A Study of the Schiff Base formed between Pyridoxal-5'-phosphate and Poly-L-lysine of low Polymerization Degree

Jose M. Sevilla,[a] Manuel Blazquez,*[,a] Manuel Dominguez[b] and Francisco García-Blanco[c]
[a] *Departamento de Quimica Física y Termodinámica Aplicada, Facultad de Ciencias, Univ. Córdoba, 14004 Córdoba, Spain*
[b] *Departamento de Química Física, Univ. Sevilla, Prof. García Gonzalez s/n, 41071 Sevilla, Spain*
[c] *Departamento de Fisicoquimica, Fac. Farmacia, Univ. Complutense, 28040 Madrid, Spain*

The interaction between pyridoxal-5'-phosphate (PLP) and poly-L-lysine of low polymerization degree has been studied in aqueous solutions. Formation of a Schiff base by a reversible reaction is observed according to absorption spectra and electrochemical results. The apparent equilibrium constant has been calculated as a function of the pH under conditions which favour addition of one molecule of the coenzyme to the polypeptide. A comparison of the poly-L-lysine:PLP Schiff base and Schiff bases from PLP and amino acids or amines is considered. From the study of the influence of the lysine:PLP ratio a characterization of the binding of PLP to the matrix is obtained in the pH interval 5–10. The response of the Schiff base to changes in pH or poly-L-lysine concentration point to an important role of the supporting polypeptide in the environment of the coenzyme favouring, under determined conditions, stabilization of the enolimine form or a carbinolamine intermediate.

Pyridoxal-5'-phosphate (PLP) is the coenzyme of many enzymes which have different substrate specificity and which catalyse a wide spectrum of reactions.[1,2] Information on the properties of the Schiff bases of PLP is basic to an understanding of the catalytic role of the coenzyme. The binding of the coenzyme and the physicochemical properties of the site are still being elucidated. Crystallographic studies have shown a multipoint interaction between apoenzyme and coenzyme in the aspartate aminotransferase.[3–5] Recently, a microcalorimetric study on Schiff base formation between PLP and model compounds of increasing complexity such as poly-L-lysine, and between PLP and the apoenzyme of aspartate aminotransferase has suggested a multiple interaction between the polymer and the ligand.[6] On the other hand, from electrochemical and spectrophotometric studies of the Schiff base of PLP and analogous compounds a quantitative characterization of microequilibria in solution as well as the stability of the imine under different conditions has been carried out.[7–12] The Schiff base and aldehyde are electroactive species with different potentials, which allows their equilibrium concentrations, to be determined.

The present work deals with an application of these studies to the reaction between PLP and poly-L-lysine of low polymerization degree. This model reaction is more sophisticated than those used in previous studies. The poly-L-lysine was chosen because its polypeptide skeleton is more similar to the protein site than those obtained with primary amines. This allows the possibility that PLP binds to it by its aldehyde group and through electrostatic interactions with lysine residues that could reveal effects of conformational changes in the polymer. Electrochemical and spectrophotometric studies of the Schiff base derived from PLP and poly-L-lysine (DP = 17) under equilibrium conditions have been carried out. The apparent formation constant under conditions which favour the reaction of only one molecule, on average, of the coenzyme with poly-L-lysine has been obtained as a function of the pH. A characterization of the stoichiometry of the binding of the ligand to the macromolecule as a function of the lysine:PLP ratio has been determined. A comparison of this Schiff base and the Schiff bases derived from PLP and primary amines or amino acids is considered. The differences in behaviour are analysed in relation to the formation of a reaction intermediate favoured by the side chains of lysine. The conclusions of this study are of interest in the analysis of the properties of the catalytic site in different enzymes.

Experimental

Poly-L-lysine hydrobromide was purchased from Sigma. The molecular weight (based on viscosity determination) of the sample used was 3600 (DP = 17). PLP was purchased from Sigma. All the other chemicals used were of Merck p.a. grade. As the supporting electrolyte, buffered solutions consisting of 0.02 mol dm^{-3} acetic acid and 0.02 mol dm^{-3} phosphoric acid for pH < 8.5 and 0.02 mol dm^{-3} phosphoric acid and 0.02 mol dm^{-3} K_2CO_3 for pH > 8.5 were used. In the spectrophotometric study buffer of 0.02 mol dm^{-3} piperazine and buffer of 0.02 mol dm^{-3} HEPES were also used. The pH was adjusted with KOH and the ionic strength was adjusted to 0.1 mol dm^{-3} with KCl.

