

Influence of the Polarity of the Medium on the Catalysis of Formation, Rate of Hydrolysis and Stability of the Schiff Bases Formed by Pyridoxal 5′-Phosphate with L-Tryptophan

Gerardo R. Echevarría Gorostidi,* José G. Santos,† Andrea Basagoitia,† Marta Castillo,† and Francisco García Blanco††

Department of Physical Chemistry, University of Alcalá, E-28871 Alcalá de Henares, Spain

†Faculty of Chemistry, Pontificia Universidad Católica de Chile, Casilla 306, Santiago 22, Chile

††Department of Physical Chemistry, Faculty of Pharmacy, Complutensian University, E-28040 Madrid, Spain

(Received July 8, 2002)

The apparent rate constants of formation (k_1) and hydrolysis (k_2), and the equilibrium constant (K_{pH}), of the Schiff bases formed by pyridoxal 5′-phosphate with L-tryptophan in water and different aqueous ethanol mixtures at a variable pH, 25 °C and an ionic strength of 0.1 M (1 M = 1 mol dm^{-3}) were determined. The individual rate constants of formation and hydrolysis of the Schiff bases of the systems corresponding to the different chemical species present in the medium, as a function of its acidity, were also determined, as were the pK values for the Schiff bases. The influence of the solvent medium on the formation and hydrolysis rate constants of the Schiff bases is discussed.

Pyridoxal 5′-phosphate (PLP) is one form of vitamin B_6 that plays a central role as a coenzyme in a wide range of reactions involved in amino acid metabolism (e.g. decarboxylations, transiminations, dealdolations, eliminations).[1–3] Its forms a aminomethanol intermediate by bonding of its carbonyl group to the ε-amino group in the L-lysine residue of the peptide chain.[1,4] The aminomethanol then releases one molecule of water to give the Schiff base in an acid-catalyzed process.[4,5]

In virtually all PLP-dependent enzymes, the first step of the catalytic process is a transimination reaction, viz. the conversion of the PLP–lysine imine into a PLP–substrate imine.[6] In other words, the covalent linkage in the Schiff base must be broken in the course of the catalytic cycle, and a new base is formed between the coenzyme and the substrate (usually an amino acid).[1] PLP is the essential component of the enzyme active site, as shown by the fact that this substance also slowly catalyzes many of these reactions in the absence of protein.[1,7] There is evidence that PLP sites in enzymes are less polar than water.[8] The literature abounds with references to the Schiff bases formed by PLP and various compounds bearing amino groups, such as amines,[9] amino acids,[10] and polypeptides.[11] However, few kinetic studies have considered solvent effects on the reactions of PLP with amine-group bearers as a function of the pH.[12–14]

In this paper, we report on the results of a study on the stability and kinetics of the formation and hydrolysis of the Schiff bases of pyridoxal 5′-phosphate with L-tryptophan (the PLP–TRP system), as a function of the pH, in ethanol–water mixtures. The results were examined in terms of the individual rate constants for the species involved in the process (see Scheme 1).

