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Kinetics of Metal Ion- and Pyridoxal-catalyzed Transamination and Dephosphonylation of 2-Amino-3-phosphonopropionic Acid

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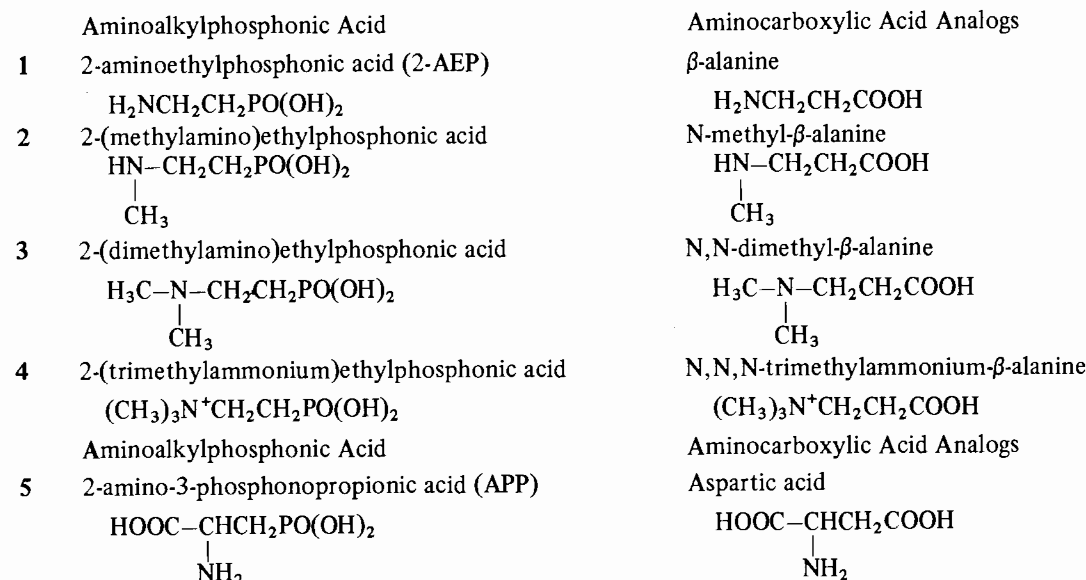
Abstract

Transamination and dephosphonylation reactions of the Schiff bases of pyridoxal(PL) with aminomethylphosphonic acid (AMP), 2-aminoethylphosphonic acid (2-AEP), and 2-amino-3-phosphonopropionic acid (APP) were studied in the absence and in the presence of Al(III), Zn(II), and Cu(II) ions. Transamination does not occur at measureable rates for the Schiff bases of AMP- and 2-AEP, and for their metal chelates. In the case of APP Schiff bases extensive transamination followed by dephosphonylation were found to occur as successive reactions. The ketimine reaction intermediate was not formed in sufficient concentration to be detected. The formation of alanine as the final product indicates that ketimine to aldimine conversion follows the dephosphonylation step. Since the molar amount of inorganic phosphate produced is considerably greater than that of pyridoxal present, the reaction may be considered to be the conversion of APP to alanine and phosphate with pyridoxal and metal ions as catalysts. The relative catalytic activities of the metal ions is Al(III) > Cu(II) > Zn(II). A proposed mechanism for β-dephosphonylation is compared with the generally accepted mechanism of pyridoxal and metal ion-catalyzed β-decarboxylation.

Introduction

Aminophosphonic acids are analogs of aminocarboxylic acids in which the carboxyl group has been replaced by a phosphonyl group ($-PO(OH)_2$). Biological interest in aminophosphonic acids began with the isolation and identification of 2-aminoethylphosphonic acid (2-AEP), $H_2NCH_2CH_2PO(OH)_2$, in living organisms [1]. Since the original isolation of this amino acid from lipid fractions of rumen protozoa, it has been found to exist in sea anemone, bovine brain, abalone, and in protein fractions of various marine invertebrates [2, 3]. The discovery of 2-AEP in living systems was the first indication that the carbon-to-phosphorus bond exists in nature. Other aminophosphonic acids have since been identified in biological systems [4]. These, along with their aminocarboxylic acid analogs, are compared in Scheme 1.



Scheme 1.

