figure 5, the equilibrium fraction of the Schiff base complex varies as a function of pH. The absorbance maximum that is observed between $-\log [H^+]$ values of 4 and 5 corresponds to the highest degree of formation of the protonated Schiff base complex having a 1:1:1 molar ratio of pyridoxal, threonine, and copper(II), and having a proton on the pyridyl nitrogen. This complex then undergoes deprotonation in the following higher pH region, and is nearly completely deprotonated at $-\log [H^+] \simeq 7$. The dissociation constant for this deprotonation process was estimated to be $10^{-5.7}$ from the curve shown in Figure 5. This sort of behavior has been described for other pyridoxal–metal–amino acid systems.[17,21] In the higher pH range ($-\log [H^+] > 7$), the fourth coordination site of copper(II), which was previously occupied by a water molecule, is most likely replaced by hydroxide ion. The aqueous solution containing pyridoxal, threonine, and copper(II) provided a *g* value and a hyperfine splitting constant of 2.149 and 73.1 G, respectively, in a lower pH range ($-\log [H^+] = 4.2$), while in a higher pH region ($-\log [H^+] = 9.1$) these values were somewhat changed, to 2.132 and 69.3 G, respectively. Also, the ligand-field band observed at 685 nm in the lower pH range shifted to 640 nm in the higher. These spectral changes are consistent with alteration of the ligand field as suggested by the changing composition of the complex with increasing pH.

An increase of Schiff base formation due to the presence of copper ion was observed for the pyridoxal–2-aminoethyl phosphate system as well. As shown in Figure 6, the Schiff base complex was formed in a higher pH range than that of the threonine system indicating lower stability of the complex when the stabilizing carboxylate group is absent. Apparently the phosphate moiety, which is probably bound to the Cu(II) ion in the Schiff base, is not as effective a ligand under the steric influence of a larger chelate ring, as the carboxylate group. In this system the protonated complex seems to be formed in a pH region below 7

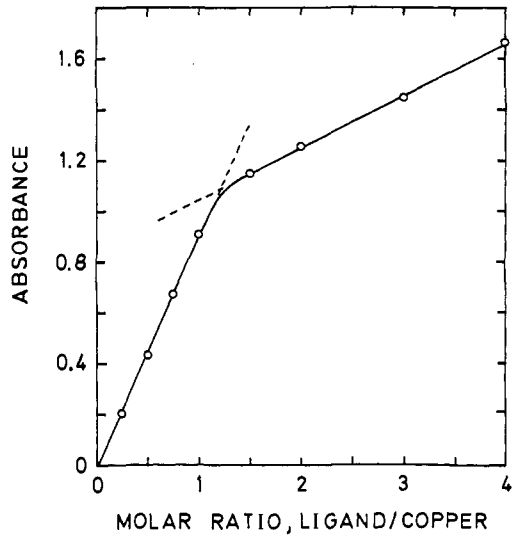


Figure 7. Mole ratio study for the pyridoxal–threonine–copper(II) system at 385 nm and $-\log [H^+] = 9.1$ (0.01 *M* borate buffer): copper(II) 2.00×10^{-4} *M*, pyridoxal–threonine (Schiff base ligand) system $0.50\text{–}8.00 \times 10^{-4}$ *M*.

with its maximum reached at around $-\log [H^+] = 6$. The proton dissociation process seems to proceed at a higher pH range in a manner similar to that described for the threonine system.

The oxovanadium ion apparently yields a 1:1 protonated complex with the Schiff base derived from pyridoxal and threonine in a manner analogous to that described above. As can be seen from Figure 5, the maximum concentration of the pyridine nitrogen-protonated species is reached at $-\log [H^+] = 4$.

