Effect of Phosphate on Stability of Pyridoxal in the Presence of Lysine

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The stability of the biologically active compound vitamin B_6 in aqueous solution was investigated. Schiff base formation is the major reaction between the ϵ-amino group of lysine and the aldehyde group of both pyridoxal and pyridoxal phosphate. Model systems composed of equal molar concentrations of lysine with either pyridoxal or pyridoxal phosphate were used to study the effect of proton transfer on Schiff base formation. Pyridoxylidenelysine was found to be the major product in both lysine/pyridoxal and lysine/pyridoxal phosphate systems. Quantitation of residual pyridoxal and pyridoxal phosphate was conducted using an HPLC to evaluate the degradation of pyridoxal and pyridoxal phosphate. The results indicate both the free phosphate ion in the buffer system and the bound phosphate on pyridoxal phosphate can enhance the formation of the Schiff base. The phosphate group serves as both proton donor and acceptor, which catalyzes the Schiff base formation. The aldehyde group on pyridoxal phosphate was found to be much more reactive than that on pyridoxal. The bound phosphate group on pyridoxal phosphate, with proton donating and accepting groups in close proximity, can simultaneously donate and accept protons, thus enhancing Schiff base formation between the aldehyde group and the ϵ-amino group. The deterioration rate of pyridoxal phosphate was faster than that of pyridoxal in an aqueous system.

Keywords: *Schiff base; pyridoxallysine; pyridoxallysine phosphate; phosphate*

INTRODUCTION

Considerable research has focused on the stability of vitamin B_6 and lysine from foods. The thermal degradation of lysine has been extensively studied (*1*), whereas less research has been conducted to establish mechanisms for vitamin B_6 degradation (*2*, *3*).

Various extents of losses of vitamin B_6 resulting from the processing and preservation of foods were studied. Main losses of vitamin B_6 were reported to vary from 57.1 to 77.4% in canned vegetables and from 42.6 to 48.9% in canned fish, seafood, meat, and poultry (*4*). Conflicting data have been reported concerning the effects of processing on the stability of naturally occurring vitamin B_6 in food products.

The stability of vitamin B_6 in foods is believed to be affected by the thermal formation of ϵ-pyridoxyllysine (*5*). The stability of pyridoxal and pyridoxal phosphate in the presence of peptides has been studied. Pyridoxal phosphate (PLP) was found to be more reactive than pyridoxal (PL) (*6*). Gregory and Hiner (*7*) revealed that in an model composed of 10% (w/v) potassium caseinate and 60 μM vitamin B_6 at pH 7, pyridoxal phosphate was 1.5−2.0-fold less stable than pyridoxal. However, opposite results were obtained by the same research group (*3*). They found that PLP exhibited slightly greater stability than PL toward caseinate under the reaction condition of autoclaving at 121 °C for 45 min. In both model systems, relatively high concentrations of the amino-group-containing reactants were used. The comparison of the relative reactivity of the aldehyde group on either pyridoxal or pyridoxal phosphate under low reactant concentration similar to that of physiological conditions has not been established.

The five major forms of vitamin B_6 are pyridoxal 5′-phosphate (PLP), pyridoxal (PL), pyridoxamine (PM), pyridoxamine 5′-phosphate (PMP), and pyridoxine (PN). Among them, PLP is an active coenzyme in many metabolic transformations of amino acids and may play a role in their absorption and transport. Plasma pyridoxal phosphate concentrations of <34.4 nM were observed in 26% of girls and values from 34.4 to 40.5 nM in 14% (*8*). The effect of aging upon vitamin B_6 status has been examined (*9*). The results of a study of plasma PLP levels in various age groups (3 h old to >80 years old) show a gradual decrease in plasma PLP from 137 to 23.1 nmol/L. Blood plasma was collected from ∼2500 elderly subjects born between 1913 and 1918 living in 17 small towns in 11 European countries, and the plasma levels of carotene, retinol, α-tocopherol, vitamin B_{12}, folic acid, and PLP were determined (*10*). The vitamin status for retinol and folic acid was adequate in all countries, whereas the prevalence of biochemical vitamin B_6 deficiency was widespread and reached >50% in some countries.

Cereals may supply nearly 60−70% of the protein in diets. Wheat has higher protein among cereals, but lysine is still the limiting amino acid in wheat protein. Studies carried out by several workers showed that fortification of white flour with synthetic lysine brings about a marked improvement in the nutritive value of wheat proteins. Fortification with synthetic lysine mono-

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hydrochloride helps to enhance the quality of the products (*11*). Postprocessing fortification of wheat flakes with 0.28% lysine significantly improved protein quality parameters, protein efficiency ratio (PER), and net protein utilization (NPU) of the product compared to the control (*12*). Increased PER values were obtained by adding 0.3−0.5% L-lysine HCl or by combining peanut with soy proteins (*13*).