Material and Methods

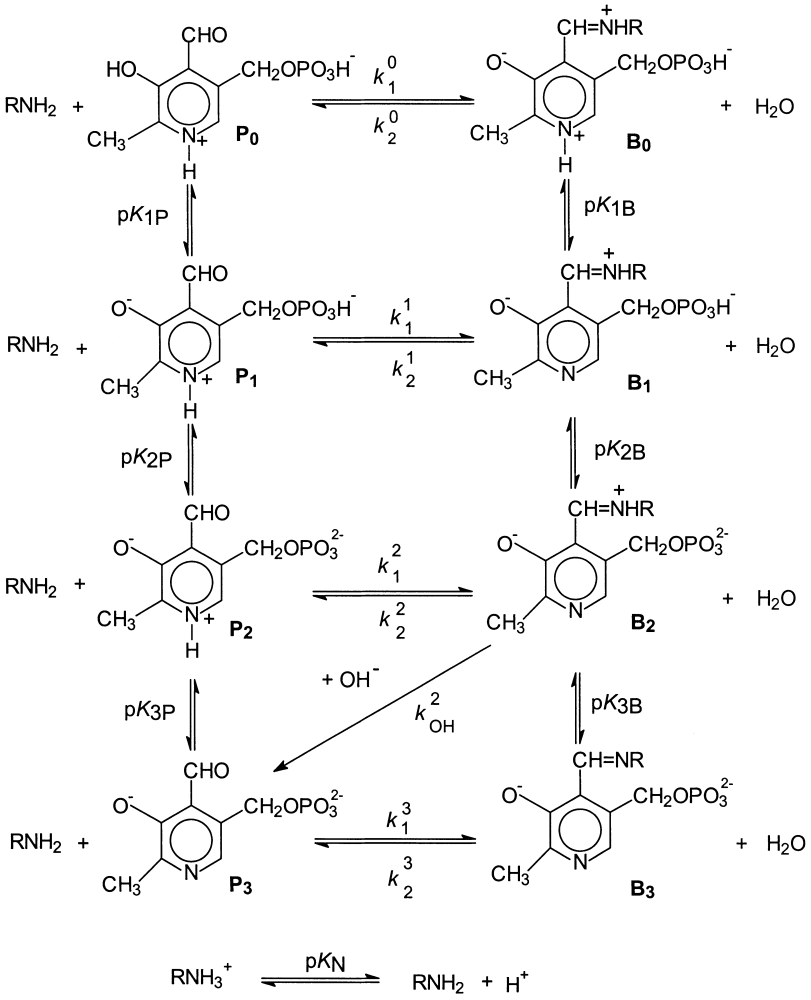
L-Tryptophan (TRP) was purchased from Sigma Chemical Co. Pyridoxal 5′-phosphate (PLP) and all other chemicals used were of reagent-grade and were purchased from Merck.

Acetate, phosphate and carbonate buffers were used over appropriate pH ranges. The buffer concentration used was typically 0.02 M and the ionic strength was maintained at 0.1 M by adding appropriate amounts of KCl to the medium.

PLP solutions were made as required in the buffers and stored in the dark. Their exact concentrations were determined by dilution with 0.1 M HCl,[15] and found to be in the region of 2×10^{-5} M. TRP solutions spanning the concentration range from 5×10^{-4} to 2×10^{-2} M were also prepared on a daily basis by diluting appropriate amounts of stock solutions in the corresponding buffer and solvent medium.

Kinetic measurements were made at a variable pH using a Hewlett-Packard 8453 diode array spectrophotometer and a Uvikon 941-Plus spectrophotometer furnished with thermostatted cells of 1-cm light path. In each case, the reaction was started by adding a known volume of PLP-buffered solution to prethermostatted TRP solutions at (25 ± 0.05) °C. The difference between the initial and final pH in the reaction cell never exceeded 0.03 units. The pH measurements were made with a Crison pH-meter equipped with a Metrohm EA120 electrode that was previously calibrated with aqueous buffers at 25.0 °C. The pH measurements were not corrected because the difference in measured pH due to the ethanol concentration was negligible based on the data reported by Gelsema et al.[16]

The overall reaction between an aldehyde and an amine can be schematized as follows:



Scheme 1.

$$R_1-CHO + NH_2-R_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} R_1-CH{=}N-R_2 + H_2O, \quad (1)$$

where k_1 and k_2 are the overall rate constants of formation and hydrolysis, respectively, of the Schiff base. The procedure used to calculate these two constants from the observed *pseudo* first-order rate constants (k_{obs}) is described in detail elsewhere.[9–14] Their ratio coincides with the equilibrium constant ($K_{pH} = k_1/k_2$).

The deprotonation equilibrium constants (K_N) for TRP in different ethanol–water mixtures studied were determined potentiometrically (by titration with 0.1 M NaOH of 0.01 M solutions of TRP and 0.01 M HCl, using a Radiometer autotitrator equipped with a PHM-62 pH-meter, an ABU-11 autoburette, a TTT-60 titrator, an REA-160 recorder, a TTA-60 thermostatic support, a G-2040 glass electrode and a K-4040 calomel electrode). The experimental conditions used were the same as those for the kinetic measurements. The thus found pK_N values for the NH_3^+ group in TRP were 9.40, 9.45, and 9.60 in 24 wt%, 44 wt% and 62 wt% aqueous ethanol, respectively.

