Determination of the Rates of Formation and Hydrolysis of the *Schiff* Bases Formed by Pyridoxal 5′-Phosphate with Copolypeptides Containing L-Lysine and Aromatic L-Amino Acids

by **Gerardo R. Echevarría-Gorostidi*** and **M. Piedad Martín Pérez**

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

and **José G. Santos**

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

and **Francisco García Blanco**

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

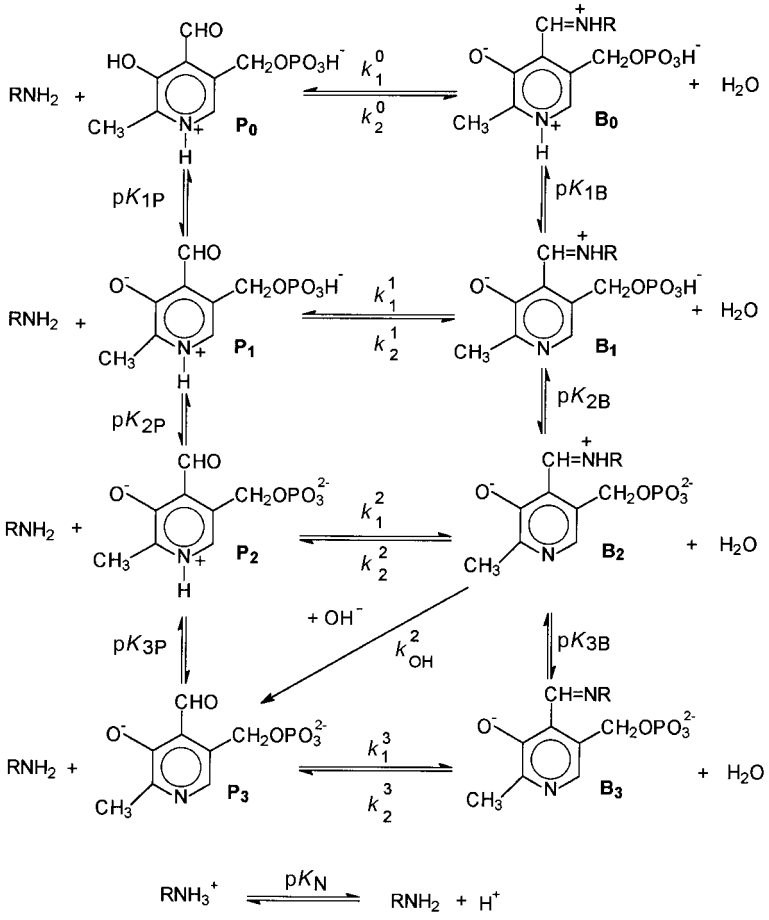
The apparent rate constants of formation (k_1) and hydrolysis (k_2) of the *Schiff* bases formed between pyridoxal 5′-phosphate and the poly(L-Lys,L-Trp)4:1 copolymer at different pH values, a temperature of 25 °C and an ionic strength of 0.1 M were determined. The individual rate constants of formation and hydrolysis of the *Schiff* bases of pyridoxal 5′-phosphate with poly(L-Lys,L-Trp)4:1, poly(L-Lys,L-Tyr)4:1, and poly(L-Lys,L-Phe)1:1 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 significance of the interactions between the pyridine ring in pyridoxal 5′-phosphate and the aromatic ring in the L-phenylalanine, L-tyrosine, and L-tryptophan side chains is demonstrated.

Introduction. – In recent papers, we reported a model for pyridoxal 5′-phosphate (PLP) enzyme binding based on studies of the formation and hydrolysis of the *Schiff* bases of PLP with poly-L-lysine in a variable degree of polymerization [1][2] and those of PLP with various L-lysine copolymers (*viz.* poly(L-Lys,L-Ala)1:1, poly(L-Lys,L-Glu)4:1, poly(L-Lys,L-Tyr)4:1, and poly(L-Lys,L-Phe)1:1) [2–4]. We found the *Schiff* bases of PLP with polypeptides containing L-lysine to be much more stable than those with primary amines as a result of H-bonding interactions between PLP groups and the peptide skeleton [3].