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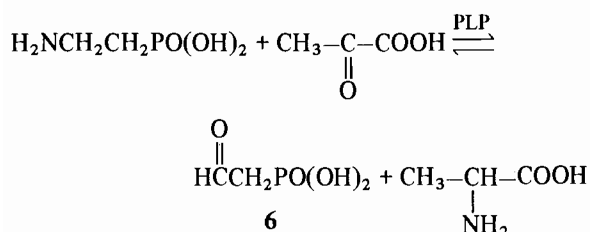
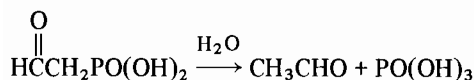
Microorganisms can utilize the phosphorus of aminoalkylphosphonic acids for growth [5], thus indicating that the carbon-phosphorus bond can be cleaved. Roberts *et al.* [6] demonstrated an enzymatic transamination of 2-AEP and 2-amino-3-phosphonoproprionic acid (APP). The transamination of 2-AEP acid with pyruvate:

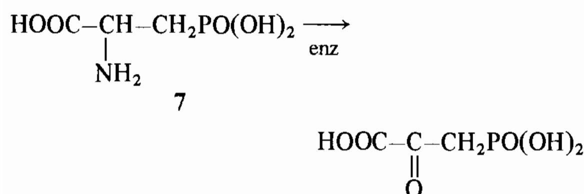


is dependent on the presence of pyridoxal phosphate (PLP), and the 2-phosphonoacetaldehyde, **6**, produced is further degraded enzymatically to inorganic phosphate and acetaldehyde [7].



This carbon–phosphorus bond cleavage has been observed to be catalyzed by phosphonatase in cell-free preparations from *Bacillus Cereus* [8]. Thus a pathway is available for the enzymatic release of phosphate from 2-AEP.

APP may also undergo enzymatic transamination to form phosphonopyruvic acid, 7 [6].



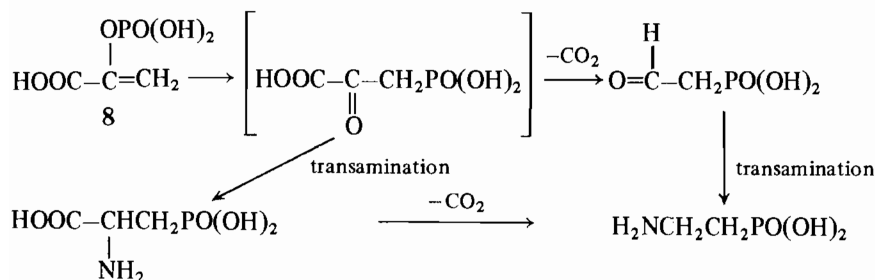
It has been proposed that phosphoenolpyruvate, **8**, which may rearrange to phosphonopyruvate, is a likely precursor of 2-AEP through the pathways shown in the reactions (Scheme 2) [4, 9]. It has been noted, however, that the participation of phosphonopyruvate in the biosynthesis of 2-AEP is still open to question [9].

Some studies of nonenzymatic reactions of aminophosphonic acids have been made recently. Several aminophosphonic acids undergo metal ion catalyzed transamination in the presence of glyoxalate, pyridoxal (PL), or pyridoxal phosphate [10]. It was proposed that the reaction occurs through Schiff base complex formation by a mechanism analogous to that previously described for pyridoxal, metal ion, and aminocarboxylic acid systems [11].

A study of Schiff base formation in aqueous solution between pyridoxal and aminomethylphosphonic acid (AMP), 2-AEP, and APP has been described [12]. A brief communication reporting the tendencies of these aminophosphonic acids to undergo pyridoxal-catalyzed and metal ion catalyzed transamination and dephosphonylation reactions, has been published [13]. The purpose of this paper is to describe the experimental conditions under which this reaction takes place, and to discuss the structural requirements of the amino acids which may undergo pyridoxal-catalyzed dephosphonylation.

Experimental

Pyridoxal hydrochloride was obtained from Sigma Chemical Company. The aminophosphonic acids employed were purchased from Calbiochem. The solutions of the metal salts employed were standardized by conventional complexometric titration using standard solutions of the disodium salt of EDTA [14]. Aqueous stock solutions of pyridoxal (5.0×10^{-3} M) were stored in a refrigerator and aliquots were diluted to provide concentrations of the order of 1.0×10^{-4} M. The stock solutions were discarded and fresh ones prepared after intervals of about a week to ten days. Solutions of the aminophosphonic acids ranging from 1.00×10^{-4} to 1.00



Scheme 2.

to 10^{-1} M were prepared either by direct weighing or by quantitative dilution from stock solutions.

Measurements of pH were made with glass and calomel reference electrodes. A small Beckman combination electrode was used for pH readings on small-volume samples. The pH meter was calibrated with standard buffers, and the pH meter readings were converted to hydrogen ion concentration through the use of activity coefficients published by Harned and Owen [15].