The in vitro synthesis of 3-carboxy-1-pyridoxyl-1,2,3,4-tetrahydro-β-carboline and 3-carboxy-1-pyridoxyl-1,2,3,4-tetrahydro-β-carboline 5′-phosphate from tryptophan with PL and PLP, both 0.1 mM, respectively, under physiological conditions, pH 7.0 at 37 °C, is presented by Argoudelis (*14*). Interestingly, in vivo formation of similar cyclic compounds was reported from L-dopa and PLP or PL in the liver and blood of rats (*15*) and from histamine and PLP or PL in the tissues and urine of mice (*16*) when large quantities of L-dopa or histamine were given to the animal. Although plasma PLP was as low as 45 ± 2 nmol/L initially, it reached 377 ± 12 nmol/L after 7 days of supplementation (*17*). The possibility for the interaction between L-lysine and PLP to proceed in vivo increases as the concentration of L-lysine increases in blood when excess L-lysine is given the animals in the lysine-fortified cereal foods. In this paper, the in vitro synthesis of pyridoxyllysine under physiological conditions, pH 7.2 and 37 °C, is presented. The impact of the bound phosphate group in the proper alignment on the PLP molecule in the catalysis of Schiff base formation remains to be thoroughly investigated. The objective of the current study is to study the high reactivity of the aldehyde group with an adjoining phosphate ion in a low-concentration model system. This information will be valuable to investigators studying the bioavailability and reactivity of PL and PLP toward amino acids, especially lysine, in drugs, animals, or humans and the possible interaction of certain PLP-requiring enzymes.

EXPERIMETAL PROCEDURES

Reagents. Sodium phosphate, phosphoric acid, and L-lysine free base were purchased from Riedel-DeHaen Co. Pyridoxal, pyridoxal phosphate, and pyridoxine were purchased from Sigma Chemical Co.

Sample Preparation. Reaction mixtures were composed of equal molar solutions of pyridoxal or pyridoxal phosphate and L-lysine (0.1 M) in deionized water, adjusted to pH 7.2 with sodium hydroxide. To study the effect of the buffer capacity on pyridoxyllysine formation, solutions containing pyridoxal (0.1 M) and L-lysine (0.1 M) with different proportions of anion phosphate (0.1 and 0.2 M) at an initial pH of 7.2 were prepared. Potassium chloride was utilized to maintain a constant ion strength for both models. All of the reactions were run at 37 °C for 5 h, and the reacted mixtures were diluted 10 times after they had been brought to room temperature.

HPLC Analysis. Each reaction mixture was routinely analyzed by HPLC with a Hitachi model 6000A liquid chromatograph and a model L-4500 HPLC photodiode array detector (220−400 nm) with a 25 × 0.46 cm Shimpak (C-18) column under isocratic conditions at ambient temperature. The solvent was 0.033 M potassium phosphate, pH 2.2, at a flow rate of 1.0 mL/min, which yielded resolution of all components (Figure 1). Remaining pyridoxal and pyridoxal phosphate and also pyridoxylidenelysine were detected spectrophotometrically at 295 nm and quantified against an internal standard (pyridoxine) by following the method of Gregory and Hiner (*7*). The sample size injected was 5 μL. To collect enough pyridoxylidenelysine for FAB mass analysis, a preparative HPLC (Hitachi L-6250 Intelligent Pump) equipped with a UV−vis detector L-7420 and a Hibar prepacked column RT 250-25 (Cica-Merck) was used.

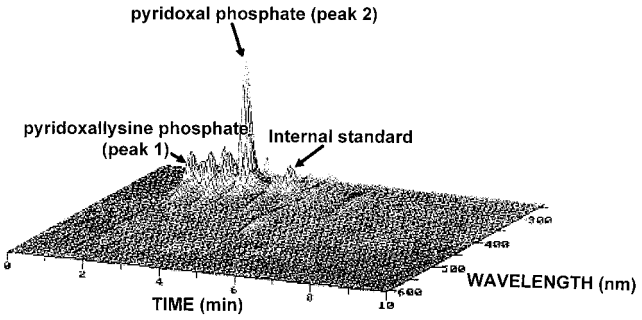

Figure 1. Three-dimensional photodiode array chromatogram of pyridoxylidenelysine (peak 1) and pyridoxal (peak 2) incubated at pH 7.2 in 0.2 M buffer, 37 °C for 5 h.