Results

Figures 1–3 show the obtained experimental results, in the form of the variation of the logarithmic overall rate constants of formation (k_1) and hydrolysis (k_2), and the equilibrium constant (K_{pH}), for the Schiff bases of the PLP-TRP system as a function of the pH in the different solvent mixtures. The figures also include the values in water media.[10b]

Reaction measurements could only be made up to pH 10.2 because more alkaline media resulted in secondary imine intramolecular cyclization by an attack of the indole group on the azomethine carbon, which hindered the reaction (as previously observed in that between PLP and histidine[17,18]) and precluded an examination beyond this alkalinity level.

In the aqueous ethanol media studied, k_1 increased with increasing pH and peaked at pH 9.0; in the pure water medium, however, k_1 increased with increasing pH, but no peak was observed.[10b]

On the other hand, k_2 behaved identically in water[10b] as in ethanol-water mixtures; it exhibited a minimum in both types of solvent (Fig. 2). The position of the minimum changed with the polarity: it occurred at pH 8.8 in water and near pH 9.3 in aqueous ethanol.

Figure 1 shows the differences in k_1 among solvents over the pH range 8–10; this suggests an increased reactivity in less-polar media.

As shown in Scheme 1, the overall rate constants of formation and hydrolysis of the Schiff bases can be described in terms of the individual constants for the different chemical

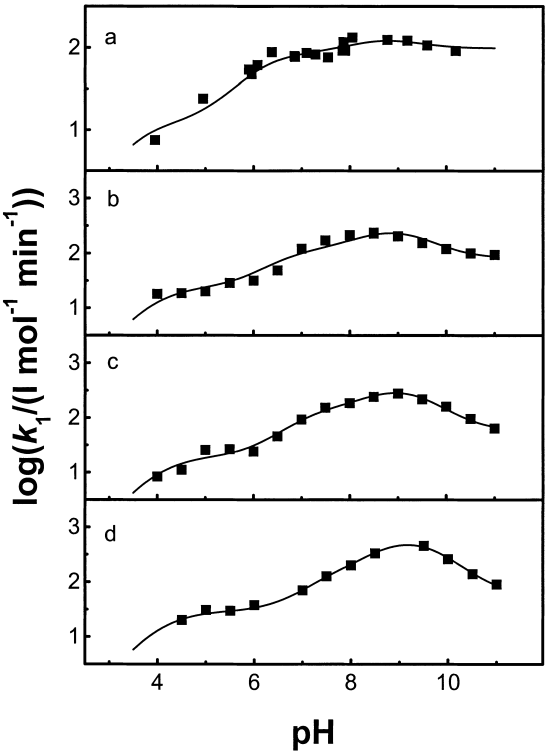


Fig. 1. Plot of log k_1 vs pH for the PLP–TRP system in a) water, b) 24 wt% ethanol, c) 44 wt% ethanol and d) 62 wt% ethanol. Curves calculated using Eq. 5 and data from Table 1.

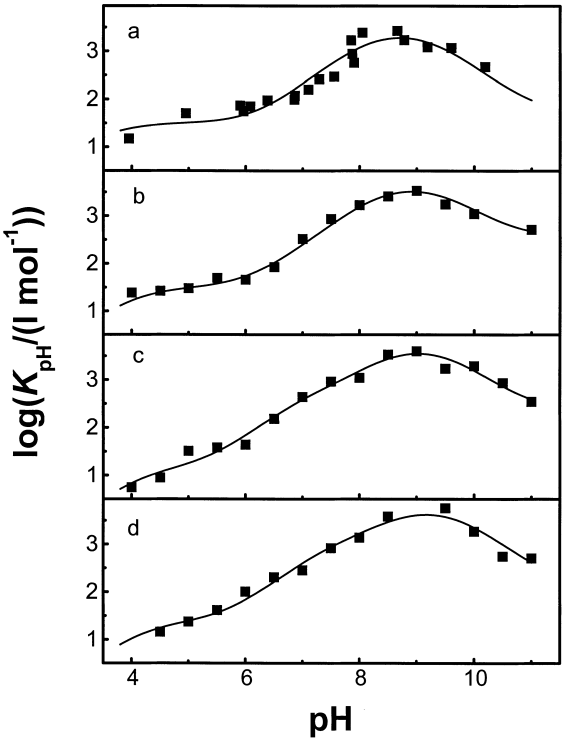


Fig. 3. Plot of log K_{pH} vs pH for the PLP–TRP system in a) water, b) 24 wt% ethanol, c) 44 wt% ethanol and d) 62 wt% ethanol. Curves calculated using Eq. 7 and data from Table 1.