Electronic absorption spectra were measured with a Cary Model 14 recording spectrophotometer or a Beckman DB spectrophotometer with a recorder attachment. Matched 1.000 cm quartz cells were employed. Solutions for spectrophotometric study were 1.00×10^{-4} M PL and metal ion with varying concentrations of aminophosphonic acid; or 1.00×10^{-4} M PL and metal ion with varying concentrations of amino acid. Ionic strength was maintained approximately at 0.10 M, where possible, by the addition of KCl. Adjustments of pH were made by adding small volumes of concentrated HCl or NaOH from a 2.00 ml Gilmont screw syringe. The reference cell contained 0.10 M KCl solution, and the same concentration of amino acid as the sample cell, in experiments where large excesses of amino acid over PL were employed. On occasion a matched pair of 0.1000 cm quartz cells was used for solutions approximately ten times as concentrated as those above.

In more concentrated solutions of PL, amino acid, and metal ion (e.g., 0.010 M PL, 0.030 M APP, 0.0010 M Al(III)), heating for varying times was carried out to observe possible reactions such as transamination. PL was determined analytically by the ethanolamine procedure described by Metzler and Snell [16]. Determination of PL plus pyridoxamine (PM) was carried out by the procedure described by the same authors [16].

Ascending paper chromatography determinations were made on samples in a solvent system of 1-butanol, glacial acetic acid, and water (57%–14%–29% by volume, respectively). Areas containing amino compounds were developed with ninhydrin spray.

An amino acid analysis on a sample of the reaction product was run on a Beckman amino acid analyzer. Quantitative determination of inorganic phosphate was done spectrophotometrically with a Beckman DB spectrophotometer, in accordance with the method of Fiske and Subbarow [17].

Results and Discussion

Aldimine Chelate Formation

AMP, the aminophosphonic acid analog of glycine, condenses with PL in aqueous solution to form a Schiff base, as evidenced by the changes in the electronic spectra of PL when the amino acid is present (Fig. 1). In solutions near neutrality, new bands having maxima at 280 and 414 nm appear, and are attributed to the Schiff base. The predominant PL absorption at 317 nm, corresponding to the neutral dipolar hemiacetal species of PL, decreases in intensity as the imine is formed. Formation of the imine is rapid and appears to be complete by the time the spectrum is recorded (10 min). As the ratio of the concentrations of AMP to PL is increased the degree of imine formation increases. In solutions where a large excess of AMP is present the spectrum approximates that of the Schiff base alone. The stability constants of the AMP–PL Schiff base, and the formation of its Al(III), Zn(II), and Cu(II) chelates, have been reported previously [12].

The PL–AMP imines and their metal chelates with Cu(II), Zn(II), and Al(III) showed no evidence of metal catalyzed reactions since spectral changes corresponding to reactions other than imine formation were not observed. To further investigate the possibility of transamination of AMP, concentrated solutions of metal ion, PL, and AMP were prepared, adjusted to pH 5 (pH optimum for transamination) and heated in a water bath (95 °C). The loss of PL concentration and the total concentrations of PL and PM were measured as a function of heating time. With Cu(II), an effective transamination catalyst, no transamination of AMP was observed, as indicated

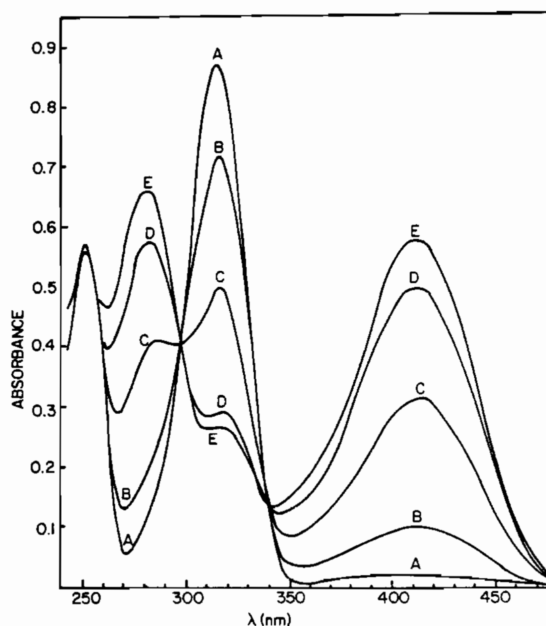


Fig. 1. Spectral changes occurring in the conversion of pyridoxal to the pyridoxal-aminoethylphosphonic acid Schiff base; t = 25.0 °C; pH = 7.50; μ = 0.10 M (KCl); [PL] = 1.00×10^{-3} M; [AMP] = A, 0.0 M; B, 5.00×10^{-3} M; C, 2.50×10^{-2} M; D, 8.00×10^{-2} M; E, 0.100 M.

by no loss of PL concentration. Al(III) and Zn(II) were also ineffective in catalyzing transamination or any other reaction of the AMP–pyridoxal Schiff base.