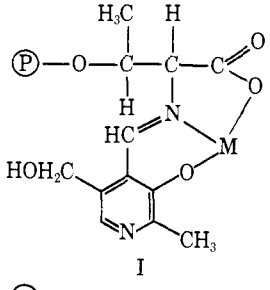
The dephosphorylation of *O*-phosphothreonine proceeds rapidly in the presence of pyridoxal and copper(II) above $-\log [H^+] = 7$. On the basis of the above equilibrium analysis it may now be concluded that the deprotonated Schiff base–copper(II) complex is the reactive species in the β-elimination reaction. The rate of the β-elimination reaction of *O*-phosphothreonine was also much enhanced by the presence of oxovanadium(IV) above $-\log [H^+] = 4$. Although the Schiff base complex is considerably protonated at pH 4, the catalytic effect was observed to increase as the pH was further increased thus increasing the concentration of the deprotonated form. The decline of the rate of dephosphorylation above pH 6 is attributed to the dissociation of the vanadyl complex at higher pH range, as described above.

Nickel(II) also forms complexes with the Schiff bases pyridoxylidenethreonine and pyridoxylidene-2-aminoethyl phosphate as indicated in Figures 5 and 6. Although the nickel complexes are less stable than the corresponding copper complexes, the extent of Schiff base formation was considerable at higher pH. On the basis of this lower stability, a weaker catalytic effect would be expected. The observed complete absence of catalysis under the conditions employed might at first seem surprising, but an attempt to rationalize this observation will be given below. The formation of aluminum(III) complexes with *N*-pyridoxylidenealanine in mono and bis forms has been detected recently by nmr spectroscopy in deuterium oxide.[22] The *N*-pyridoxylidenevaline–iron-

(21) H. N. Christensen, *J. Amer. Chem. Soc.*, **79**, 4073 (1957).

(22) O. A. Gansow and R. H. Holm, *ibid.*, **91**, 573 (1969).

(III) complex at a 2:1 molar ratio of Schiff base to metal ion has been isolated and characterized by potentiometric titration.[21] The pyridoxal-*O*-phosphothreonine and other related Schiff base systems in the present study must yield metal complexes with iron(III) and aluminum(III) in a similar manner and one would expect catalytic effects in these complexes that are similar to those observed with copper(II). As reported above, no catalysis was observed with these metal ions. Thorium(IV) ion was also studied in the present work because of the fact that the thorium(IV) has been observed to be an effective catalyst for the hydrolysis of organic phosphates, where thorium attacks from the site of the phosphate moiety.[23,24] In the present systems no thorium(IV) catalysis was observed. In view of the known coordinating ability of these four metal ions, it would seem that the explanation for the observed lack of catalytic activity must rest on unfavorable structures and conformations of the Schiff base complexes for the promotion of the β-elimination reaction. Judging from the spacial arrangement of functional groups in the aldimine Schiff bases derived from pyridoxal and amino acids these ligands must be effectively terdentate, as indicated in the above discussion of the coordinating role of the carboxylate group. This conclusion is supported by the X-ray structural determination[25] of the bis(pyridoxylidenevalinato)manganese(II) complex. It is now suggested that the effectiveness of the copper(II) and oxovanadium(IV) ions as dephosphorylation catalysts is related to the strong tendencies of these ions toward limited coordination (effectively four-coordination) with a planar positioning of ligand donor groups. Since the ligand in the Schiff base complex system I



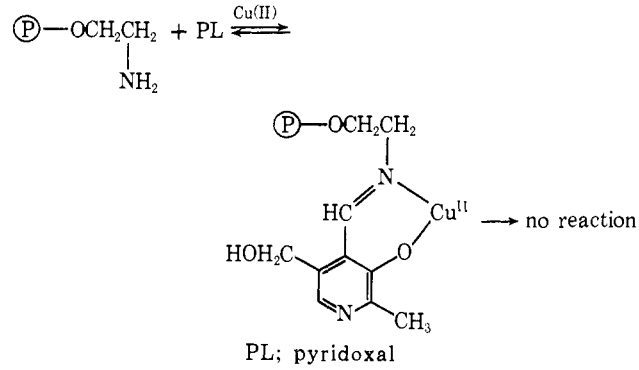
I

Ⓟ, phosphate moiety

occupies three of these positions, it follows that there would be little tendency for these metal ions to coordinate effectively with the phosphate group, which cannot approach the fourth planar coordination position of the metal ion. The strong coordination tendencies of these metal ions are responsible for the observed strong electronic effect on the α-hydrogen abstraction in the resulting Schiff base complexes.