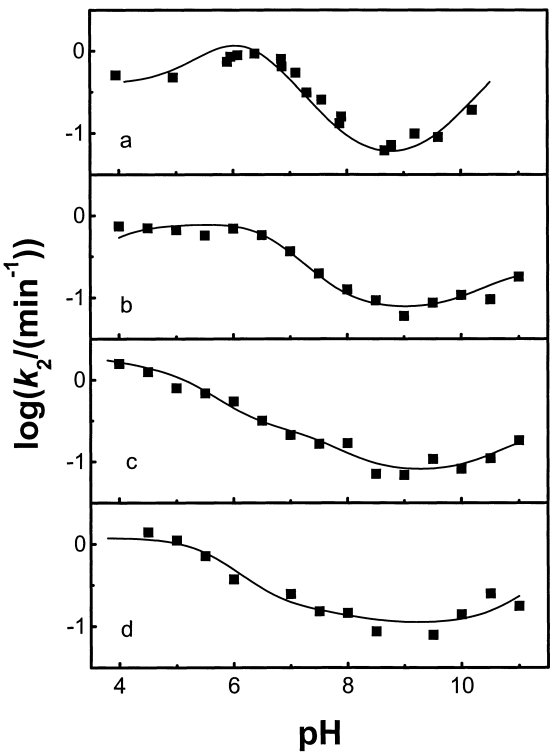


Fig. 2. Plot of log k_2 vs pH for the PLP–TRP system in a) water, b) 24 wt% ethanol, c) 44 wt% ethanol and d) 62 wt% ethanol. Curves calculated using Eq. 6 and data from Table 1.

species present in the medium at each pH.

Thus, k_1^i and k_2^i (with $i = 0$, 1, 2 or 3) are the individual rate constants of the formation of the Schiff bases and of their hydrolysis by H_2O; k^2_{OH} is the rate constant of hydrolysis of species B_2 (a Schiff base with a net charge of -2) by OH^- ions. P_i (with $i = 0$, 1, 2 or 3) denote the chemical species of PLP, and pK_{3P}, pK_{2P} and pK_{1P} the pK values that relate to them. B_i (with $i = 0$, 1, 2 or 3) are the different chemical species of the Schiff bases, and pK_{3B}, pK_{2B} and pK_{1B} are the pK values that relate to them. Finally, K_N is the deprotonation equilibrium constant of the $-NH_3^+$ group in TRP.

The hydrolysis reactions of the forms B_i ($i = 0$, 1) for OH^- have been omitted from Scheme 1 because of the very low concentration of OH^- present at the pH where the concentration of B_i ($i = 0$, 1) was substantial.

The rate of formation of the Schiff base is given by

$$v_1 = [RNH_2]_T \sum_{i=0}^{3} k_1^i[P_i] = k_1[RNH_2]_T[PLP]_T, \quad (2)$$

where T denotes the concentration of all species.

The hydrolysis of the Schiff base conforms to

$$v_2 = k^2_{OH}[B_2][OH^-] + \sum_{i=0}^{3} k_2^i[B_i] = k_2[\text{Schiff Base}]_T, \quad (3)$$

The equilibrium constant, K_{pH}, is given by

$$K_{pH} = [\text{Schiff Base}]_T/([RNH_2]_T[PLP]_T), \quad (4)$$

Taking into account the equilibria in Scheme 1 and the fact

that the equilibrium formation constant for the Schiff base at very high pH values is given by $K_M = k_1^3/k_2^3$, Eqs. 2–4 can be readily transformed into the following:

$$k_1 = \frac{k_1^3 + k_1^2 \cdot a/K_{3P} + k_1^1 \cdot a^2/(K_{3P} \cdot K_{2P}) + k_1^0 \cdot a^3/(K_{3P} \cdot K_{2P} \cdot K_{1P})}{(1 + a/K_N)(1 + a/K_{3P} + a^2/(K_{3P} \cdot K_{2P}) + a^3/(K_{3P} \cdot K_{2P} \cdot K_{1P})}, \quad (5)$$

