The Schiff Base between Pyridoxal-5'-Phosphate and Hexylamine. Equilibria in Solution

Manuel Blazquez,* Jose M. Sevilla, Juana Perez, Manuel Dominguez,† and Francisco Garcia-Blanco‡
Departamento de Química Física y Termodinámica Aplicada, Facultad de Ciencias, Universidad de Córdoba, 14004 Córdoba, Spain

Electrochemical and spectroscopic studies of the pyridoxal-5'-phosphate–hexylamine Schiff base (PHSB) over a wide pH range have been carried out. This compound has been used as a simple model of the binding of pyridoxal-5'-phosphate (PLP) to protein. In this work different equilibria involved in the formation reaction have been considered and a quantitative distribution of the species in solution has been obtained. The method is based on the analysis of the reduction wave and absorption bands of the PHSB. Protonation equilibria constants (PHSB pK) have been obtained. Absorption spectra as a function of the medium composition have been resolved by a log normal distribution. Tautomeric equilibria have been considered and microscopic pK have been evaluated. Fluorescence results show that Schiff-base species bearing a protonated ring nitrogen are the most fluorescent. However, the fluorescence decreases in an acid medium due to an hydrolysis reaction. An important conclusion of this investigation is that the combined use of electrochemical and spectroscopic techniques is a valuable tool for the quantitative characterization of the pyridoxal-5'-phosphate Schiff bases.

Pyridoxal-5'-phosphate (PLP) binds to protein by the formation of Schiff bases.[1–4] To clarify the catalytic behaviour of PLP it is important to establish whether there is a relationship between the enzymatic activity and stability of the imine bond. The Schiff bases of PLP with amino acids or amines have been shown to have formation constants dependent on the acidity and relative permittivity of the medium.[5–17] Stability profiles indicate that protonation equilibria can be responsible for this behaviour. Thus, the pK values of the Schiff base and the different molecular species occurring in solution must be clearly established.

It is widely known that PLP Schiff bases undergo hydrolysis in an acid medium.[12–16] Any study carried out in this medium should take into account all the species occurring in solution. Potentiometric and u.v.–visible measurements do not give unequivocal results owing to the influence of PLP and the amine, respectively.

Vitamin B_6 derivatives have been shown to exist in different tautomeric forms.[18–21] Absorption spectra are complicated by the occurrence of several very close bands. However, the u.v.–visible bands of compounds of the vitamin B_6 group can be described on the basis of a log normal plot.[22–25] In these cases, curve-fitting methods are a suitable tool for evaluation of the different molecular species.

A study of the model reactions between amines or amino acids and PLP to simulate the PLP site in the enzyme is limited by the obtaining of the equilibrium concentration of the species involved in the reaction. The quantitative distribution of species, mainly in conditions approaching physiological environments, is required to extrapolate the results to biological systems.

The adduct formed on reaction of PLP with hexylamine is one of the simplest Schiff bases used as a model to illustrated binding. The kinetics of hydrolysis were studied by a spectroscopic procedure.[13–16] In addition, electrochemical reduction was used to calculate the equilibrium formation constant.[26,27]

This paper deals with an electrochemical and spectroscopic study of the PHSB to obtain a quantitative distribution of the ionic species in solution. In our investigation the combined use of these techniques has been shown as a valuable tool for study of the equilibrium Schiff base pyridoxal-5'-phosphate and it is possible to extend their application to binding models of increasing complexity. Results, in progress, with poly-L-lysine support this conclusion.

Experimental

Pyridoxal-5'-phosphate was purchased from Sigma. All other chemicals were supplied by Merck and were of reagent grade. Acetate and phosphate buffer for pH <8.5 and phosphate and carbonate buffer for pH >8.5 were used. Ionic strength was adjusted with KCl. All measurements were made at 25 $\pm$ 0.1 °C.

DC and DP polarographic curves were recorded by means of a 626 Metrohm polarograph. A saturated calomel electrode was used as reference electrode. The working electrode was a mercury capillary. In DP polarography the drop time was 2 s, the pulse amplitude, ΔE, 10 mV and the pulse duration 60 ms. All measurements were made in a nitrogen atmosphere. Spectrophotometric measurements were performed on a Varian Cary 219 spectrophotometer with 1 cm quartz cuvettes.

The spectra of the Schiff base were recorded by using as blank a solution containing the equilibrium concentration of PLP. This concentration was obtained by DC or DP polarography [Figure 1(*a*)]. If reduction processes appeared very close a procedure based on the resolution of overlapped peaks in DP polarography was used[28,29] [Figure 1(*b*)].

The fluorescence spectra were recorded on an MPF 66 Perkin-Elmer Spectrophotofluorimeter furnished with a 150 W xenon lamp. The microcomputer used for the calculation was a 48K Apple II+.