Glycine, the aminocarboxylic acid analog of AMP, undergoes transamination with PL and Al(III), but only with about 4% conversion to products at equilibrium [18]. Although some α-aminocarboxylic acids are about 50% converted to transamination products at equilibrium if the reactants are in equimolar concentration initially, for glycine the equilibrium strongly favors the aldimine, which is much more stable than the ketimine under the reaction conditions employed. For AMP a similar equilibrium effect could explain the lack of transamination observed, with the ketimine present at concentrations that are too low to be detected by the methods employed in this study.

The extent of formation of 2-AEP aldimines and of their Al(III), Zn(II), and Cu(II) chelates, was found to be somewhat lower than those of AMP [12], but pH dependence and spectra of the Schiff bases and their metal chelates were otherwise quite similar. With Cu(II), Al(III), and Zn(II) ions as potential catalysts for the transamination of 2-AEP and PL, no reactions other than chelate formation were observed. Evidence for this comes from lack of change in the absorption spectra once the Schiff base chelate had formed completely. Over a period of several days no spectral changes were noted, even when samples were later heated for two hours at 95 °C. Determinations of the change in PL concentration with heating time for a sample of 0.010 M 2-AEP 0.0010 M PL, and 0.0010 M Al(III), at pH 5 showed negligible change over a period of two hours.

For comparative purposes the possible transamination of β-alanine was briefly re-investigated under conditions employed in this study. A solution similar to that described above but containing β-alanine in place of 2-AEP revealed no change in the original concentration of PL upon heating for four hours. Thus a detectable extent of transamination does not occur in this system.

The failure to observe transamination of 2-AEP is in contrast to the enzymatic transamination reaction that has been reported [6]. It is also noted that pyridoxal-catalyzed transamination of β-alanine also has not been observed [10], even though this amino acid can undergo transamination in the presence of a suitable enzyme. However, transamination reactions of simple PL or PLP Schiff bases in model systems are generally recognized as involving conditions far different from those that prevail in enzymatic systems.

The fact that 2-EAP, β-alanine, and other amino acids, in which the amino group is not attached to the alpha carbon atom, do not undergo transamination via chelated imines in model systems is understandable. In α-amino acid Schiff bases the removal of the α-proton in the first step of the reaction is promoted by electronic effects of the adjacent carboxylate and imine groups. An additional effect in the Schiff bases results from the proton on the azomethine nitrogen of the ketoenamine form of the monoprotonated species [19]. In the diprotonated species there is also the additional electron withdrawal effect of the positively charged (protonated) pyridine nitrogen. In Schiff base metal chelates, the electron-withdrawal effect of the metal ion also promotes α-proton labilization. However, for β- or γ-amino acids this effect is greatly diminished, since the chelate ring is too large to form readily and the carboxyl group is too distant from the hydrogen requiring labilization to exert its effect. Thus in β-amino acids such as 2-AEP and β-alanine, loss of the β-hydrogen as the first step in the transamination is much more difficult due to the lower degree of activation of the β-C–H bond.

Unlike AMP and 2-AEP, APP was found to undergo transamination in the presence of Al(III), Cu(II), or Zn(II) ions. Evidence for transamination comes from the following observations: (a) changes in the electronic absorption spectra of solutions of PL, APP, and metal ion with time; (b) loss of PL with time and the appearance of PM as a reaction product; (c) appearance of inorganic phosphate and alanine as reaction products.