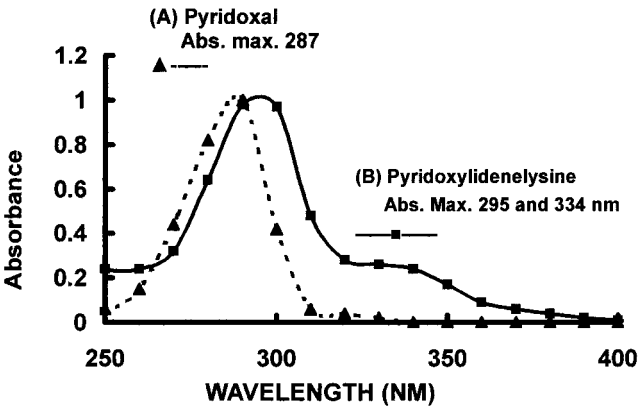

Figure 2. Absorption spectra of pyridoxal (A) and pyridoxylidenelysine (B).

FAB Mass. A VG 7070 mass spectrometer equipped with an Ion Tech saddle field FAB gun was used with 3-nitrobenzyl alcohol (NBA) (copper probe) and 8 kV of xenon.

RESULTS AND DISCUSSION

Interaction of Pyridoxal and Lysine. A typical three-dimensional HPLC profile of a heated pyridoxal/lysine mixture is shown in Figure 1. Peak 2 was characterized as pyridoxal by spiking with an authentic standard. To confirm the structure of peak 1 eluted by HPLC analysis, one of the reacted mixtures was subjected to HPLC with photodiode array used as the detector. The presence of bound pyridoxal in lysine was demonstrated by UV difference spectra versus its respective blank. Figure 2 shows typical absorption spectra of pyridoxal and the Schiff base derived from the interaction of pyridoxal and lysine. A characteristic shoulder of pyridoxyl amino absorption maximum at 334 nm was observed in the Schiff base, indicating that a conjugated double bond formed after the coupling of the carbonyl moiety of the pyridoxal molecule onto the lysine anino group. In addition to the evolution of the shoulder in the absorption spectrum of the Schiff base, the absorption maximum shifted from 295 to 287 nm when they exited in the mobile phase used in HPLC analysis. The binding of pyridoxal to lysine was confirmed by a FAB-MS spectrum. The Schiff base was collected by using a preparative HPLC. The molecular weight of this compound was found to be 295, *m/z* 296 for $[M + H]^+$. The molecular ion was confirmed by the presence of *m/z* 318 for $[M + Na]^+$ and *m/z* 334 for $[M + K]^+$. Thus, peak 1 was tentatively characterized as pyridoxylidenelysine. Similar pyridoxylideneamino acids

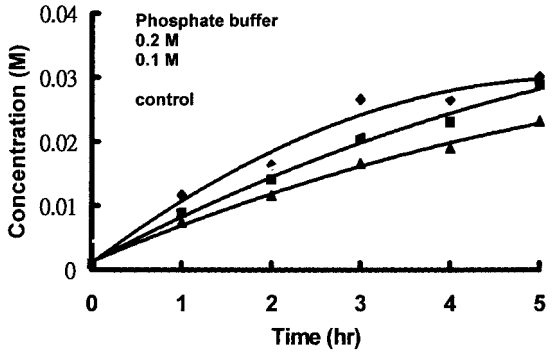

Figure 3. Effect of phospate on pyridoxylidenelysine formation in aqueous pyridoxal−lysine system with various concentrations of phosphate buffer at pH 7.2 (37 °C).

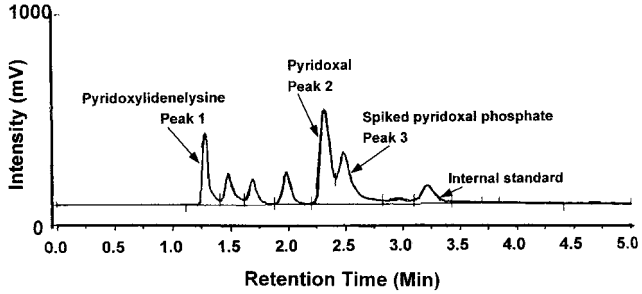

Figure 4. Typical HPLC profile of heated PLP/lysine system in aqueous solution at pH 7.2 (37 °C).

have been synthesized: pyridoxylideneglutamic acid and pyridoxylidenealanine (*18*), *N*6-(*P*-pyridoxylidene)aminocaproic acid (*19*), and pyridoxylidenevaline (*20*).