The Schiff base was obtained by adding known amounts of hexylamine to PLP solutions of known concentrations. Thus

† Departamento de Química Física, Universidad de Sevilla, Prof. Garcia Gonzalez, 41071 Sevilla, Spain.
‡ Instituto Rocasolano, CSIC, 28006 Madrid, Spain.

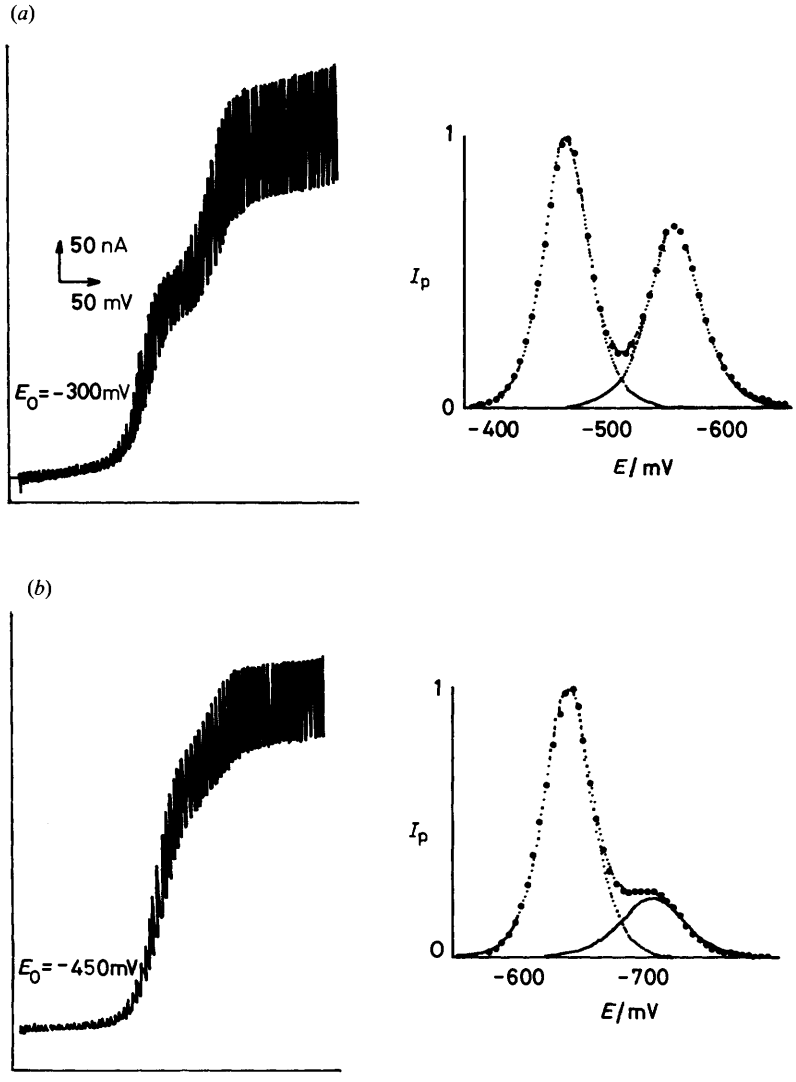


Figure 1. DC and DP polarography. (*a*) pH 4.0, 40% ethanol vol., at 2 000:1 mol ratio of hexylamine:PLP. (*b*) pH 6.0, 60% ethanol vol., at 300:1 mol ratio of hexylamine:PLP. DP polarography: ■ Experimental polarogram. · · · Theoretical profiles calculated according to ref. 28.

solutions with different molar ratios were prepared. The mixtures were kept in the dark to avoid photolysis reactions.[30]

The overall reaction between PLP and hexylamine can be represented by Scheme 1.

$$RCHO + R'NH_2 \rightleftharpoons RCH{=}NR' + H_2O$$

Scheme 1.

K_F, the apparent formation constant, is defined in equation (1),

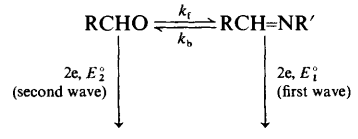
$$K_F = \frac{c_B}{c_P c_A} \qquad (1)$$

where c_A, c_B, and c_P are the equilibrium concentrations of amine, Schiff base, and PLP, respectively.

PHSB has four proton-accepting groups and therefore shows four macroscopic pK values. In addition, different tautomeric equilibria are involved and there are different neutral and ionic species in solution (Scheme 2).