$$k_2 = \frac{k_{OH} + k_2^2 \cdot a/K_{3B} + k_2^1 \cdot a^2/(K_{3B} \cdot K_{2B}) + k_2^0 \cdot a^3/(K_{3B} \cdot K_{2B} \cdot K_{1B})}{1 + a/K_{3B} + a^2/(K_{3B} \cdot K_{2B}) + a^3/(K_{3B} \cdot K_{2B} \cdot K_{1B})}, \quad (6)$$

$$K_{pH} = \frac{(1 + a/K_{3B} + a^2/(K_{3B} \cdot K_{2B}) + a^3/(K_{3B} \cdot K_{2B} \cdot K_{1B}) K_M}{(1 + a/K_N)(1 + a/K_{3P} + a^2/(K_{3P} \cdot K_{2P}) + a^3/(K_{3P} \cdot K_{2P} \cdot K_{1P}))}, \quad (7)$$

where $k_{OH} = k_2^3 + k_{OH}^2 (K_W/K_{3B})$ and $a = 10^{-pH}$, K_W being the ionic product of H_2O.

The experimental values of k_1, k_2 and K_{pH} were fitted simultaneously to Eqs. 5–7 by using a nonlinear regression method involving minimization of the following functions:

$$U_1 = \Sigma(\log k_{1,e} - \log k_{1,t})^2, \quad (8)$$

$$U_2 = \Sigma(\log k_{2,e} - \log k_{2,t})^2, \quad (9)$$

$$U_{pH} = \Sigma(\log k_{pH,e} - \log K_{pH,t})^2, \quad (10)$$

where subscripts *e* and *t* denote the experimental and theoretical data, respectively.

The pK_{iP} values for PLP in water were taken from Ref. 19; those for PLP in ethanol-water mixtures were obtained by interpolating the data in Ref. 12; those for TRP in the different reaction media were determined in this work (see under Material and Methods).

Table 1 gives the individual rate constants of formation (k_1^i) and hydrolysis (k_2^i, k_{OH}), as well as the pK values obtained by fitting k_1, k_2 and K_{pH} to Eqs. 5, 6 and 7, respectively.

At this point, it is interesting to note that the reaction mechanism involves a aminomethanol intermediate, as in Eq. 11 [R_1-CHO is P_i (with i = 0, 1, 2 or 3) and R_1-CH=N-R_2 is B_i (with i = 0, 1, 2 or 3); therefore, the rate constants $k_1{}^i$ in Scheme 1 involve the formation of aminomethanol (k_{ci}) and the reactions by which the intermediate aminomethanol yields the Schiff base through water elimination (k_{di}) and reverts to reactants by amine elimination (k_{-ci}).

$$R_1 - CHO + NH_2 - R_2 \underset{k_{-ci}}{\overset{k_{ci}}{\rightleftharpoons}}$$

$$R_1 - CH(OH) - NH - R_2 \underset{k_{-di}}{\overset{k_{di}}{\rightleftharpoons}} R_1 - CH{=}N - R_2 + H_2O \quad (11)$$

If K_{ci} ($= k_{ci}/k_{-ci}$) is defined as the equilibrium formation constant for the aminomethanol, provided this forms and splits fairly rapidly, and there is little accumulation of the aminomethanol, then $k_1^i = K_{ci}k_{di}$ and $k_2^i = k_{-di}$. This is consistent with the linear k_{obs} vs [amine] plots obtained at different solvents and pH values.

Discussion

As can be seen from Table 1, the k_1^i values for the reactions of PLP with TRP in different reaction media decrease in the sequence $k_1^0 > k_1^1 > k_1^2 > k_1^3$, which is consistent with previous results for the Schiff bases of PLP with various amine group bearers.[9–11] This behavior has been discussed in the light of a mechanistic scheme, such as that of Eq. 11, where the rate-determining step of the formation of a Schiff base is the dehydration of an intermediate aminomethanol formed by an attack of the amine on the carbonyl group.[4,5] Also, the dehydration is subject to intramolecular acid catalysis and the phenol group at

Table 1. Best Kinetic Constant Values Obtained in the Fitting of Experimental k_1 (L mol^{-1} min^{-1}), k_2 (min^{-1}) and K_{pH} (L mol^{-1}) Values to Scheme 1