Effect of Phosphate Buffer on Pyridoxylidenelysine Formation. As expected, addition of phosphate ion into the aqueous model system of pyridoxal/lysine increased the Schiff base formation rate significantly, as shown in Figure 3. The pyridoxylidenelysine formation increased with increasing concentration of phosphate ion from 0.1 to 0.2 M. The free phosphate may catalyze the Schiff base formation between the ϵ-amino group of lysine and the aldehyde group of pyridoxal. A similar buffer effect has been observed in the catalysis of the Schiff base formation in thiazolidine from aldehydes and cysteamine (*21a*,*b*) and diketopeperazine from aspartame (*22*).

Interaction of Pyridoxal Phosphate and Lysine. A typical HPLC profile of a heated pyridoxal phosphate/lysine mixture is shown in Figure 4. Peaks 1 and 2 were characterized as pyridoxylidenelysine and pyridoxal, respectively. When the reaction mixture (37 °C, 1 h) was spiked with authentic standard, peak 3 was characterized as pyridoxal phosphate. The data revealed that pyridoxal phosphate is an effective catalyst for PLP deterioration. As shown in Figure 5, pyridoxal phosphate was much more unstable than pyridoxal in aqueous solution in the presence of lysine at pH 7.2. Within 1 h, all of the pyridoxal phosphate had been converted to pyridoxal completely. Pyridoxal phosphate in the system was bound to lysine through Schiff base linkage, followed by a depletion of the phosphate group, leading to the formation of pyridoxal. The interaction between pyridoxal and lysine in the system leads to the formation of pyridoxylidenelysine.

The bound phosphate on pyridoxal phosphate could enhance Schiff base formation between the aldehyde group of pyridoxal phosphate and the amino group of lysine. A higher yield in the synthesis of 3-carboxy-1-pyridoxyl-1,2,3,4-tetrahydro-β-carboline 5′-phosphate compared to that of 3-carboxy-1-pyridoxyl-1,2,3,4-tetrahydro-β-carboline 5′-phosphate was observed, indicating that cyclic compounds (1-tetrahydrocarbolines) are formed from L-tryptophan and pyridoxal or pyridoxal 5′-phosphate. Both sets of data reveal that the aldehyde group on PLP was much more reactive than that on PL. Gregory and Kirk (*6*) reported that PLP was much more reactive than PL. Quantitative results obtained from using a PLP/peptide system were compared with those obtained using a PL/peptide system. The results showed that the yield is much higher for peptide-bound PLP at 20% compared to a value of 3% for peptide-bound PL.

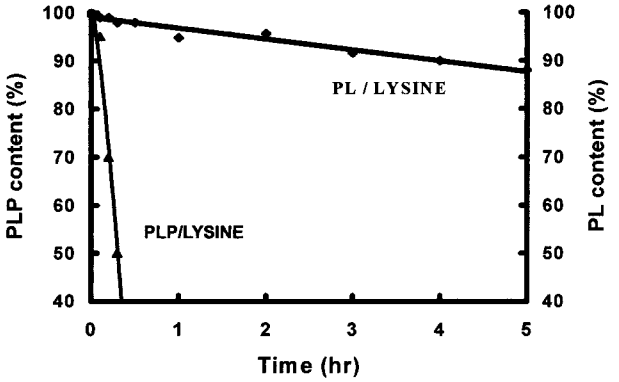

Figure 5. Stability of pyridoxal (PL) and pyridoxal phosphate (PLP) in aqueous solution at pH 7.2 (37 °C).

Kinetic Analysis of the Degradation of Pyridoxal and Pyridoxal Phosphate. As shown in Figure 4, at least five peaks were formed in either lysine/pyridoxal or lysine/pyridoxal phosphate model systems. The degradation of pyridoxal increased with increasing reaction time and temperature. To calculate the Arrhenius activation energy (E_a), we regressed the remaining pyridoxal concentration with time at a constant temperature to determine the rate constant and, then, regressed ln K with the reciprocal temperature to determine E_a. The degradation rates for pyridoxal in the presence of lysine at 40, 50, and 60 °C were 0.0001, 0.0005, and 0.0011 $M \cdot h^{-1}$, respectively. A similar tendency was observed for pyridoxal phosphate degradation in the lysine/pyridoxal phosphate aqueous model system. The degradation rates for pyridoxal phosphate in the presence of lysine at 30, 40, and 50 °C were 0.0003, 0.0011, and 0.0017 $M \cdot h^{-1}$, respectively. The activation energy values were calculated from the Arrhenius plots for pyridoxal at pH 7.2 in aqueous solution, in 0.1 M phosphate buffer, and in 0.2 M phosphate buffer, as well as for pyridoxal phosphate in aqueous solutions of 23.9, 12.4, 10.5, and 5.7 kcal/mol, respectively. The presence of a phosphate ion decreased the activation energy for pyridoxal degradation. The activation energy for pyridoxal degradation also decreased with increasing phosphate buffer concentration from 0 to 0.2 M, whereas the activation energy value for pyridoxal phosphate degradation was the lowest, even in the absence of free phosphate ions in the reaction mixture. Kinetic analyses showed a difference in temperature dependence on the relative degradation rate of the B_6 vitamers. The activation energy values for the loss of pyridoxine, pyridoxamine, and pyridoxal during processing were 27.3, 23.7, and 20.8 kcal/mol, respectively (*7*). Relatively high activation energy values for the loss of PN, PL, and PM during processing in neutral phosphate buffer are reported to be 54, 50, and 85 kcal/mol, respectively (*4*).