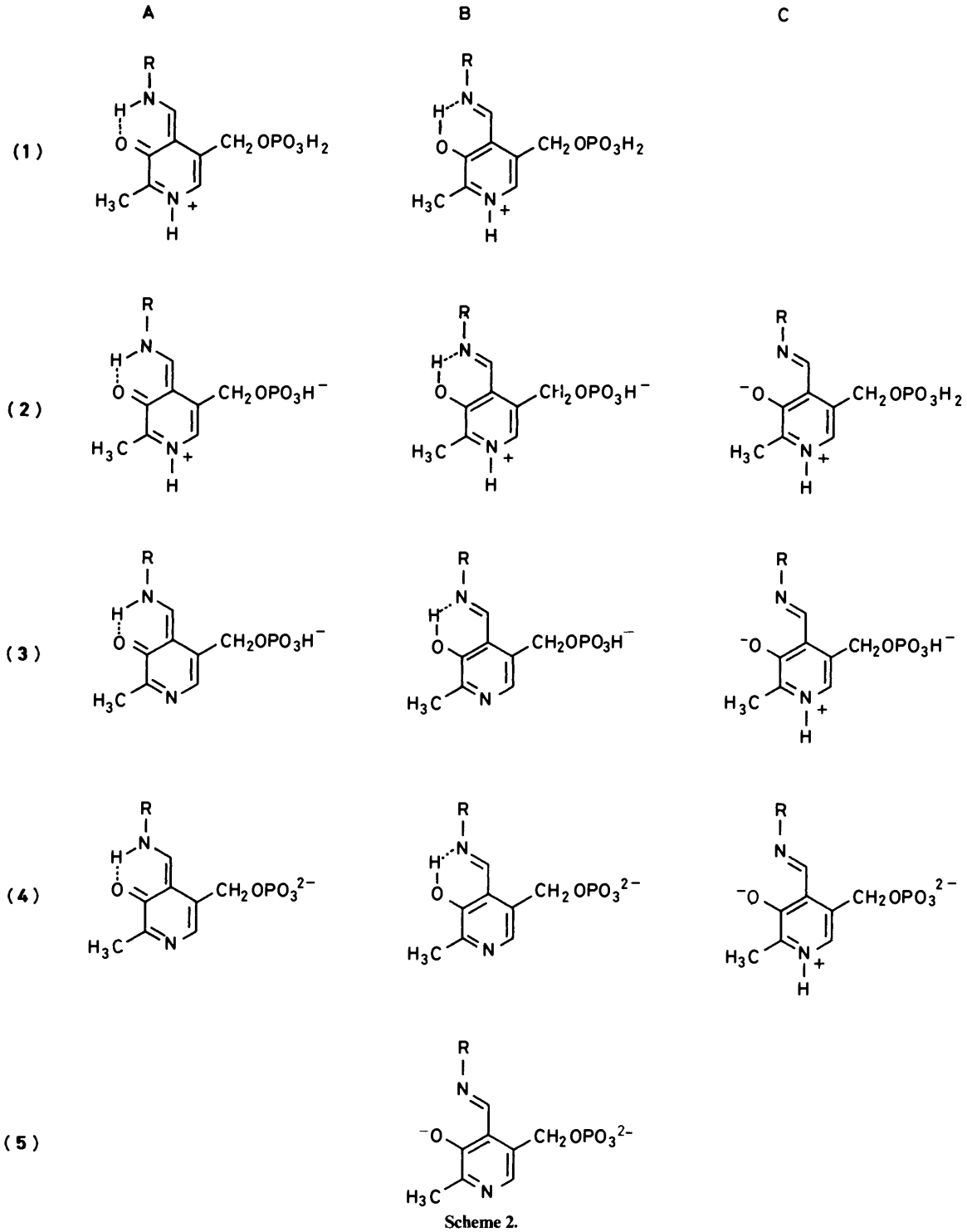
This reaction mixture shows, by DC polarography, one or two reduction waves.[31] This fact is a function of the experimental conditions, such as pH and PLP:amine ratio.

Under the experimental conditions of this work, a formation equilibrium due to pseudo-first chemical reactions can be assumed. The electrochemical behaviour is shown in Scheme 3.



Scheme 3.

The first wave corresponds to a two-electron reduction of the imine group of PHSB and is represented by the standard potential, E°_1. The second wave corresponds to a two-electron reduction of the carbonyl group of the PLP, at E_2°, E_2° being more cathodic than E_1°. If the term kt (where $k = k_f + k_b$ and t is the drop time in polarography) is sufficiently low, the perturbation of the equilibrium concentrations near the electrode surface due to the reduction of the Schiff base is not compensated for by the chemical reaction. In these conditions the process is controlled by diffusion of the Schiff base from the bulk solution. The experimental results at pH <11 (diffusion control in the limiting zone of the first wave) indicate such behaviour. In this limiting case, K (defined as $K = k_f/k_b = c_B/c_P = K_F c_A$) can be obtained from



Scheme 2.

equation[32] (2), where i_L and i_D are the limiting current of the

$$\frac{i_L}{i_D} = \frac{K}{K+1} \quad (2)$$

first wave and the diffusion current of the initial analytical concentration of PLP, respectively.