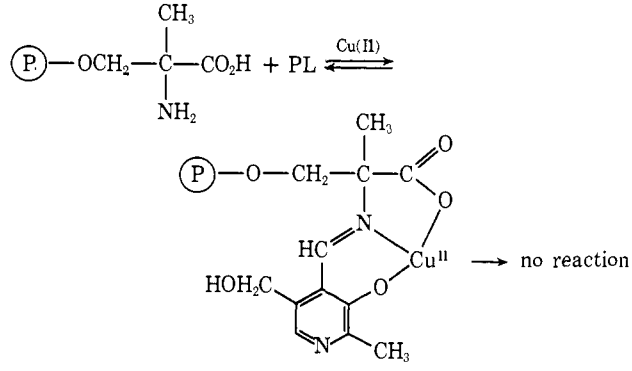
Reaction Mechanisms. From the preceding discussion, it is quite certain that metal-catalyzed β-elimination of *O*-phosphothreonine proceeds through Schiff base complex formation. In order to clarify the nature of the reaction mechanism, the following three reactions were examined under the same experimental conditions in the presence of copper(II).

(A) 2-Aminoethyl phosphate system

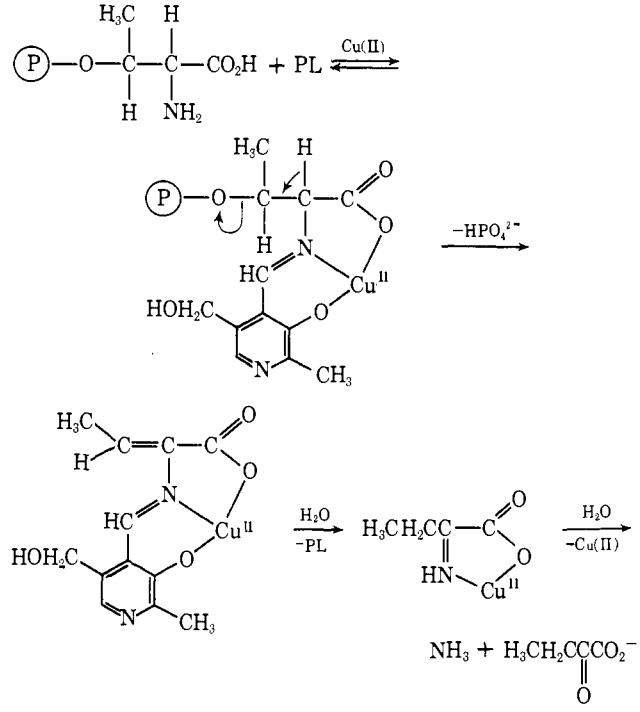


PL; pyridoxal

(B) *O*-Phospho-α-methylserine system



(C) *O*-Phosphothreonine system

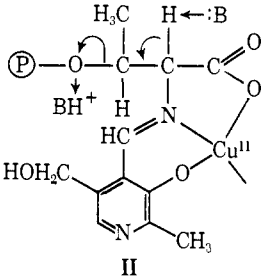


The presence of both the α-hydrogen and carboxylate groups of the amino acid moiety seems to be essential for β-elimination of the phosphate group. Similar requirements have been observed for the β-elimination of *O*-sulfoserine.[6] Since all the metal ions used in this investigation form aldimine Schiff base complexes of type I quite rapidly in solution, Schiff base formation must be a preequilibrium step, and the rate-determining step for the reaction must be the removal of

(23) Y. Murakami and M. Takagi, *Bull. Chem. Soc. Jap.*, **42**, 3478 (1969).
(24) Y. Murakami and J. Sunamoto, *ibid.*, **44**, 1827 (1971).
(25) E. Willstadter, T. A. Hamor, and J. L. Hoard, *J. Amer. Chem. Soc.*, **85**, 1205 (1963).

the phosphate residue. The zero-order kinetics observed for the pyridoxal–*O*-phosphothreonine–copper(II) system support this conclusion, since the rapid preequilibrium formation of the Schiff base complex in the presence of a large excess free substrate would be expected to maintain a constant concentration of the intermediate Schiff base.