The presence of residues containing aromatic side chains near the active site in PLP-dependent enzymes such as Tyr70 and Tyr225 in ArAT (aromatic amino acid transferase) [5] or Tyr573 in glycogen phosphorylase [6] is known to promote other types of interaction that alter the stability of the C=N bond in the *Schiff* base formed by PLP and the L-lysine residue in the peptide skeleton, or between PLP and an amino-acid substrate. The micro-environment where the *Schiff* base is formed has a critical influence on its rate constants of formation and hydrolysis. In the absence of non-aqueous solvents, the *Schiff* base is located in a partially hydrophobic environment. The interaction of PLP with L-tryptophan is essential for D-amino acid transferase (Trp139) to exert an optimal catalytic function [8] and also for aspartate amino transferase in the same respect (Trp140) [9].

In this work, the stability and kinetics of formation and hydrolysis of the *Schiff* bases of pyridoxal 5′-phosphate with poly(L-Lys,L-Trp)4 : 1 were determined and the results compared with those previously obtained for other copolymers bearing various aromatic side chains. To shed additional light on the interactions between the aromatic ring in PLP and those in the side chains of the copolymers, the kinetic results for the reaction of PLP with various copolymers bearing aromatic side chains (*viz*. poly(L-Lys,L-Tyr)4 : 1, poly(L-Lys,L-Phe)1 : 1, and poly(L-Lys,L-Trp)4 : 1) were examined in terms of the individual rate constants involved (see *Scheme 1*).

Scheme 1



Material and Methods. – The polypeptide was purchased from *Sigma Chemical Co*. The molecular weight of poly(L-Lys,L-Trp)4 : 1, as determined viscosimetrically, was 38000 (DP = 187) Da. Pyridoxal 5′-phosphate and all other chemicals used were of reagent-grade and purchased from *Merck*.

Acetate, phosphate, and carbonate buffers were used over appropriate pH ranges. The buffer concentrations used were typically 0.02 mol/l, and the ionic strength was kept constant at 0.1M by adding appropriate amounts of KCl to the medium.

PLP Solns. were made in appropriate buffers and stored in the dark. Their exact concentrations were determined by dilution with 0.1M HCl [10] and were found to be in the region of 2×10^{-5} M. Polypeptide solns. 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 polypeptide in the corresponding buffer.

Kinetic measurements were made at a variable pH by using a *Uvikon 941-Plus* spectrophotometer furnished with thermostated cells of 1-cm lightpath. In each case, the reaction was started by adding a known volume of PLP buffered soln. to prethermostated polypeptide solns. at $25 \pm 0.05°$. The difference between the initial and final pH in the reaction cell never exceeded 0.03 units. pH Measurements were made by using a *Crison* pH-meter furnished with a *Metrohm EA120* electrode that was previously calibrated with aqueous buffers at 25.0°.

The overall reaction between an aldehyde and an amine is given in *Eqn. 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 is described in detail in [11]. Their ratio coincides with the equilibrium constant ($K_{pH} = k_1/k_2$).

Results. – *Figs. 1–3* show the experimental results of the logarithmic overall rate constants of formation (k_1) and hydrolysis (k_2), and the equilibrium constant (K_{pH}), respectively, as a function of pH for the *Schiff* bases of PLP with poly(L-Lys,L-Trp)4 : 1. They also include the results for the *Schiff* bases of PLP with poly(L-lysine) homopolymers (degree of polymerization, DP = 277 and 1150) [1] and poly(L-Lys,L-Phe)4 : 1 at the same temperature and ionic strength [4].

The k_1 values for the *Schiff* bases of PLP with poly(L-Lys, L-Trp)4 : 1 exceed those of its bases with poly(L-lysine) throughout the pH range studied [1]. The sole difference is the presence or absence of side chains containing aromatic groups, so these must be the origin of the differential behavior observed.

As can be seen from *Fig. 2*, the hydrolysis rate constant, k_2, for the *Schiff* bases of PLP with poly(L-Lys,L-Trp)4 : 1 in alkaline media are greater than those of the bases with poly(L-lysine) [1]. The minimum in the k_2 *vs.* pH plot for the *Schiff* bases of PLP with poly(L-Lys,L-Phe)1 : 1 and poly(L-lysine) at pH 5 and 8, respectively, is absent in the plot for the base with poly(L-Lys, L-Trp)4 : 1 over the pH range studied (*Fig. 2*).