According to this approach an apparent formation constant, K_F, as a function of pH was reported.[26,27] This indicates that i_L is proportional to the equilibrium concentration of the Schiff base. Assuming that the diffusion coefficients of two electroactive species are the same, i_L can also be expressed by equation (3), where χ_B is the molar fraction of the Schiff base

$$i_L = i_D\chi_B \quad (3)$$

for all species in solution.

The normalized limiting current of the first wave coincides with χ_B as indicated in equation (4). Due to the proton-

$$I_L = \frac{i_L}{i_D} = \chi_B \quad (4)$$

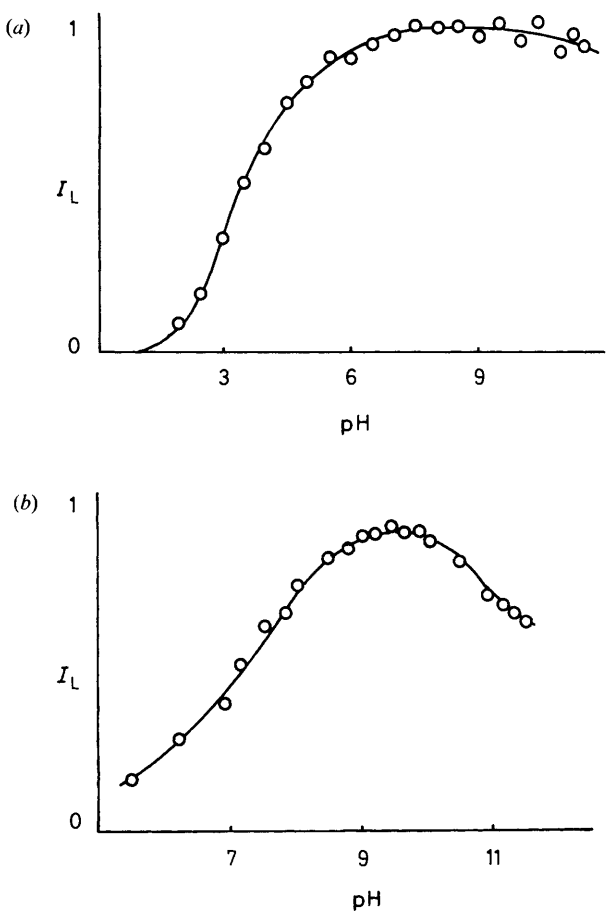


Figure 2. DC polarography. First wave. Plot of I_L *vs.* pH. Molar ratio hexylamine:PLP; (*a*) 2 000:1; (*b*) 40:1.

Table 1. Fitting results of the curves I_L *vs.* pH. Input parameters: pK_{P_i}, pK_N, and K_M. Optimized parameters: pK_{B_i}.

Input		Output	
pK_{P_1}	2.50	pK_{B_1}	2.80 ± 0.14
pK_{P_2}	3.80	pK_{B_2}	5.17 ± 0.06
pK_{P_3}	6.17	pK_{B_3}	7.37 ± 0.03
pK_{P_4}	8.44	pK_{B_4}	11.55 ± 0.01
pK_{P_N}	10.70		
pK_M	2.40		

accepting groups of the PLP, Schiff base, and hexylamine, different species are involved in the reaction in Scheme (1), depending on the pH. Therefore, K_F can be expressed by equation (5), where $[B_i]$, $[P_i]$, and $[A_i]$ represent any species

$$K_F = \frac{\sum_{i=1}^{5} [B_i]}{\left\{\sum_{i=1}^{5} [P_i]\right\}\left\{\sum_{i=1}^{2} [A_i]\right\}} = \left(\frac{S_B}{S_P S_A}\right) K_M \qquad (5)$$

according to the protonation equilibria of the Schiff base, PLP, and hexylamine, and S_B, S_P, S_A, and K_M are given for equations (6)–(9).

$$S_B = \sum_{i=1}^{4} \prod_{i=1}^{4} \frac{[H^+]}{K_{B_i}} + 1 \qquad (6)$$

$$S_P = \sum_{i=1}^{4} \prod_{i=1}^{4} \frac{[H^+]}{K_{P_i}} + 1 \qquad (7)$$

$$S_A = \frac{[H^+]}{K_N} + 1 \qquad (8)$$

$$K_M = \frac{[B_5]}{[P_5][A_2]} \qquad (9)$$

In equations (6)–(9), K_{B_i} and K_{P_i} are the macroscopic ionization constants of the Schiff base and PLP, K_N is the ionization constant of hexylamine, and B_5, P_5, and A_2 represent the most deprotonated species of the substances involved in the reaction in Scheme 1.