The addition of piperidine and morpholine to the pyridoxal–*O*-phosphothreonine–copper(II) system resulted in further enhancement of the reaction rate. In the pH range of the present study, both organic bases are in equilibrium between protonated and deprotonated forms. As indicated in the discussion of results, a reasonable mechanism must allow for catalytic effects by both the acid and basic forms of the amine. It is now suggested that the catalysis involves attack by the free base on the α hydrogen, concomitant with attack by the corresponding protonated base on the phosphate ester oxygen as indicated in formula II.



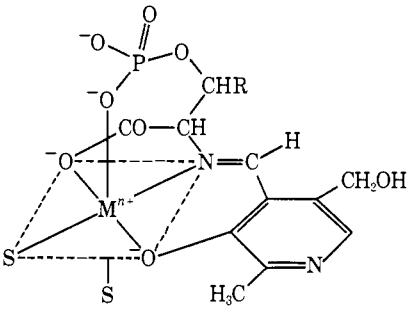
II

B, organic base

The lack of catalysis by imidazole deserves comment in view of the report by Bruice, *et al.*,[26] of catalysis of the transamination reaction by imidazole. It is noted, however, that the mechanism suggested here for β-elimination of the phosphate group does not involve transamination and does not require prior dissociation of the α proton of the amino acid, a step in the generally accepted mechanism of the transamination reaction. Thus a catalyst for the transamination reaction need not be effective in promoting β-dephosphorylation. The lower pK of imidazole relative to the bases used here indicates that there would be very little of the protonated form present under the reaction conditions employed to provide the BH^+ group in II, while the basic form, B in formula II, would not be so strong a base as would the amines in the present study. Also it should be pointed out that even in the transamination reaction imidazole is a very poor catalyst and extremely high concentrations were needed to observe a significant effect.

(26) T. C. Bruice and R. M. Topping, *J. Amer. Chem. Soc.*, **85**, 1488 (1963).

Finally, the lack of catalysis of β-elimination of the negative phosphate group by all metal ions studied excepting those of oxovanadium(IV) and copper(II) requires further explanation. It is noted that all of the metal ions that are inactive have strong axial coordination sites at right angles to the three coordination positions indicated in II, that are occupied by the Schiff base ligand. The axial coordination site should be accessible to the phosphate group, and a quadridentate complex of the type pictured in III is sug-

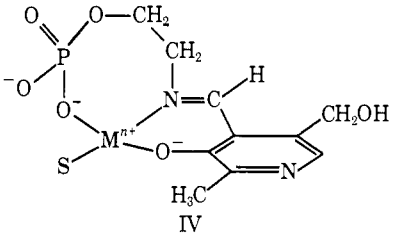


III

S, solvent

gested for these metal complexes. The coordination of the phosphate group would tend to reduce the rate of dephosphorylation in several ways, such as: (1) a chelate effect that would prevent the carbon–oxygen fission by shifting electrons throughout the chelate ring; and (2) and electronic effect that would reduce the ability of metal ion to catalyze α-hydrogen abstraction. The latter effect would result from the coordination of the negatively charged phosphate group with the metal ion thus reducing the coordination tendency of the metal ion toward the remaining ligand donor groups. In the pH range of this investigation the phosphate group exists largely in the completely deprotonated form.

The lack of catalysis by copper(II) and oxovanadium(IV) on the Schiff base complex formed with aminoethyl phosphate may be due to a similar phosphate coordination effect. In this case the phosphate group is favorably positioned for strong coordination in the planes of the copper(II) and oxovanadium(IV) ions, as indicated by IV.



IV