PLP solutions were prepared daily in a suitable buffer and kept in the dark. Poly-L-lysine solutions were also prepared daily by dissolving the appropriate amount in the buffer solution.

DC and differential pulse polarographic curves were recorded by means of a 626 Metrohm polarograph. In DP polarography the drop time was 0.5 s, the pulse amplitude ΔE, 10 mV and the pulse duration 60 ms. A saturated calomel electrode (SCE) was used as the reference electrode. The working electrode was a mercury capillary. The pH was measured with a Hanna 8418 pH-meter. The temperature of the solutions was kept constant at 25 ± 0.1 °C. An Amel polarograph was used to study the influence of the capillary characteristics. Polarographic measurements were carried out on a thermostatted Amel 494 cell equipped with a SCE, a platinum auxiliary electrode and a dropping mercury electrode (DME). Chronoamperometric measurements were carried out by using a Tacussell potentiostat PRGE and a Houston Inst. mod. 2000 graphic recorder. All measurements were carried out in a nitrogen atmosphere.

Spectrophotometric measurements were performed on a Varian Cary 219 spectrophotometer with 1 cm quartz cuvettes thermostatted at 25 ± 0.1 °C.

Fluorescence spectra were recorded on a MPF 66 Perkin-Elmer spectrophotofluorimeter furnished with a 150 W xenon lamp and cuvettes thermostatted at 25 ± 0.1 °C.

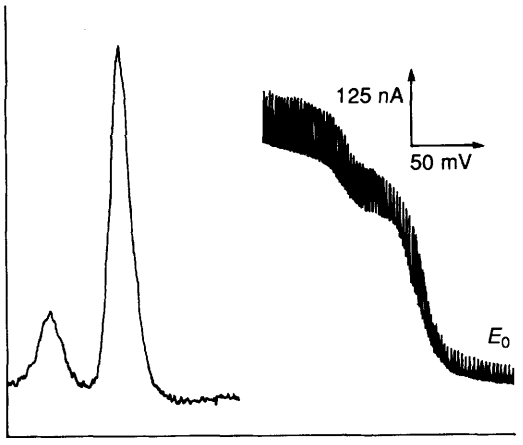


Fig. 1 DC and DP polarography. $c_{PLP} = 1.10^{-4}$ mol dm^{-3}. $c_{polylysine} = 5.9 \times 10^{-5}$ mol dm^{-3}. pH = 9.15.

The imines were formed by addition of known amounts of PLP to buffered solutions of poly-L-lysine. The reaction mixture was studied after equilibrium was reached.

Results and Discussion

Electrochemical Behaviour.—The interaction between pyridoxal-5′-phosphate (PLP) and poly-L-lysine yields a Schiff base according to the general reaction between aldehydes and amino acids. The reaction mixture was studied after the reaction reached equilibrium. The formation of the Schiff base was followed by classical polarography (CP) and differential pulse polarography (DPP). In CP two waves are observed under most of the experimental conditions (Fig. 1). The reduction of the -CH=N- group of the Schiff base yields a first wave, at less negative potentials than the second wave, which corresponds to the reduction of the carbonyl group of the free PLP in solution. The electroreduction of the PLP in the absence of poly-L-lysine agrees with this assignment. The heights of the waves are stable with time if the reaction mixture is kept in the dark. In DPP two well-resolved peaks are obtained. Under the conditions of Fig. 1, a difference between the first peak (Schiff base) and the second peak (PLP) close to 120 mV is reached. This behaviour corresponds to two electroactive species, Schiff base and PLP, coupled by the formation reaction (Scheme 1).

$$\xleftarrow[E_2^0]{2e,\,2H^+} \text{R–CHO} \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \text{R–CH=NR} \xrightarrow[E_1^0]{2e,\,2H^+}$$

(second wave) (first wave)