The large k_1 values obtained result in also very large K_{pH} values (*Fig. 3*). In the vicinity of pH 9, the K_{pH} values for the *Schiff* bases of PLP with the L-lysine homopolymers and copolymers are close to the reported values for the *Schiff* bases of PLP with primary amines [12].

As shown in *Scheme 1*, the overall rate constants of formation and hydrolysis of the *Schiff* base can be described in terms of the individual constants for the different chemical 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 formation of the *Schiff* base and of its hydrolysis by H_2O, and k_{OH}^2 is the rate constant of hydrolysis of species $\mathbf{B}_2$ (a *Schiff* base with a net charge of -2) by OH^- ions.

$\mathbf{P}_i$ (with $i = 0$, 1, 2, or 3) denotes the different chemical species of PLP, and pK_{3P}, pK_{2P}, and pK_{1P} are the different pK's that relate them. $\mathbf{B}_i$ (with $i = 0$, 1, 2, or 3) are the different chemical species of the *Schiff* base, and pK_{3B}, pK_{2B}, and pK_{1B} the pK's that relate them. K_N is the deprotonation equilibrium rate constant of the NH_2 group in the side chain of L-lysine. The formation equilibrium constant of the *Schiff* base in highly alkaline media is given by $K_M = k_1^3/k_2^3$.

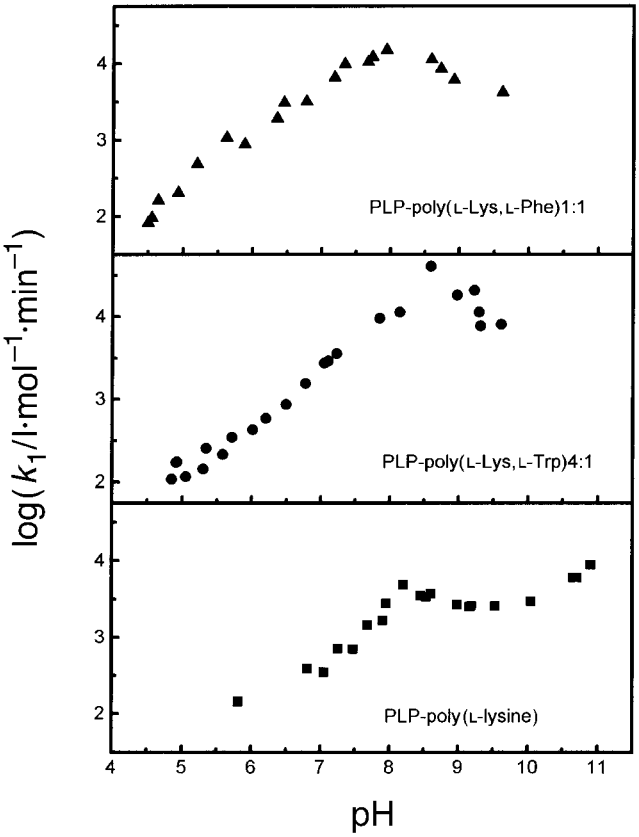


Fig. 1. *Plot of log* k_1 vs. *pH for different* Schiff *bases of PLP*

The rate of formation of the *Schiff* base can be expressed as

$$v_1 = [\mathrm{RNH_2}] \sum_{i=0,1,2,3} k_1^i [\mathbf{P}_i] = k_1 [\mathrm{RNH_2}]_\mathrm{T} [\mathrm{PLP}]_\mathrm{T} \tag{2}$$

where subscript T denotes the combined concentration off all species.