Finally, it is easy to derive equation (10) from equations (4) and (5):

$$I_L = \frac{c_A K_F}{1 + c_A K_F} \qquad (10)$$

where c_A is the molar concentration of free hexylamine, which in our experimental conditions ($c_A \gg c_P$) can be taken as the initial concentration of the amine.

In this work, equation (10) is used in relation to the polarographic results of the Schiff base and its application is given in the next section.

Results and Discussion

A polarographic study of the reaction of PLP with hexylamine was carried out over the entire pH range. The normalized limiting current, I_L, of the first reduction wave (Schiff base) was obtained [equation (4)]. Their variation with pH is shown in Figure 2 at two different molar ratios (amine:PLP). Diffusion control on the limiting current (pH <11) indicates that the kinetic effect of the reaction in Scheme 1 is not observed at the electrode–solution interphase. Therefore, in the experimental conditions equation (2) holds and the molar fraction of the Schiff base is given by equation (4).

In addition, a single wave is observed in this pH range for the reduction of the Schiff base. Thus, the fast tautomeric equilibria involved in solution (Scheme 2) are not detected in the electrode process.

The variation of χ_B with the pH is due to the different protonation equilibria involved in the reaction in Scheme 1. Equation (10) expresses a theoretical relationship between the normalized limiting current and these protonation equilibria. A fitting of this equation to experimental data was carried out by a computer. The procedure minimizes the sum of squares of deviation. Input parameters were K_{P_i} of the PLP,[33] K_N of the hexylamine[34] and the value of K_M[13,26] [see equation (9)]. Output parameters, K_{B_i} values of the Schiff base, were the same in both molar ratios (amine:PLP). These results are an estimation of the macroscopic ionization pK of the Schiff base (Table 1, Scheme 2). Other estimations have been reported using different methods.[13,14,26]

Taking into account these values, pH values which ensure 85% of a macroscopic protonation stage were chosen and u.v.–visible spectra were recorded for different ethanol–water compositions. Schiff base spectra, obtained as indicated in the Experimental section, showed several overlapping bands. We have resolved these spectra from a log normal distribution.[22–25,35]