Scheme 1

In the case of a slow chemical reaction the eqn. (1) is obtained[11]

$$I_{L1} = \frac{i_{L1}}{i_D} = \frac{K}{K+1} \qquad (1)$$

where $K = k_f/k_b$, k_f and k_b being the pseudo-first-order rate constants, i_{L1} and i_D are the limiting current of the Schiff base and the diffusion current corresponding to the initial analytical concentration of PLP, c_0, respectively, and I_{L1} the normalized limiting current of the Schiff base. In this equation identical diffusion coefficients were assumed for both electroactive species. A complementary equation (2) can be obtained,[11] in

$$I_{L2} = \frac{i_{L2}}{i_D} = \frac{1}{K+1} \qquad (2)$$

which i_{L2} and I_{L2} are the limiting current and the normalized limiting current of the second wave (PLP).

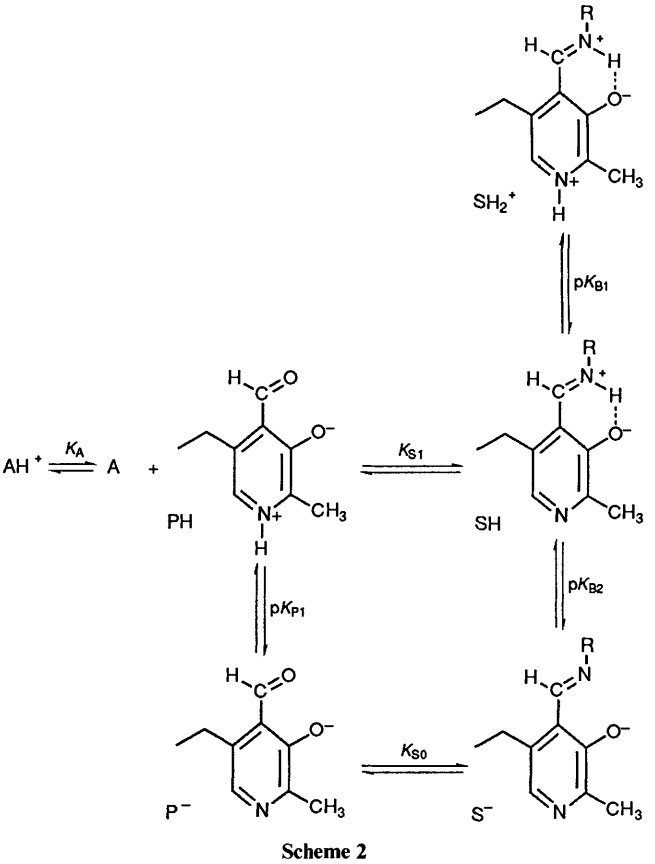
In the case of a fast chemical reaction eqn. (3) is valid,[11,13]

$$\frac{i_D}{i_{L2}} = 1.386\,[(K^2k)^{0.545}]^{-1}\,t^{-0.545} + 1 \qquad (3)$$

where $k = k_f + k_b$ and t is the drop time.

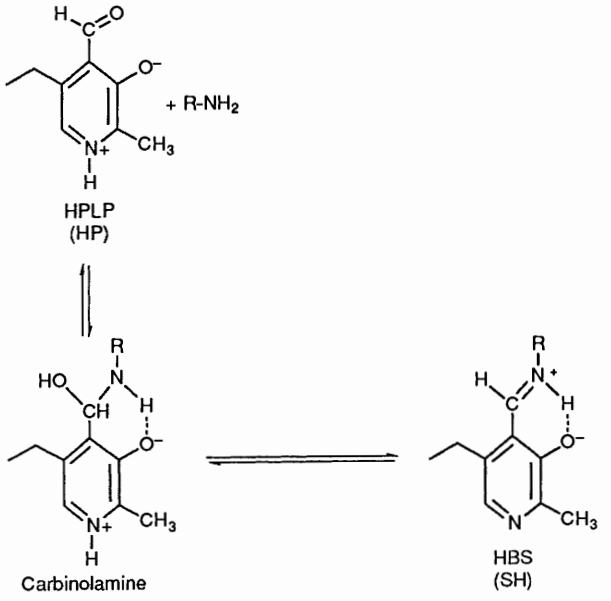
In order to characterize the electrode process, a study of the current–time curves at constant potential in the limiting zones of the first wave and the overall process, was carried out. At pH < 11, the results over a wide pH range indicate diffusion control in both cases (d log i_L/d log t = 0.19). This indicates that the limiting currents i_{L1} and i_{L2} are proportional to the equilibrium concentration of the Schiff base and PLP in the bulk of solution. In this case the eqns. (1) and (2) can be applied. At pH > 11 kinetic control was obtained (d log i_L/d log t = 0.36, at pH = 11.3) for the first wave. Furthermore, the plot of i_D/i_{L1} *vs.* $t^{-0.545}$ is linear with a slope proportional to the parameter K^2k of the chemical reaction and an intercept close to unity, indicating a good agreement with eqn. (3). This behaviour indicates that, to some extent, the calculated equilibrium concentration will be affected by the formation of the Schiff base at the electrode–solution interface.