	Ethanol content (wt%)			
	0[a)]	24	44	62
log k_1^0	6.83 ± 0.07	6.76 ± 0.05	6.68 ± 0.04	6.97 ± 0.01
log k_1^0	5.15 ± 0.03	4.84 ± 0.06	4.62 ± 0.05	4.40 ± 0.02
log k_1^2	3.14 ± 0.07	3.41 ± 0.08	3.47 ± 0.05	3.72 ± 0.02
log k_1^3	1.96 ± 0.08	1.91 ± 0.08	1.76 ± 0.08	1.78 ± 0.04
pK_{1P}	3.46[b)]	4.02[c)]	4.05[c)]	4.10[c)]
pK_{2P}	6.02[b)]	6.70[c)]	7.00[c)]	7.40[c)]
pK_{3P}	8.22[b)]	8.51[c)]	8.65[c)]	8.80[c)]
log k_2^0	−0.42 ± 0.10	−0.17 ± 0.03	0.14 ± 0.05	0.08 ± 0.07
log k_2^1	0.33 ± 0.05	−0.19 ± 0.05	−0.60 ± 0.06	−0.78 ± 0.11
log k_2^2	−1.16 ± 0.07	−1.17 ± 0.04	−1.16 ± 0.06	−1.03 ± 0.11
log k_{OH}	0.01 ± 0.17	−0.70 ± 0.06	−0.58 ± 0.09	−0.18 ± 0.17
pK_{1B}	6.04 ± 0.35	5.99 ± 0.17	5.37 ± 0.15	5.67 ± 0.15
pK_{2B}	6.33 ± 0.30	7.01 ± 0.17	7.61 ± 0.14	7.99 ± 0.13
pK_{3B}	10.58 ± 0.30	10.62 ± 0.25	10.95 ± 0.45	11.4 ± 1.0
log K_M	2.10 ± 0.28	2.55 ± 0.22	2.32 ± 0.41	2.08 ± 0.95
pK_N	9.20[d)]	9.40[d)]	9.45[d)]	9.60[d)]

a) Taken from Ref. 10b. b) Taken from Ref. 19. c) Obtained by interpolation of data in Ref. 12. d) Determined by potentiometry.

C-3 on the pyridine ring plays an especially prominent role in acid media.[20] The Brønsted plots (log k_1^i vs. p$K_{(i+1)P}$) are linear (α = 0.70–0.77), similarly to those for other α-amino acids in water[10] (α = 0.74–0.78); this indicates that changes in the solvent polarity do not alter the rate-determining step. Since the rate-determining step is aminomethanol dehydration, strictly the pK_a values of the phenol, phosphate and pyridino groups of aminomethanol should be plotted. Nevertheless, all of the Brønsted plot described with the pK_a of these groups in PLP are linear[10a,12–14,20] with slopes in the range −0.5 to −0.8, which suggests a linear correlation between the pK_a in aminomethanol and PLP. On the other hand, a plot of log k_1^i vs [pK_N − p$K_{(i+1)P}$] for the corresponding species in the different media, which is linear (see Fig. 4), has a slope of 0.7 and describes solvent and intramolecular acid catalysis in our system.[13]

The maxima in the k_1 curves (Fig. 1) are a result of the pK_N for the amino group (the mole fraction of free amine increases with increasing pH) and of the sequence $k_1^0 > k_1^1 > k_1^2 > k_1^3$ (the reactivity decreases with increasing pH). The net balance between these two opposing effects leads to the maximum in Fig. 1.

It is interesting to note that, while all individual formation rate constants for the PLP–NHA system increase[12] with increasing polarity of the solvent mixture, the k_1^2 rate constant for the PLP–TRP system exhibits the opposite trend. Taking into account that the rate-determining step of Schiff-base formation is dehydration of the aminomethanol intermediate, the k_1^i increase with the solvent polarity in the PLP–NHA system may be due to a greater stabilization of the corresponding transition state, given its higher polarity. Nevertheless, in the PLP–TRP system the corresponding aminomethanol shows an additional negative charge, due to the carboxylate group, which could stabilize reactants more than the transition state, decreasing k_1^2 with an increase of the media polarity.

It is noteworthy that as in the PLP–NHA system the pK_{3B} value increases with the polarity; in the PLP–TRP system the opposite occurs. Probably the presence of the α-carboxylate group is responsible, at least in part, for the different behavior. It is noteworthy that pK_{3B} values in both systems show the same trend, that their corresponding amino group bearers: the pK_a of hexylamine conjugate acid increases with the solvent polarity, whereas that of L-tryptophan decreases with increasing the solvent polarity.