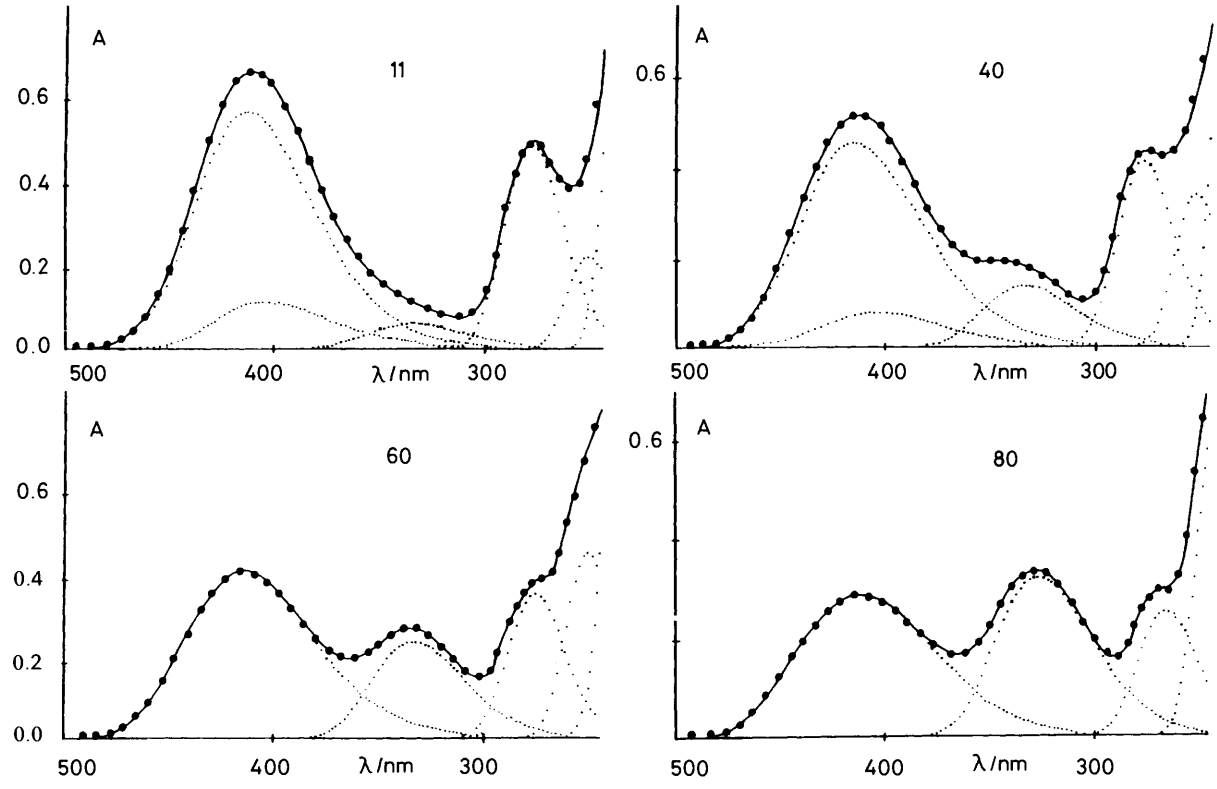


Figure 3. U.v. spectra of the Schiff base PLP–hexylamine in ethanol–water solutions. pH 6.0. ● Experimental spectrum; —— total absorbance (sum of the five log normal distributions); • • • contribution of each individual species. The ethanol content (v/v) is indicated in the spectrum.

Table 2. Molar absorptivity and molar area of the species of the Schiff base (see Scheme 2).

	A		B		C	
Species	ε_0/mol^{-1} dm^3 cm^{-1}	a_0/km mol^{-1}	ε_0/mol^{-1} dm^3 cm^{-1}	a_0/km mol^{-1}	ε_0/mol^{-1} dm^3 cm^{-1}	a_0/km mol^{-1}
(1)	—	—	—	—	—	—
(2)	11 765 ± 159	490 ± 18	3 945 ± 62	206 ± 15	—	—
(3)	13 388 ± 181	481 ± 18	3 625 ± 57	211 ± 16	4 726 ± 172	194 ± 21
(4)	8 475 ± 114	353 ± 13	4 320 ± 68	226 ± 17	6 400 ± 233	323 ± 36
(5)	7 500 ± 101	366 ± 20	4 235 ± 67	284 ± 21	—	—

A computer program was written to handle the curve-fitting procedure.[36] Input data are four parameters to describe an absorption band. These parameters are: absorption maximum wavelength (λ_i), absorbance maximum (A_i), half-width of the band (w_i), and the skewness of the band (b_i). The program minimizes the sum of squares of deviation and from the best-fit the output parameters are obtained. A comparison between experimental and theoretical spectra are shown in Figure 3, at pH = 6, and at different ethanol concentrations in solution.

Absorption bands were assigned to the ketoenamine species (415, 275 nm) and enolimine species (335, 250 nm) according to reported data[8,10,12,37] (see Scheme 2, species A and B respectively). The spectra were resolved by using five log normal curves. There are, in order of increasing energy, (*a*) band I of the ketoenamine species, (*b*) the band of the multipolar form (Scheme 2), species C), (*c*) band I of the enolimine species, (*d*) band II of the ketoenamine, and (*e*) band II of the enolimine species and uncharacterized absorption (zone < 250 nm). In this last region, the spectra were adjusted to avoid interference with other bands.

The parameters of bands 1 and 3 were obtained at high ethanol–water ratios in which they are the main absorption bands. Parameters of band 2 were obtained in aqueous media (low ethanol content).

This absorption can be ascribed to the multipolar species (iC) as was reported in analogous Schiff bases.[12] The band appears in polar media and at pH *ca.* 7.

The band area for the simple ionic form is not dependent on changes in the solvent composition, within experimental error.[38] However, our results show that the area of bands 1, 2, and 3 vary as a function of the ethanol–water composition. These results indicate that tautomeric equilibria between ketoenamine, enolimine, and multipolar species are involved (Scheme 2, *i*A, *i*B, and *i*C species).*

The molar area of an individual species, a_i°, is defined by the expression,[21,24,38]

$$a_i^\circ = a_i/\chi_i \qquad (11)$$

where a_i is the band area of the species i and χ_i is its molar fraction. The molar area of *i*A, *i*B, and *i*C species can be

* Throughout this paper, the prefix *i* in species A, B, and C refers to species at different stages of protonation.

calculated from the experimental area (curve resolution), taking into account the fact that $\chi_A + \chi_B + \chi_C = 1$. The data for molar area and molar absorptivity for different species and the standard deviations are listed in Table 2.

Mole fractions can be evaluated from equation (11) as a function of ethanol content. The results are gathered in Table 3. From these results and macroscopic pK values (Table 1), microscopic pK values between some species in solution can be evaluated. Values of pK corresponding to species in a neutral pH zone are given in Table 4. For example, a pK value of 6.9 ± 0.1 was calculated between species 3C and 4A (see Scheme 2) taking into account the third macroscopic pK of 7.37 ± 0.03 (Table 1).

Table 3. Molar fraction of the species of the Schiff base as a function of the ethanol–water composition (see Scheme 2).