Additional information on the electrode process was obtained from the plot of the half-wave potential ($E_{\frac{1}{2}}$) *vs.* pH for the first wave. The behaviour is similar to that found in the electroreduction of the Schiff base of PLP with amino acids.[11] This is assigned to a two-electron reduction of the electroactive species SH_2^+ and SH. The intersections of the linear segments of the $E_{\frac{1}{2}}$ *vs.* pH plot at pH close to 6 and 12 are estimates of pK_{B1} and pK_{B2}, respectively (see Scheme 2).



Scheme 2

Schiff Base Formation.—The reaction of PLP with primary amines or amino acids yields a Schiff base according to Scheme 3. The first reaction is the rate-determining step in an acid medium and the second reaction in a basic medium. In general the equilibrium concentration of carbinolamine is very low and can be neglected in the calculation of the apparent equilibrium constant.[14,15]



Scheme 3

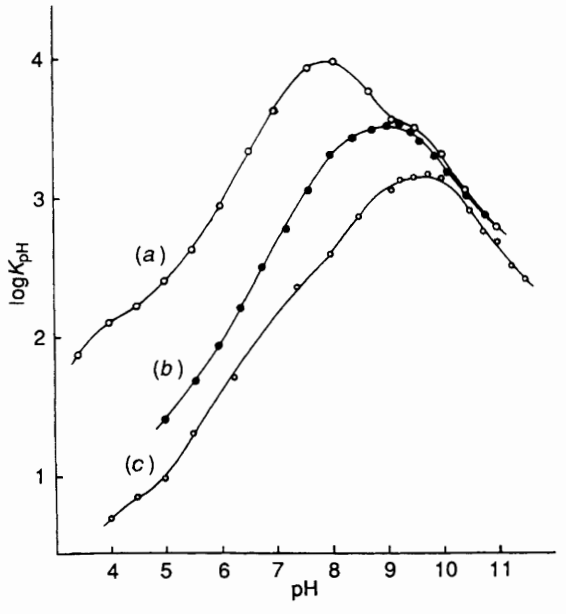


Fig. 2 Variation of K_{pH} with pH. (*a*) PLP:poly-L-lysine; (*b*) PLP:lysine; (*c*) PLP:hexylamine.

In Scheme 2 are shown the main species in neutral and basic media. The dissociation of the phosphate is not included since its influence is not detected in spectrophotometric[14,15] or electrochemical studies.[7–11] According to Scheme 2, an apparent equilibrium constant, K_{pH}, is defined by eqn. (4) where $[SH_i^{(i-1)}]$, $[PH_i^{(i-1)}]$ and $[AH_i^{(i)}]$ are the equilibrium concentrations of the species in different protonation states of the Schiff base, PLP and poly-L-lysine, respectively. This equilibrium concentration ratio varies with the pH and depends on the acid–base constants of the PLP (K_{P2}), Schiff base (K_{B1}, K_{B2}) and poly-L-lysine (K_A), respectively. K_{pH} is also a function of the equilibrium constants of the ionic species, K_{S0} and K_{S1}. The latter constants are defined between unprotonated and monoprotonated species, respectively.

$$K_{pH} = \frac{[SH_2^+] + [SH] + [S^-]}{\{[PH] + [P^-]\}\{[AH^+] + [A]\}} = K_{S0}\frac{[(c_H^2/K_{B1}K_{B2}) + (c_H/K_{B2}) + 1]}{[(c_H/K_{P2}) + 1][(c_H/K_A) + 1]} \quad (4)$$

Taking into account Scheme 1 and the relationship between K_{pH} and the apparent constant, K, eqn. (5) can be obtained,

$$K_{pH} = \frac{1}{c_A}\cdot\frac{I_{L1}}{1 - I_{L1}} \quad (5)$$

where c_A is the total concentration of the amino group.