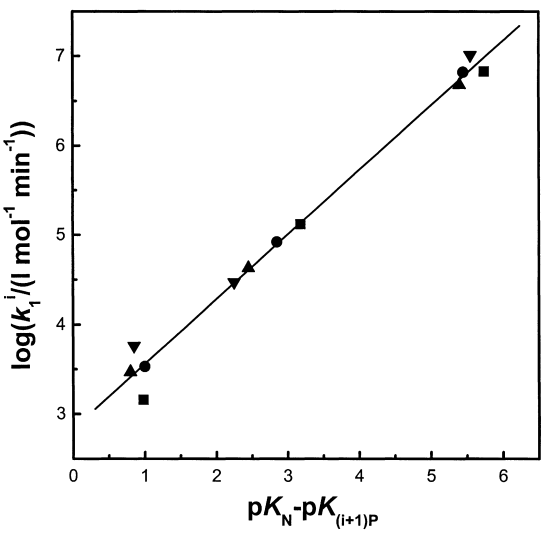


Fig. 4. Plot of log k_1^i as a function of (pK_N − p$K_{(i+1)P}$) for the corresponding species in water (■) and in aqueous media with different ethanol contents: 24 wt% (●), 44 wt% (▲) and 62 wt% (▼)

As in previously reported models,[9–11] the minimum in the log k_2 vs pH curves of Fig. 2 is consistent with the fact that k_2^2 is the smallest of the individual hydrolysis rate constants (see Table 1). The minimum shifts from pH 8.8 in water to pH 9.3 in the ethanol–water mixtures. This shift to alkaline pH values is parallel to a similar shift in pK_{2B} along with an increase in the ethanol content (Table 1). Table 1 also gives the pK_{iB} values found for the Schiff bases in the different solvent mixtures studied; as can be seen, there are no marked differences among the deprotonation equilibrium constants, with the sole likely exception of K_{2B}, which is 50-times greater in 62 wt% ethanol than in pure water, similarly to the PLP–NHA system.[12]

The shift in the minimum of k_2 for the PLP–NHA system in ethanol–water mixtures is to acid pH values[12] and parallel to that in pK_{3B}. Therefore, the shift in the minimum seems to be due to the shifts in both pK_{2B} and pK_{3B} with the solvent composition. For the PLP–NHA system in water–dioxane mixtures, the minimum occurs at pH 9 in water, but is not visible up to about pH 10 in dioxane–water mixtures.[13a] In fact, because it appears at pH 10.2 in 60:40 v/v dioxane–water mixtures,[13b] it is a shift to alkaline pH values. In contrast, in the reaction of deoxypyridoxal with hexylamine (NHA) in dioxane-water mixtures, the position of the minimum is independent of the composition of the solvent mixture.[14] There is thus no universal behavior for the shifts on the minima; however, we can conclude that they are related mainly to the effect of the solvent on the pK_{2B} and pK_{3B} values for the corresponding Schiff base. This in accord with Eq. 3, since at the pH values where k_2 is minimum, species B_2 and B_3 are responsible and their concentrations are determined by pK_{2B} and pK_{3B}.

The log K_{pH} vs. pH plot (Fig. 3) is consistent with the results for similar systems. As can be seen, K_{pH} increases with increasing pH up to a value in between that of pK_N for the amine and that of pK_{3P} for PLP[21] (see Scheme 1); the experimental maximum lies at about 8.7 for PLP–TRP in water and 9.1 in aqueous ethanol (pK_N for TRP is 9.2 in water, and ranges from 9.40 to 9.60 in ethanol-water mixtures; and pK_{3P} for PLP is 8.22 in water[19] and 8.4–8.8 in water–ethanol mixtures [Table 1]).

This work was funded by Spain's DGI (Project BQ2000-0646), FONDECYT (Chile, Projects 1990551 and 2990006) and Programa para la Cooperación con Iberoamérica.

References

1 E. E. Snell, Pyridoxal Phosphate: History and Nomenclature. In "Vitamin B-6 Pyridoxal Phosphate. Chemical, Biochemical and Medical Aspects. Part A," ed by D. Dolphin, R. Poulson, and O. Avramovic, J. Wiley & Sons, New York, (1986), pp. 1–12.