Aldimine chelate formation in solutions of 1.00 X 10^{-4} M PL and Cu(II), and 1.0 X 10^{-2} M APP (Fig. 2) is evidenced by the growth of an absorption band at 380 nm and the decrease of the free PL peak at 317 nm. The aldimine chelate absorption band was observed throughout the pH range 5 to 9. After a period of one day solutions at room temperature and at pH 5 to 6 show spectral changes in which there is a decrease in the intensity of the aldimine

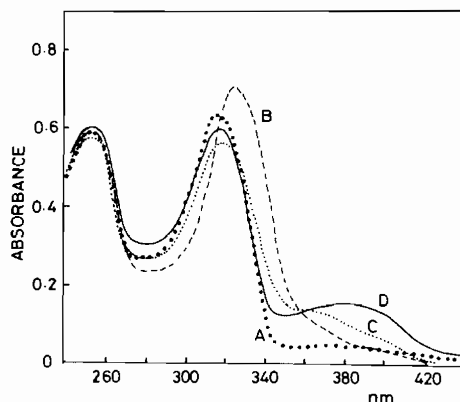


Fig. 2. Absorbance of a solution containing 1.00 × 10^{-4} M PL and Cu(II), and 1.0 × 10^{-2} M APP, as a function of time; pH = 6.0; μ = 0.10 M (KCl); t = 25.0 °C; A, 5 min; D, 1.3 h; C, 24 h; B, 24 h + 2 h at 95.0 °C.

chelate peak and a slight shift in the PL band at 317 nm to longer wavelengths. Heating the solutions accelerates these changes. Figure 2 illustrates the spectra observed at pH 6 over a 24 hour period. The peak finally formed is located at 326 nm and corresponds to the transamination product, PM (λ_{max} = 326 nm at pH 5–6 as determined from spectrum of 1.00×10^{-4} M PM). Such spectral changes are characteristic of transamination and have been observed in many analogous systems containing aminocarboxylic acids [20].

A solution of 0.010 M PL, 0.010 M APP, and 0.0010 M Cu(II) was adjusted to pH 5 and aliquots were heated at 95 °C in sealed flasks purged with nitrogen. The change in PL concentration was followed with time, as was that of the sum of the concentrations of PL and PM (total vitamin B_6 concentration). Figure 3 shows the results for APP solutions of 0.010 M and 0.030 M concentration. While PL is lost in approaching equilibrium, the total concentration of PL plus PM is essentially constant. Increasing the APP concentration is seen to result in a more rapid loss of PL.

The loss of PL and the appearance of PM demonstrates that transamination takes place in these systems. (It was found that prolonged heating of PL and metal alone results in only a slight loss of PL). Transamination of APP with PL and Cu(II) as catalyst also occurred at room temperature, equilibrium being reached after about two days. Heating of PL and APP without metal ion results in a much smaller loss of PL; therefore, the metal ion greatly enhances the rate of transamination.

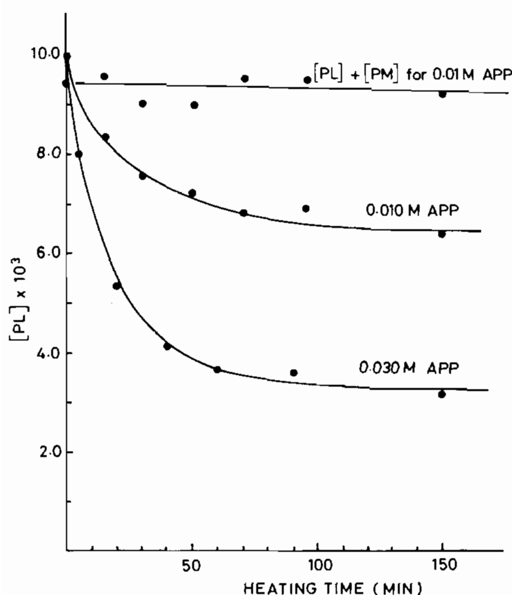


Fig. 3. Loss of pyridoxal with time at 95.0 °C in a solution containing 0.0010 M Cu(II), initially 0.010 M PL, and 0.010 M or 0.030 M APP; μ = 0.10 M K(KCl); pH = 5.0; t = 25.0 °C.

Paper chromatographs of the reaction mixtures show the presence of PM as an orange spot on reaction with ninhydrin spray. An authentic chromatographed sample of PM showed the same color and approximately the same position on the chromatogram as that noted for the solutions that had undergone transamination.

In substituting Al(III) for Cu(II) in the PL–APP systems, a small amount of aldimine chelate was formed, as indicated by weak absorbance in the 380 nm region. Changes in absorption spectra at pH 5 and 6 over three days at room temperature followed by heating consist of a shift of the PL peak (317 nm) to that of PM (326 nm) (Fig. 4). The final spectrum recorded after 120 minutes heating is practically superimposable with that of 1.00×10^{-4} M PM at the same pH. Measurement of the PL present after heating indicate transamination of about 90% at pH 5 and 6, 35% at pH 7, and less than 10% at pH 8 and 9. This is in accord with observations on aminocarboxylic acids that the transamination rate is greater in slightly acidic solutions and decreases with increasing pH [16].