Under the experimental conditions (1×10^{-4} mol dm^{-3} PLP, 5.9×10^{-5} mol dm^{-3} poly-L-lysine) it can be assumed that only one molecule of PLP binds to the matrix. The formation reaction can be treated as a pseudo-first-order chemical reaction as the concentration of the lysine residue is higher than the coenzyme concentration. Eqn. (5) is valid assuming the same diffusion coefficient for the electroactive species (PLP and Schiff base). This is a large assumption and to avoid it we have compared the limiting current of the second wave, i_{L2}, with that obtained at the same conditions in the electroreduction of PLP in absence of poly-L-lysine. From this comparison we can calculate K and K_{pH} using eqn. (6).

$$K_{pH} = \frac{1}{c_A}\cdot\frac{1}{1 - I_{L2}} \quad (6)$$

In Fig. 2 is shown K_{pH} *vs.* pH for the Schiff base of PLP and poly-L-lysine. In this figure are included results for Schiff bases PLP:hexylamine and PLP:lysine. K_{pH} varies as predicted by eqn. (4) and the highest values are reached in the poly-L-lysine Schiff base. A stability maximum appears in the plot at pH 9.5, 8.9 and 7.8 for the Schiff bases PLP:hex, PLP:lys and PLP:poly-L-lysine, respectively.

UV–VIS Absorption Spectra.—A study of the absorption spectrum of the reaction mixture (PLP–poly-L-lysine) in the 250–500 nm interval was carried out. The spectrum shows bands at 412 and 275 nm corresponding to ketoenamine tautomer and a band at 325/335 nm close to the wavelength of the enolimine in related Schiff bases.[14,15] At pH < 6.5 a shift from 412 to 395 nm and a small increase in the absorbance indicates the influence of the free aldehyde PLP (390 nm). At 6.5 < pH < 11, the influence of free PLP seems to be negligible. In Fig. 3 are shown some spectra corresponding to this pH interval. The absorbances at 412 and 335 nm, increase and decrease, respectively as the pH increases. The second band shows a shift from 335 nm to 325 nm. The variations resemble acid–base dissociation curves. In a piperazine/HEPES buffer inflection points are found between 7.6–7.8. In phosphate buffer a pK value close to 7.5 was obtained.

The absorbance at 275 nm shows a similar variation in this pH interval. However, in an acid medium and in a strong basic medium, inflections at pH 5.5 and 12.1 were obtained. This indicates a decrease in the Schiff base formation and should be related to the acid–base equilibrium of the heterocycle and the imine. At this wavelength no influence of the PLP is observed. The intermediate inflection is not observed in the Schiff base PLP:lysine.

A study of the absorption spectra of poly-L-lysine in the absence of the coenzyme in the interval 200–240 nm was also carried out. From the absorbance at 208 nm an inflection at pH 9.3–9.4 was obtained. An acid–base titration of the polymer yielded a pK value of 9.8 ± 0.1. These values are lower than the value of 10.2 observed in poly-L-lysine of high polymerization degree for the dissociation of the ε-amino group of the lysine residues. This behaviour can be explained by the influence of the terminal carboxylate in this case.[16]

Formation of the Carbinolamine.—Our results are in agreement with the formation of a Schiff base in aqueous solution (band at 412 nm).[8,12,14,15] However, the additional