On the other hand, the rate of hydrolysis of the *Schiff* base can be expressed as

$$v_2 = k_{\mathrm{OH}}^2 [\mathbf{B_2}][\mathrm{OH^-}] + \sum_{i=0,1,2,3} k_2^i [\mathbf{B}_i] = k_2 [\mathit{al}\mathrm{dim}\mathit{ine}]_\mathrm{T} \tag{3}$$

The equilibrium constant can be expressed as

$$K_\mathrm{pH} = [\mathit{al}\mathrm{dim}\mathit{ine}]_\mathrm{T} / ([\mathrm{RNH_2}]_\mathrm{T} [\mathrm{PLP}]_\mathrm{T}) \tag{4}$$

Taking into account the equilibria of *Scheme 1*, *Eqns. 2* and *3* can readily be transformed into the following:

$$k_1 = \frac{k_1^3 + \frac{k_1^2 a}{K_{3\mathrm{P}}} + \frac{k_1^1 a^2}{K_{3\mathrm{P}} K_{2\mathrm{P}}} + \frac{k_1^0 a^3}{K_{3\mathrm{P}} K_{2\mathrm{P}} K_{1\mathrm{P}}}}{(1 + K_\mathrm{N})\left(1 + \frac{a}{K_{3\mathrm{P}}} + \frac{a^2}{K_{3\mathrm{P}} K_{2\mathrm{P}}} + \frac{a^3}{K_{3\mathrm{P}} K_{2\mathrm{P}} K_{1\mathrm{P}}}\right)} \tag{5}$$

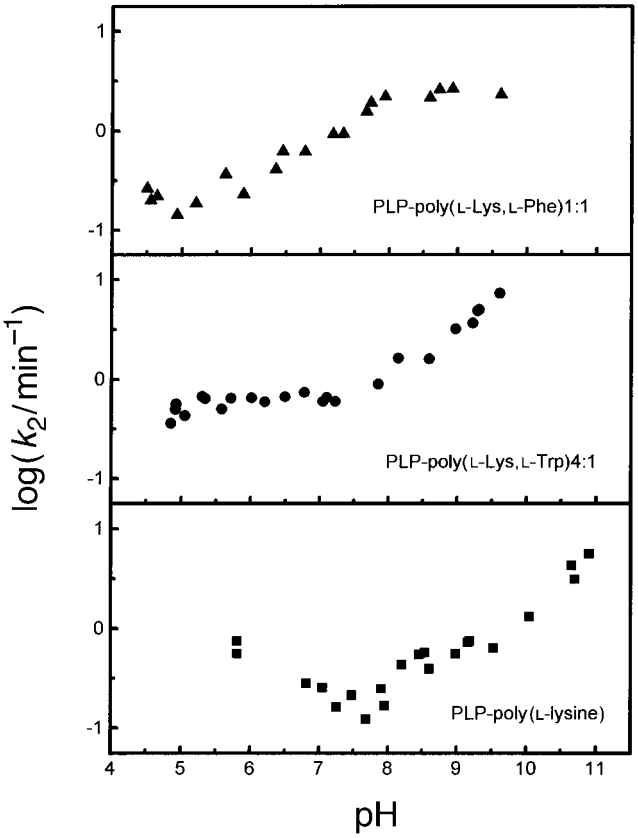


Fig. 2. *Plot of log* k_2 vs. *pH for different* Schiff *bases of PLP*

$$k_2 = \frac{k_{OH} + \frac{k_2^2 a}{K_{3B}} + \frac{k_2^1 a^2}{K_{3B}K_{2B}} + \frac{k_2^0 a^3}{K_{3B}K_{2B}K_{1B}}}{1 + \frac{a}{K_{3B}} + \frac{a^2}{K_{3B}K_{2B}} + \frac{a^3}{K_{3B}K_{2B}K_{1B}}} \tag{6}$$

where $a = 10^{-pH}$, $k_{OH} = k_2^2 + \frac{k_{OH}^2 K_W}{K_{3B}}$ and K_W is the ionic product of water.

The experimental results of k_1 for the *Schiff* bases studied in this work and those of PLP with poly(L-Lys,L-Tyr)4 : 1 and poly(L-Lys,L-Phe)1 : 1 [4] were fitted to *Eqn. 5* up to pH 8.5, using a non-linear regression that minimizes the following function

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

where subscripts e and t denote experimental and theoretical data, respectively. The protonation constants for PLP and L-lysine in the copolymers were obtained from the literature [7][13]. The *Table* gives the individual rate constants of formation, k_1^i, and the pK values obtained in the fitting. For comparison, the values for the *Schiff* bases of PLP with poly(L-lysine) are also listed [2].