2 V. C. Emery, M. Akhtar, "Pyridoxal Phosphate Dependent

Enzymes," in "Enzyme Mechanisms," ed by M. I. Page and A. Williams, The Royal Society of Chemistry, London (1989), pp. 345–389.

3 E. Braunstein, "Transamination and Transaminases," in "Transaminases," ed by P. Christen and D. E. Metzler, J. Wiley & Sons, New York (1985), pp. 2–19.

4 W. P. Jencks, in "Catalysis in Chemistry, and Enzymology," McGraw-Hill, New York (1969), pp. 490–496.

5 S. Rosemberg, S. M. Silver, J. M. Sayer, and W. P. Jencks, *J. Am. Chem. Soc.*, **96**, 7986 (1974).

6 R. B. Silverman, in "The Organic Chemistry of Drug Design and Drug Action," Academic Press Inc., San Diego (1992), pp. 116–125.

7 E. E. Snell, "Pyridoxal Phosphate in Nonenzymic and Enzymic Reactions," in "Transaminases," ed by P. Christen and D. E. Metzler, J. Wiley & Sons, New York (1985), pp. 19–35.

8 R. G. Kalllen, T. Korpela, A. E. Martell, Y. Matshusima, C. M. Metzler, D. E. Metzler, Y. V. Morozov, I. M. Ralston, F. A. Savin, Y. M. Torchinsky, and H. Ueno, "Chemical and spectroscopic properties of Pyridoxal and Pyridoxamine Phosphates," in "Transaminases," ed by P. Christen and D. E. Metzler, J. Wiley & Sons, New York (1985), pp. 37–108.

9 G. R. Echevarría Gorostidi, A. Basagoitia, E. Pizarro, R. Goldsmitd, J. G. Santos, and F. García Blanco, *Helv. Chim. Acta*, **81**, 837 (1998), and references therein.

10 a) M. A. Vázquez, F. Muñoz, J. Donoso, and F. Garcia Blanco, *Int. J. Chem. Kinet.*, **22**, 905 (1990). b) G. R. Echevarria Gorostidi, J. G. Santos, A. Basagoitia, and F. Garcia Blanco, *Bull. Chem. Soc. Jpn.*, **75**, 2471 (2002).

11 G. R. Echevarría Gorostidi, M. P. Martín Pérez, J. G. Santos, and F. García Blanco, *Helv. Chim. Acta*, **82**, 769 (1999). and references therein.

12 M. A. García del Vado, G. Echevarría J. Donoso, F. Muñoz, A. García-Espantaleón, and F. García Blanco, *J. Mol. Catal.*, **44**, 313 (1988).

13 a) I. M. Plaza del Pino, J. Llor, and J. M. Sánchez Ruiz, *J. Chem. Soc. Perkin Trans. 2*, **1993**, 581. b) I. M. Plaza del Pino, Ph.D. Thesis, University of Granada, Spain (1990).

14 M. A. Vázquez, J. Donoso, F. Muñoz, F. Garcia Blanco, M. A. García del Vado, and G. Echevarría, *J. Mol. Catal.*, **59**, 137 (1990).

15 E. A. Peterson, and H. A. Sober, *J. Am. Chem. Soc.*, **76**, 169 (1954).

16 W. J. Gelsema, C. L. de Ligny, and M. G. F. Wisserman, *Rec. Trav. Chim.*, **84**, 1129 (1965).

17 T. C. Bruice, and A. Lombardo, *J. Am. Chem. Soc.*, **91**, 3009 (1969).

18 M. Coll, J. Frau, J. Donoso, F. Muñoz, and F. García Blanco, *An. Quim.*, **87**, 179 (1991).

19 G. Echevarría, M. A. García del Vado, F. García Blanco, M. Menéndez, and J. Laynez, *J. Sol. Chem*. **15**, 151 (1986).

20 J. M. Sánchez-Ruiz, J. M. Rodríguez Pulido, J. Llor, and M. Cortijo, *J. Chem. Soc. Perkin Trans. 2*, **1982**, 1425.

21 C. M. Metzler, A. Cahill, D. E. Metzler, *J. Am. Chem. Soc*. **102**, 6075 (1980).