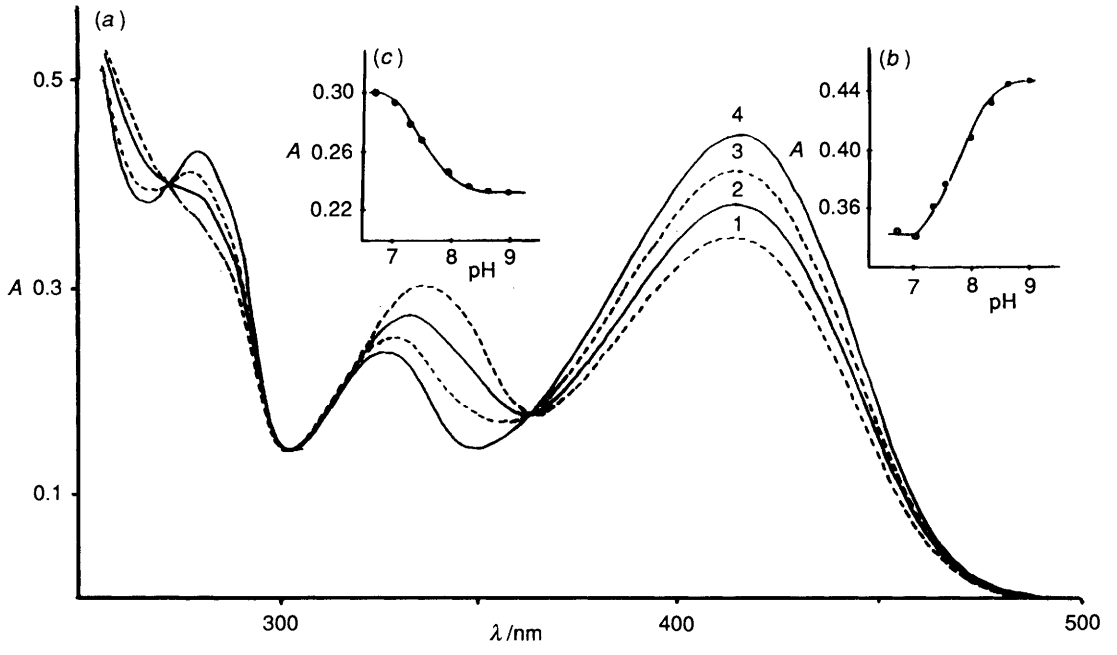
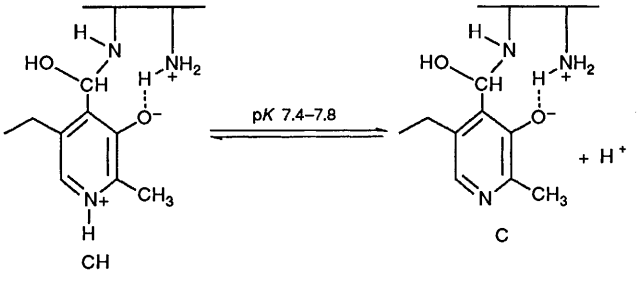


Fig. 3 UV-VIS absorption spectra. Initial concentrations: PLP 1 × 10^{-4} mol dm^{-3}; poly-L-lysine 1.18 × 10^{-4} mol dm^{-3}. (*a*) Absorption spectra 1–4, pH: 7.03, 7.50, 7.94 and 8.62. (*b*) Absorbance (412 nm) *vs.* pH. (*c*) Absorbance (335/325 nm) *vs.* pH.

absorption band at 335/325 nm suggests that an appreciable amount of the carbinolamine intermediate or enolimine form of the Schiff base is also formed. This tautomeric form has been observed in appreciable concentrations in Schiff bases of PLP even in polar media.[14,15] The high apparent formation constant of the Schiff base PLP:poly-L-lysine should be related to either the presence of this intermediate or a shift in the microenvironment of the coenzyme, favouring the enolimine. In fact, the equilibrium concentration of a possible intermediate is not considered in the calculation according to eqn. (6). However, the second reduction wave, i_{L2}, gives the concentration of the free aldehyde and, by subtracting from the initial concentration of PLP, the amount of combined coenzyme as Schiff base in all its forms. The stabilization as carbinolamine under determined conditions could explain the low concentration of PLP in aqueous solution in the reaction mixture with polylysine in comparison with PLP:hex or PLP:lys mixtures. Since the carbinolamine is a non-electroactive species its electroreduction can only be achieved indirectly by previous transformation of the Schiff base or PLP respectively. The first possibility is not probable since the limiting current of the wave is diffusion controlled in the pH interval when the above behaviour is observed and this indicates that no chemical reaction prior to the electrode process is observed in the window time of polarography. The second possibility, *i.e.* an hydrolysis to yield PLP is unreasonable since a low equilibrium concentration of free PLP (measured as i_{L2}) was observed.