An experiment similar to that indicated in Fig. 3, but with Al(III) in place of Cu(II) was carried out. The results were similar, with the exception that the transamination rate was found to be higher with Al(III) as catalyst. The total concentration of PL plus PM remained constant.

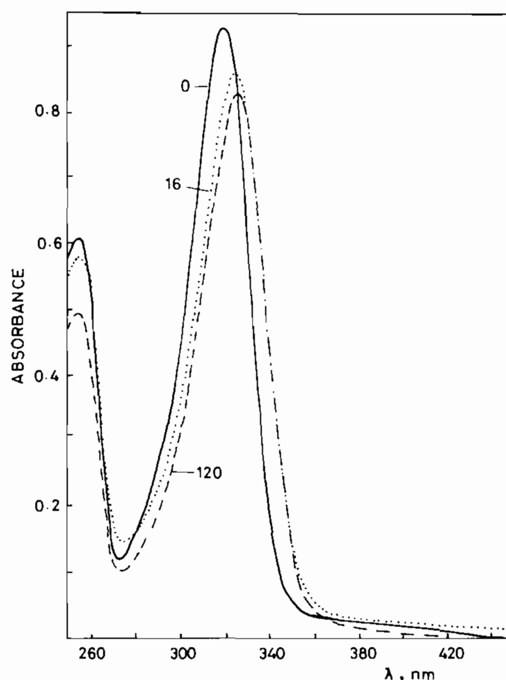


Fig. 4. Absorbance of 1.00×10^{-2} PL, 0.050 M APP, and 1.00×10^{-3} Al(III) at pH 5.0; μ = 0.10 M (KCl), after heating at 95.0 °C for the times (in minutes) indicated on curves.

Zn(II) showed much lower catalytic activity than either Cu(II) or Al(III). While the latter two metal ions are strong catalysts in the transamination of aminocarboxylic acids, Zn(II) has typically been found to be a much weaker catalyst [16, 21].

During the course of transamination of APP, phosphate was detected in heated reaction mixtures. A spectrophotometric assay for phosphate was run on solutions of PL, APP, and Cu(II) or Al(III). A plot of molar concentration of phosphate *vs.* heating time is shown in Fig. 5 for the PL (0.010 M)–APP (0.030 M)–Al(III) (0.0010 M) system. Solutions containing APP alone, APP and PL without metal, and APP plus metal, show no phosphate formed upon heating. Thus all three components, PL, APP, and metal ion, are required for the dephosphonylation of the APP. It was observed that the amount of phosphate formed was considerably greater than the total (initial) concentration of PL, which is the limiting reagent for a stoichiometric reaction.

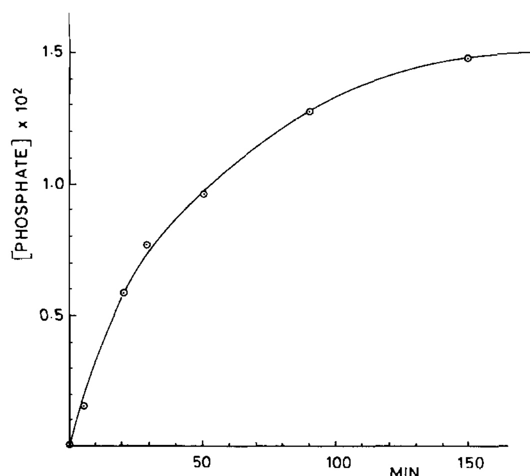


Fig. 5. Increase in phosphate concentration at 95.0 °C in a solution initially containing 0.10 M PL, 0.030 M APP, and 0.0010 M Al(III) at pH = 5.0.

This evidence indicates that a reverse transamination reaction occurs subsequent to initial transamination, to regenerate pyridoxal, thus accounting for the greater than stoichiometric yield of phosphate. In the development of paper chromatograms of the heated solutions a third constituent, in addition to PM and APP, was detected after an interval of heating time. The positions and color with ninhydrin (light purple) suggested this third species to be alanine. To confirm this a heated sample of Cu–PL–APP (after removal of Cu(II) as the sulfide) was run on a Beckman amino acid analyzer and the peaks were shown to co-chromatograph with a known standard sample of APP plus alanine. Measurement of peak areas indicated about a 20% yield of alanine from a reaction mixture originally containing 0.010 M PL, 0.030 M APP, and 0.0010 M Cu(II) after 2.5 hours of heating at 95°.