	Species	χ_A	χ_B	χ_C
Aqueous medium	(1)	—	—	—
(11% ethanol vol.)	(2)	0.81 ± 0.04	0.19 ± 0.02	—
	(3)	0.57 ± 0.03	0.16 ± 0.01	0.27 ± 0.03
	(4)	0.73 ± 0.04	0.10 ± 0.01	0.17 ± 0.02
	(5)	—	—	—
(40%) ethanol vol.)	(1)	—	—	—
	(2)	0.68 ± 0.04	0.32 ± 0.03	—
	(3)	0.44 ± 0.02	0.36 ± 0.02	0.20 ± 0.03
	(4)	0.64 ± 0.04	0.35 ± 0.03	0.01 ± 0.001
	(5)	0.61 ± 0.03	0.39 ± 0.04	—
(60% ethanol vol.)	(1)	—	—	—
	(2)	0.58 ± 0.03	0.41 ± 0.05	0.01 ± 0.001
	(3)	0.39 ± 0.02	0.60 ± 0.06	0.01 ± 0.001
	(4)	0.51 ± 0.03	0.49 ± 0.05	—
	(5)	0.52 ± 0.03	0.48 ± 0.04	—
(80% ethanol vol.)	(1)	—	—	—
	(2)	—	—	—
	(3)	0.25 ± 0.01	0.75 ± 0.07	—
	(4)	0.40 ± 0.02	0.60 ± 0.04	—
	(5)	0.44 ± 0.02	0.56 ± 0.05	—

Fluoroscence techniques are widely used in protein studies. It is known that the Schiff base formed by PLP in protein (phosphorylase b, for example [3]), by excitation at 425 nm, shows a pH-dependent emission at 535 nm. This variation is similar to an acid-base titration curve. Understanding the role of the proton-accepting group in the coenzyme site requires parallel studies on model compounds. Thus, fluorescence studies of different Schiff bases were reported.[6,39–41] In this work, the fluorescence of PHSB was investigated. The study was carried out both in an aqueous medium and in ethanol–water solutions. In polar media the absorption spectra of the Schiff base show a main band at 415 nm. This band corresponds to ketoenamine species (Scheme 2); an excitation wavelength of 415 nm was therefore used. Different molar ratios amine: PLP were studied. Our results show that fluorescence is pH-dependent (Figure 4).

In solutions of ethanol (60%, v/v), an analogous variation of fluorescence intensity and emission were observed. However, in these conditions, a lower intensity was obtained. The maximum wavelength varies in the range 490–530.

This behaviour can be explained by taking into account the protonation equilibria of the Schiff base. A theoretical expression of the fluorescence as a function of pH was derived. In this approach, the observed fluorescence intensity is considered to be due to the contribution of all fluorescent species in solution. There are five species of the Schiff base related *via* macroscopic ionization equilibria. Therefore, the fluorescence intensity can be expressed as equation (12),

$$F = \sum_{i=1}^{5} F_i \qquad (12)$$

where

$$F_i = q_i[B_i] \qquad (13)$$

and

$$q_i = I_0 f(\theta) g(\lambda) \xi_{\lambda exc} \varphi_i l \qquad (14)$$

Table 4. Microscopic pK values of the some of the Schiff base species depicted in Scheme 2.

Equilibrium species	pK_{B_i}	pK_{AA}	pK_{AB}	pK_{AC}	pK_{BA}	pK_{BB}	pK_{BC}	pK_{CA}	pK_{CB}	pK_{CC}
(2) ⇌ (3)	5.17 ± 0.06	5.3 ± 0.1	5.9 ± 0.1	5.6 ± 0.1	4.7 ± 0.1	5.2 ± 0.2	5.0 ± 0.2	—	—	—
(3) ⇌ (4)	7.37 ± 0.03	8.5 ± 0.1	8.1 ± 0.1	7.9 ± 0.1	6.7 ± 0.1	7.5 ± 0.1	7.3 ± 0.1	6.9 ± 0.1	7.7 ± 0.1	7.6 ± 0.1