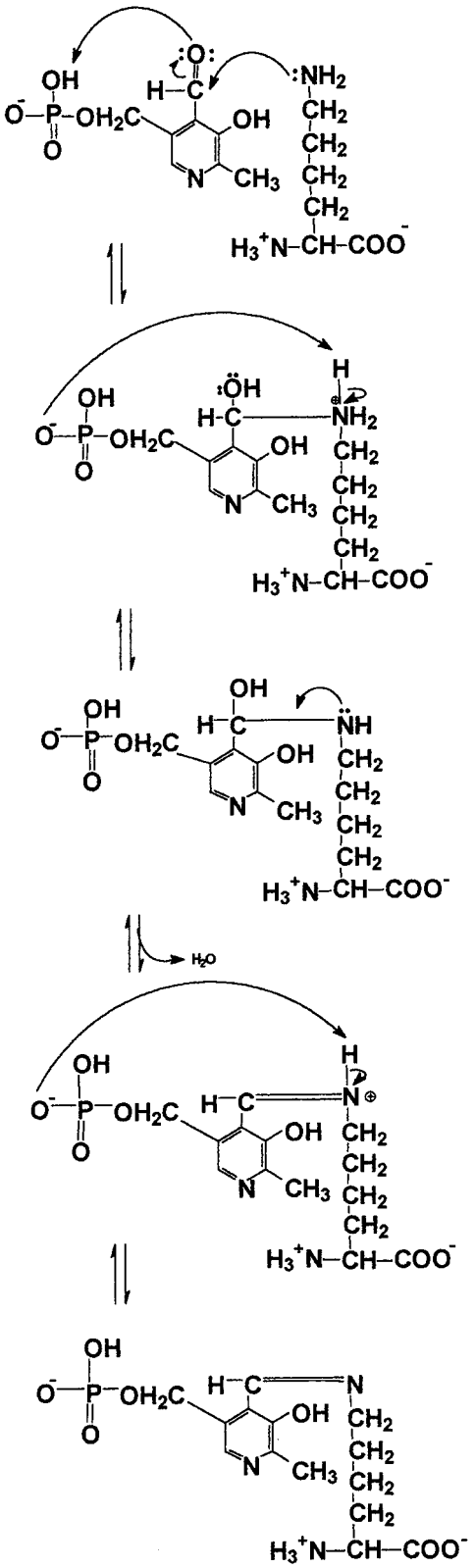

Figure 6. Proposed mechanism for pyridoxylidenelysine formation.

Proposed Mechanism for Pyridoxylidenelysine Formation. The interaction of PL and PLP with the free amino group of amino acids, amines, peptides, and proteins has been extensively studied. Reactivity of PLP was greater than that of PL was attributed to the blocking of internal hemiacetal formation by the bound phosphate moiety (*23*). Argoudelis (*14*) explained the higher reactivity of aldehyde in PLP than in PL as being due to the fact that PLP exists in a free aldehyde form rather than as a hemiacetal. On the basis of kinetic analysis and the series studies on phosphate-mediated catalysis of Schiff base formation (*21a,b*, *24*), a mechanism for pyridoxylidenelysine formation was proposed, as shown in Figure 6. Pyridoxal may react with the ϵ-amino group of lysine to form a Schiff base and subsequently rearrange to pyridoxylidenelysine, which was catalyzed by the nearby phosphate group. The phosphate group acts as both proton donor and acceptor. The bound phosphate group on pyridoxal phosphate was found to be much more reactive than the free phosphate in the buffer system. The bound phosphate group on pyridoxal phosphate, being both proton donating and accepting groups in close proximity, can simultaneously donate and accept protons, thus enhancing Schiff base formation between the aldehyde group and the ϵ-amino group. A similar reaction of α-amino group on lysine was observed in the study of glucosylysine formation from the Maillard reaction of glucose and the lysine ϵ-amino group (*25*).

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