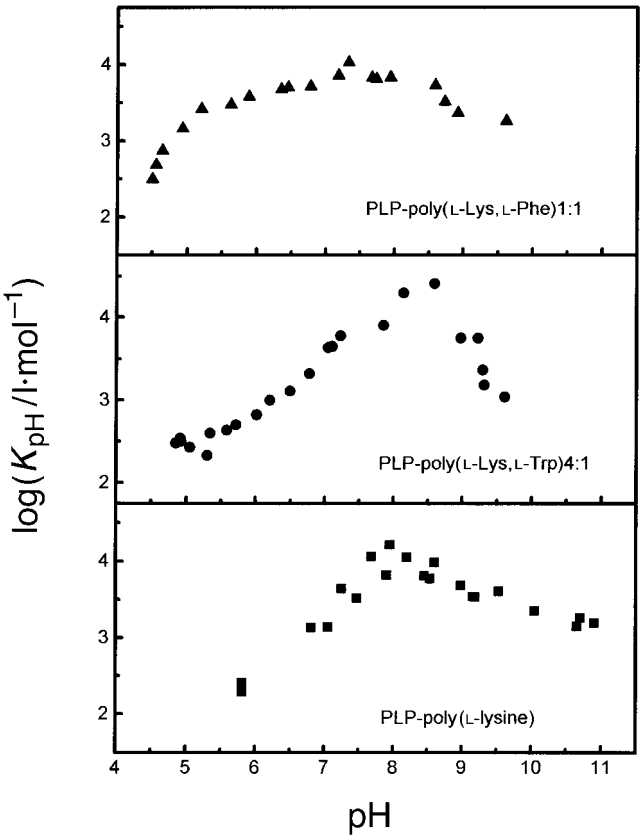


Fig. 3. *Plot of log* K_{pH} vs. *pH for different* Schiff *bases of PLP*

The experimental k_2 values for the *Schiff* bases studied and those of PLP with poly(L-Lys,L-Tyr)4 : 1 and poly(L-Lys,L-Phe)1 : 1 [4] were fitted to *Eqn. 6* over the pH range examined, again using a non-linear regression method that minimized the following function:

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

The deprotonation constants for the *Schiff* bases, required in the initial fitting, were estimated from reported data for related systems [7]. The *Table* lists the individual rate constants of hydrolysis (k_2^i, k_{OH}) and the pK values obtained from the fitting. For comparison, the values for the *Schiff* bases of PLP with poly(L-lysine) are also given [2].

Discussion. – As can be seen from *Fig. 1*, the presence of side chains containing aromatic groups in L-lysine copolymers favors the formation of *Schiff* bases with PLP. In previous work, the number of charges in the side chains, their sign, and their distribution were found to have no effect on the formation of such bases. Any interactions with the peptide skeleton are identical in all cases. Therefore, the

Table. *Best Kinetics Constants and p*K_a *Values Obtained in the Fitting of Experimental Values of* k_1 *and* k_2 *to* Scheme 1 *for Various* Schiff *Bases of PLP with Copolypeptides Bearing Aromatic Side Chains* and *with Poly*(L-*lysine) (*k_1 in $l \cdot mol^{-1} \cdot min^{-1}$, k_2 in min^{-1})

	Schiff base			
	PLP + Poly(L-lysine)	PLP + Poly(L-Lys,L-Tyr)4 : 1	PLP + Poly(L-Lys,L-Trp)4 : 1	PLP + Poly(L-Lys,L-Phe)1 : 1
log k_1^0	8.74	8.88	8.52	8.70
log k_1^1	6.13	6.39	6.85	7.40
log k_1^2	5.45	5.92	6.41	6.52
log k_1^3	3.53	3.95	2.45	2.42
pK_{1P}	3.46	3.58	3.58	3.58
pK_{2P}	6.02	5.86	5.86	5.86
pK_{3P}	8.22	8.59	8.59	8.59
log k_2^0	– 0.17	– 0.42	– 0.26	–
log k_2^1	– 2.29	– 0.45	– 0.19	– 0.56
log k_2^2	– 0.42	0.75	0.05	0.43
log k_{OH}	1.04	0.25[a])	1.25	–
pK_{1B}	6.62	5.64[b])	6.62	5.08[b])
pK_{2B}	7.74	8.90[b])	7.74	7.60[b])
pK_{3B}	10.92	10.29[b])	9.83	10.42[b])
pK_N	10.03	10.05	10.06	10.06