Therefore, our results suggest a stabilization of the species CH (protonated on the ring nitrogen) because of a withdrawing effect of the lysine side chains (Scheme 4) (hydrogen bonding with a neighbouring lysine residue could be involved). Pyridoxal-phosphate derivatives with sp^3 hybridization of the C'_4 carbon have absorption maxima at 325/310 nm for protonated and unprotonated forms of the heterocycle, respectively (for example, hydrate of PLP and pyridoxamine-5′-phosphate[15,17]). The observed shift from 335 to 325 agrees with the variation in these derivatives.

Moreover, the Schiff bases derived from PLP show a p*K* for the heterocycle of *ca.* 5.5–6.5,[14,15] lower than the same p*K* in PLP (8.4–8.6). This fact is related to the C'_4 atom which has configurations sp^2 and sp^3 in the Schiff base and PLP



Scheme 4

respectively. According to these results the p*K* values at 7.6 and 5.5–6 (from UV–VIS and electrochemical results) can be assigned to the dissociation of the heterocycle of the carbinolamine and of the Schiff base, respectively.

Above pH 7.8, the stability of the species C of the carbinolamine shows a relative decrease in comparison with the species SH of the Schiff base. This fact agrees with the stabilization of species SH (Scheme 2) observed in Schiff bases of PLP.[11,14,15]

The above conclusion is in agreement with an acid–base equilibrium of the carbinolamine species coupled with a third-species (Schiff base) for the model PLP:polylysine. This represents a difference in behaviour from other Schiff bases where no appreciable concentrations of carbinolamine were observed.[11,14,15] This fact would explain the variation of the spectra in the pH interval 6–10.5 in this case.

Tobias *et al.*[14,15] have established that the stability maximum for a PLP Schiff base lies in the pH range between the pK_a of the conjugate acid of the amine and the pK_a for the ionization of the last PLP ring proton. In the cases studied here, the intervals are 8.6–10.7, 8.6–10.2 and 8.6–9.8 for the Schiff bases PLP:hex, PLP-lys and PLP-poly-L-lysine, respectively. The values 9.5 and 8.9 for the hex and lys Schiff bases are in agreement with this rule. The value of 7.8 in the poly-L-lysine Schiff base is lower than the p*K* of the PLP. This behaviour, indicates that the maximum is not characteristic of a PLP Schiff base and suggests the influence of a different species, which could be the intermediate carbinolamine.

Table 1 Fluorescence parameters

Compound	λ_{exc}/nm	λ_{em}/nm	pK_{ap}	Ref.
PLP hydrate	320–310	401–363	8.50	This work
	330–315	400–370	8.75	17
PMP	327–310	392–370	8.6–8.9	19
PLP:poly-L-lysine	322–320	408–372	7.10	This work