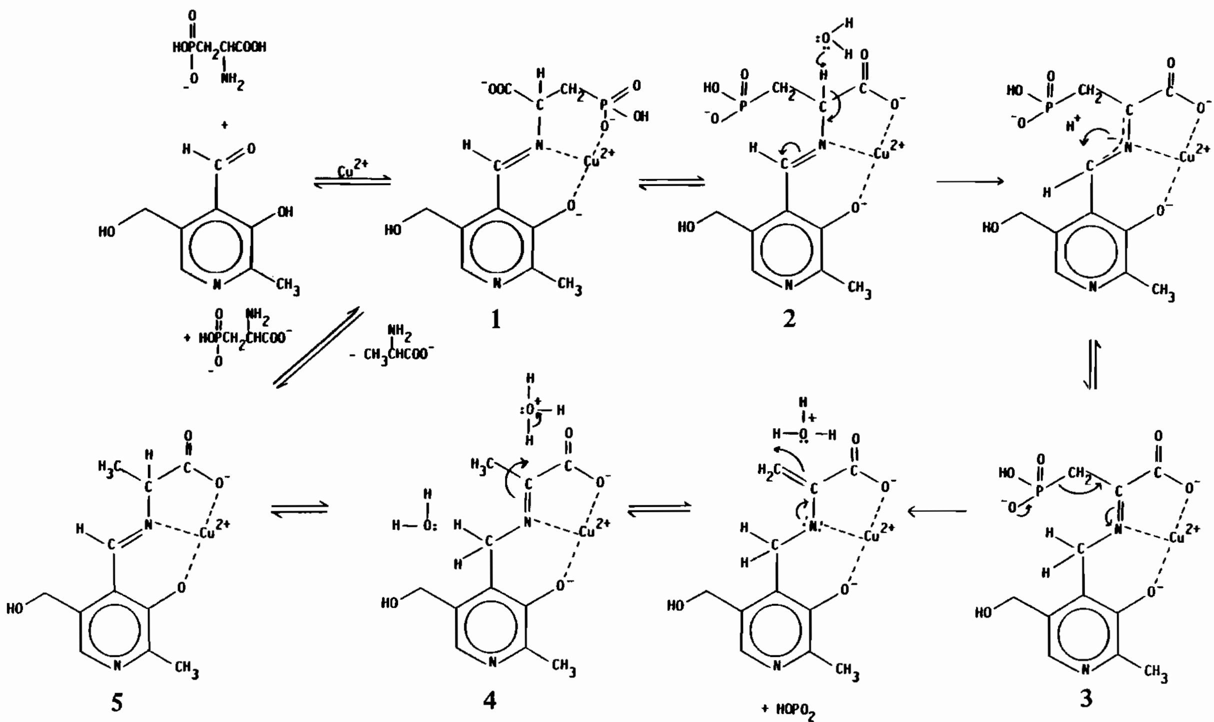
A reaction mechanism that accounts for the species detected in PL–APP–metal ion solutions would involve initial transamination of the APP Schiff base, followed by loss of the phosphonate group of the amino acid which hydrolyzes to phosphate. The Schiff base species formed is that of PM and pyruvate which then undergoes metal ion catalyzed transamination from ketimine to aldimine to form alanine and PL. It is therefore evident that PL and metal ion function as true catalysts in being regenerated at the end of the reaction sequence. The proposed reaction mechanism is indicated in Scheme 3.

Initial Schiff base chelation is suggested as occurring through the formation of two species, one involving coordination of the carboxylate group while the other involves metal ion coordination of the phosphonate group. Coordination through the latter would require a less stable six membered chelate ring, but would be favored somewhat by the greater negative charge of the phosphonate group. The loss of the α-proton in the initiation of the transamination step is more highly favored if the metal ion can exert its catalytic effect through the carboxylate group. The Schiff base metal chelate in which the carboxylate group is coordinated to the metal ion is therefore suggested as the reactive species in the reaction pathway.