[a]) Estimated value from experimental data in [4].
[b]) Spectroscopie data from [4].

differences observed in *Fig. 1* can only be ascribed to the presence of aromatic groups in the side chains. There may be some stacking between the aromatic ring in L-tryptophan, L-phenylalanine, or L-tyrosine, and the pyridine ring in PLP. The k_1 values for the *Schiff* bases of the copolymer containing L-tryptophan are greater than those for the bases containing L-tyrosine (*Fig. 4*) [4]. Both systems possess the same proportion of aromatic side chains (4 : 1), so their differential behavior results from the aromatic ring in L-tryptophan being larger than that in L-tyrosine. On the other hand, the fact that k_1 is greater for the copolymer containing L-phenylalanine than for that containing L-tryptophan (*Fig. 1*) must be a result of the former possessing a greater number (50%) of side chains including an aromatic ring.

The k_1 values for the *Schiff* bases of PLP with poly(L-Lys,L-Trp)4 : 1 peak at a pH of *ca.* 8.5 (*Fig. 1*). This is consistent with the behavior of other *Schiff* bases of PLP with copolymers containing L-lysine (*Fig. 1*), and is related to a conformational change in the copolymer. Above pH 8.5, the formation of the α-helix, which is the most stable conformation of poly(L-lysine) under these conditions [14], makes k_1 dependent on the copolymer conformation. Above pH 8, some of the protonable groups in the different side chains of the *Schiff* bases of PLP with poly(L-lysine), poly(L-Lys,L-Trp)4 : 1, and poly(L-Lys,L-Phe)1 : 1 are uncharged, so the conformation is stable. This is also the case with the *Schiff* bases of PLP and poly(L-Lys,L-Ala) [2]. It is interesting to note that the stabilization of the α-helix conformation starts at roughly the same pH in all these systems.

In acidic media, the PLP molecule has a zero net charge ($\mathbf{P_0}$); also, the rate constant of formation of the *Schiff* base, k_1^0, is very similar to those for the different bases (*Table*), whether or not side chains bearing aromatic rings are present in the