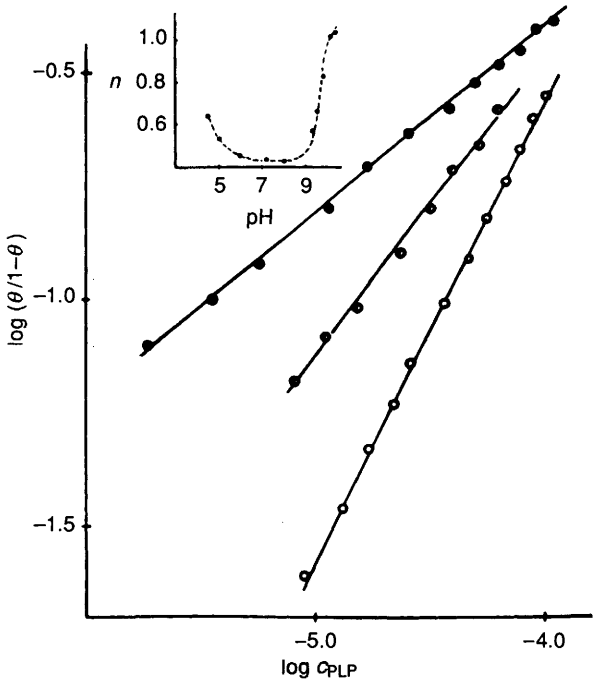


Fig. 4 Variation of log $\theta/(1-\theta)$ with log c_{PLP}. pH: ● 8.02; ◑ 9.5; ○ 10.15. Inset: Variation of n_H with pH.

Fluorescence Study.—Fluorescence emission of the reaction mixture by excitation at 415 nm was studied as a function of the pH. A maximum appears at pH 5.8, similar to that obtained in PLP:hex.[8] The results indicate that species bearing a protonated ring nitrogen are most fluorescent, as was shown for the Schiff base of PLP.[8,18] However, in acid medium a low fluorescence is observed because of the hydrolysis of the Schiff bases. An inflection at pH 6.6–6.7 is obtained from the variation of λ_{em} with pH. A shift from 490 nm (pH < 5) to 515 nm (pH > 8) is obtained. In the same interval, the PLP:hex Schiff base shows a shift from 490 nm to 525–535 nm.

Fluorescence emission by excitation at 325/335 nm has also been studied. An inflection close to 7 is obtained. A shift from 408 nm (pH < 6) to 372 nm (pH > 8) is observed. Moreover, the excitation spectrum shows a band at 322 nm (pH < 6) and 320 nm (pH > 8). In Table 1 are shown results of the fluorescence of coenzymes PLP and PMP.[17,18]

These results correspond to species with a sp^3 C_4' carbon atom. From this comparison, the fluorescence by excitation at 325/335 nm of the PLP:poly-L-lysine mixture should be assigned to an intermediate with sp^3 configuration, such as carbinolamine. The possibility of a geminal diamine is not considered since the intermediate species is favoured as the amine group is still protonated. The geminal diamine has been observed in kinetic studies of the reaction of fully unprotonated diamine with PLP.[20]

Binding Constant and Stoichiometry.—A quantitative treatment of the interaction of PLP with poly-L-lysine was carried out on the basis of the electrochemical study. The Scatchard plot in this case is curved concave upwards. For the simple case of one class of identical sites (ε-amino group) this indicates an anticooperative interaction between sites. A characterization of the interaction was carried out by the semi-empirical Hill's approach using eqn. (7) where θ is the ratio of the ligand

$$\frac{1}{1-\theta} = K^{n_H} c_{PLP}^{n_H} \qquad (7)$$

concentration bound to polymer to the concentration of initial lysine residues, K is the binding constant and n_H the Hill constant. In Fig. 4 is shown log $\theta/(1-\theta)$ *vs.* log c_{PLP} at different pH. Linear relations are obtained and from the slope and the intercept the value of K and n_H may be calculated. A plot of n *vs.* pH is also shown in Fig. 4. In the 6 < pH < 8.5 interval a constant value of n lower than unity (average 0.43) is obtained. This value indicates negative cooperativity. Under such conditions, the binding of each substrate molecule (PLP) decreases the intrinsic affinities of the vacant sites. At pH > 10, the value of n is close to unity indicating no cooperativity. These results are in agreement with an interaction of the *o*-hydroxy group with protonated lysine residues, favouring the intermediate species, although interaction of the phosphate could also be possible, yielding a multiple interaction between polymer and ligand.

The results and conclusions reached in the study of the Schiff base of PLP:polylysine may help in the understanding of the binding site, and the method could be applied in the study of the catalytic role of the coenzyme.

Finally, the involvement of an intermediate carbinolamine or enolimine species was elucidated from the above results, although no direct structural evidence could be found. However, experimental differences concerning the variation with pH of the 412 and 335/325 nm absorption bands in comparison to the Schiff bases of simple amines or amino acids were observed. If a carbinolamine species is assumed to be involved, a reinvestigation of some enzymes would be appropriate to clarify if this intermediate plays a role in the enzymatic catalysis.

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