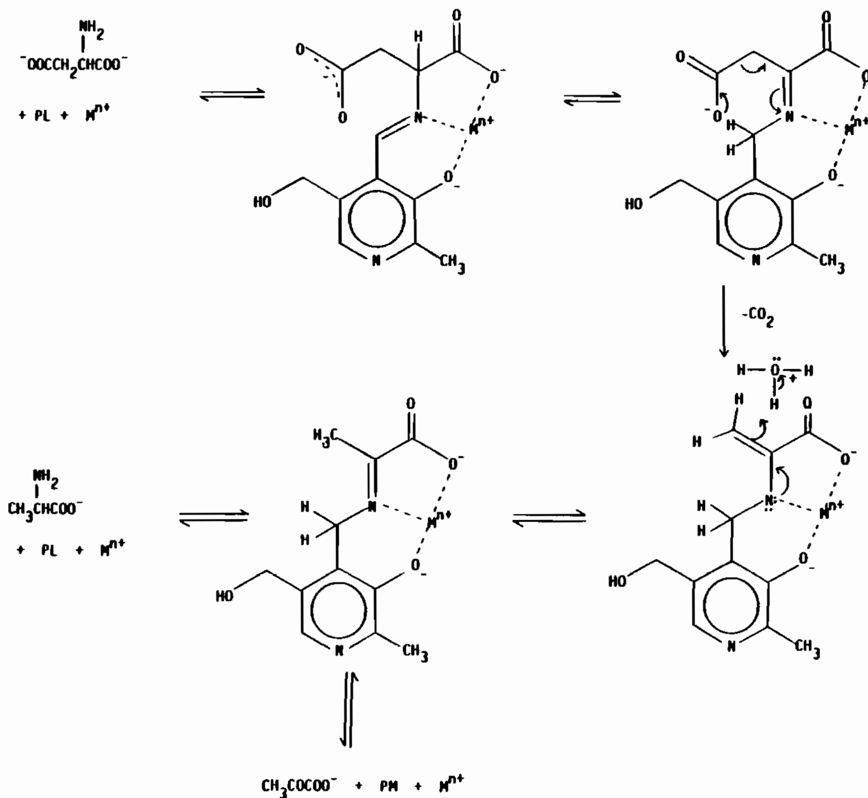
Following initial transamination, the ketimine species may undergo cleavage of the C–P bond to yield the dephosphonylated species. The C–P bond of aminophosphonic acids is ordinarily very stable but if an electron accepting group is on the carbon beta to the phosphonate group cleavage of the C–P bond can occur because of the favorable electronic pathway for bond breaking that exists in the ketimine. This dephosphonylation is analogous to decarboxylation of β-keto acids, yielding instead of carbon dioxide, monomeric metaphosphate, HPO_3, which is then rapidly hydrated to phosphate. The metaphosphate intermediate is thought to be involved in the hydrolysis of acyl phosphates [22] and methyl dihydrogen phosphate [23].

In the proposed enzymatic pathway for the biosynthesis of 2-AEP from phosphonopyruvic acid discussed above it was suggested that phosphonopyruvic acid could undergo decarboxylation to yield 2-phosphonoacetaldehyde. In the model system consisting of PL, APP and metal ion no decarboxylation is observed. This is reasonable if the metal ion is coordinated to the carboxylate group, thus opposing the electronic shift necessary for decarboxylation.

The aminocarboxylic acid analog of APP is aspartic acid. Enzymatically aspartic acid is decarboxylated



Scheme 3. Mechanism of metal and pyridoxal-catalyzed dephosphonylation of 2-amino-3-phosphonopropionic acid.



Scheme 4. Mechanism of metal and pyridoxal-catalyzed decarboxylation of aspartic acid.

to alanine by aspartate β-decarboxylase. Nonenzymatic β-decarboxylation of aspartic acid has been studied in model systems containing Al(III) and PL. Evidence for PL catalyzed β-decarboxylation of L-aspartic acid at elevated temperatures in the presence of Al(III) at pH 5 has been obtained through NMR and UV spectroscopy [24]. Nearly quantitative conversion of PL to PM indicates a transamination step followed by decarboxylation of the ketimine Schiff base chelate. The use of α-methyl-L-aspartic acid in place of L-aspartic acid did not lead to PM formation, thus confirming the necessity of the presence of an α-hydrogen in the transamination of aspartic acid.

A model system reaction mechanism analogous to that suggested for dephosphonylation of APP is indicated in Scheme 4 for the PL and Al(III)-catalyzed β-decarboxylation of L-aspartic acid. The relative effectiveness of metal ions in catalyzing the model system reactions of aspartic acid is similar to that observed in the present work for APP *i.e.*, Al(III) > Cu(II) > Zn(II) [25].

Finally, it is noted that in the case of APP no enzyme has been reported that catalyzes a dephosphonylation reaction analogous to the decarboxylation of aspartic acid by aspartate-β-decarboxylase. The logical nature of the reaction mechanism in Scheme 3, and the analogy to the β-decarboxylation mechanism of aspartic acid, suggests however, that a search for enzymes that catalyze β-dephosphonylation in natural systems would probably be a productive field for future biochemical studies.

Acknowledgement

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