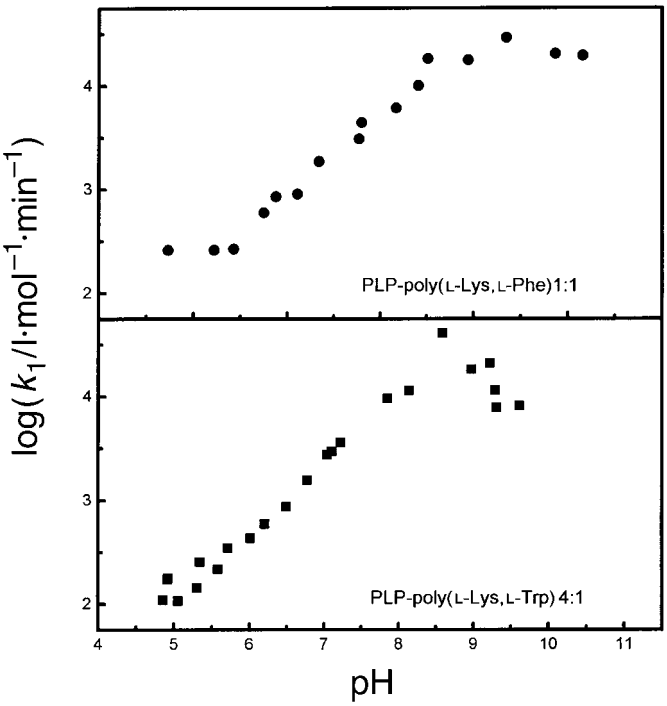


Fig. 4. *Plot of* k_1 vs. *pH for different* Schiff *bases of PLP*

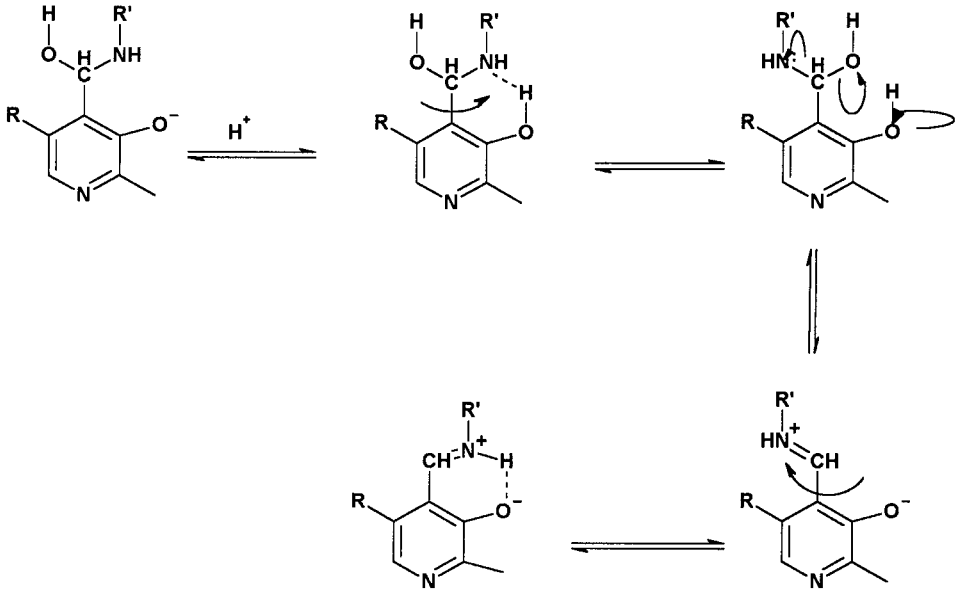
copolymer. Consequently, this PLP form, with a protonated 3-hydroxy group, does not seem to interact with the aromatic side chains.

As the pH is raised, the presence of forms $\mathbf{P_1}$ and/or $\mathbf{P_2}$ of PLP in the medium increases, and so do k_1^1 and k_1^2 to a marked extent, by virtue of the presence of aromatic side chains (*Table*). The 3-hydroxy group in both $\mathbf{P_1}$ and $\mathbf{P_2}$ is deprotonated and the pyridine N-atom protonated. The largest individual rate constant is that of the *Schiff* base of PLP with poly(L-Lys,L-Phe)1 : 1, which is the copolymer with the greatest number of aromatic side chains. The *Schiff* bases of PLP with poly(L-Lys,Tyr)4 : 1 and poly(L-Lys,Trp)4 : 1 contain the same proportion of aromatic side chains; however, the individual rate constants of formation for the *Schiff* bases of poly(L-Lys,L-Trp) are greater, because the aromatic ring in L-tryptophan is larger than that in L-tyrosine.

In alkaline media, the formation of *Schiff* bases becomes independent of the copolymer composition again. k_1^3 values depart from the trends of k_1^1 and k_1^2, since, under these pH conditions, the pyridine N-atom in $\mathbf{P_3}$ of PLP is deprotonated.

As can be seen from the *Table*, the rate constants for all the *Schiff* bases studied conform to the sequence $k_1^0 > k_1^1 > k_1^2$, which is consistent with the occurrence of intramolecular acid catalysis in the rate-determining step of the *Schiff* base formation [15] (*Scheme 2*). Our *Brønsted* plots are not linear, because, as noted earlier, the presence of side chains containing aromatic groups affects the individual rate constants to a varying extent.

Scheme 2



The presence of aromatic rings in the side chains of the copolymers favors the hydrolysis when the 3-hydroxy group and the pyridine N-atom are deprotonated (forms $\mathbf{B_1}$ and $\mathbf{B_2}$ of the *Schiff* base). As a result, the k_2^1 and k_2^2 values for the *Schiff* bases of PLP with poly(L-lysine) are the smallest. At lower pH values, the *Schiff* bases have a protonated pyridine N-atom, and the corresponding individual rate constants, k_2^0, are very similar (*Table*).

In summary, in the formation of the *Schiff* bases of pyridoxal 5′-phosphate with copolymers containing L-lysine and amino acids possessing side chains with aromatic rings are involved interactions with the 3-hydroxy group deprotonated and the pyridine N-atom protonated. This may be the origin of the fact that the rings in Trp140 and PLP lie virtually parallel at the active site of aspartate aminotransferase [9].

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