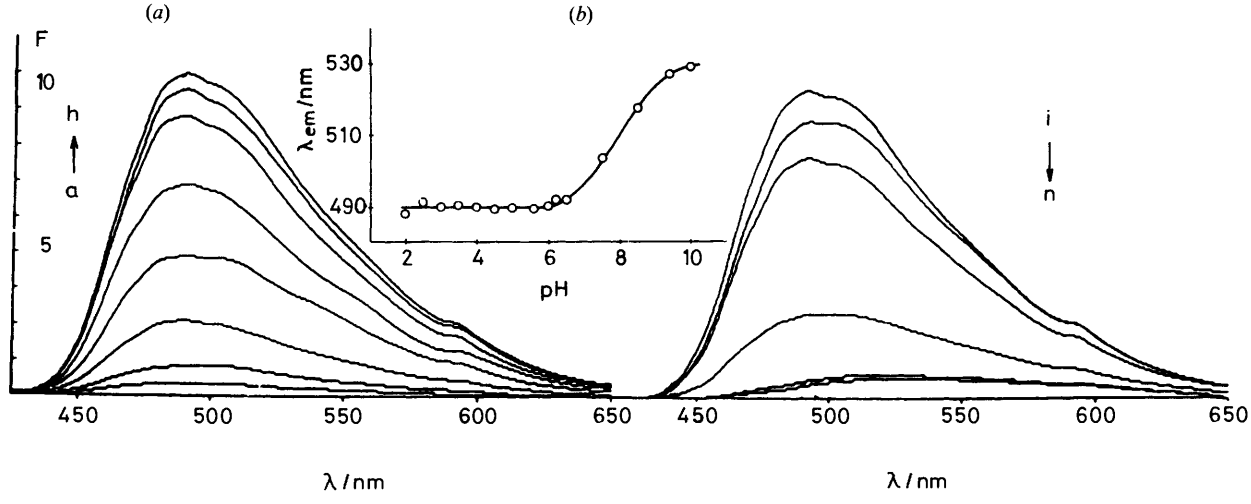


Figure 4. Fluorescence of the Schiff base PLP:hexylamine. Influence of pH. λ_{exc} = 415 nm, $c_P = 10^{-5}$ mol dm^{-3}. Molar ratio hexylamine:PLP (2 000:1). (*a*) Spectra at pH: (a) 2.0, (b) 2.5, (c) 3.0, (d) 3.5, (e) 4.0, (f) 4.5, (g) 5.0, (h) 5.5, (i) 6.0, (j) 6.2, (k) 6.5), (l) 7.5, (m) 8.5, and (n) 9.4 (*b*) Emission wavelength.

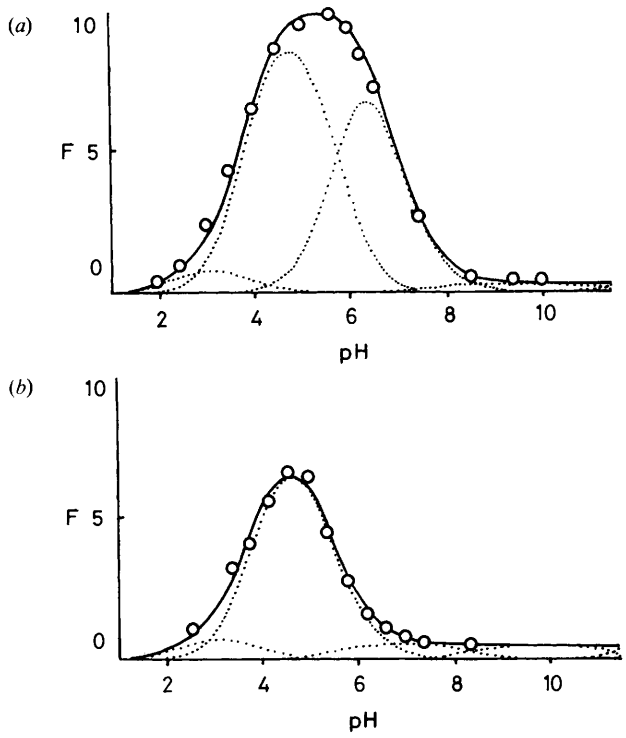


Figure 5. Comparison of experimental and calculated fluorescence intensity. Influence of the pH. ○ Experimental values; —— theoretical values calculated from equation (15); ... contribution of the species of the Schiff base. (*a*) Aqueous media. (*b*) 60% ethanol vol.

Table 5. Parameters of the curve-fitting fluorescence *vs.* pH.

pK_{B_1}	pK_{B_2}	pK_{B_3}	pK_{B_4}	
2.80 ± 0.15	5.24 ± 0.02	6.86 ± 0.02	11.57 ± 0.02	
Species	(1)	(2)	(3)	(4)	(5)
$q_i \times 10^{-4a}$	500.0	500.0	151.0	2.30	2.30
Standard deviation	±0.2	±0.2	±0.1	±35	±82

[a] Values obtained with the concentration expressed in mol dm^{-3}.

where I_0 is the intensity of the exciting radiation, $f(\theta)$ is the geometric factor, $g(\lambda)$ is the detector response, $\xi_{\lambda exc}$ is the molar absorptivity at the exciting wavelength, φ_i is the quantum efficiency of species i, l is the sample path length, and $[B_i]$ is the molar concentration of species i of the Schiff base. From equations (12)–(14), and taking into account equations (5)–(9), equation (15) can be easily derived.

$$F = \frac{K_F c_P c_A}{1 + K_F c_A}\left(\frac{S_F}{S_B}\right) \qquad (15)$$

where

$$S_F = \sum_{i=1}^{4} q_i \prod_{j=1}^{4} \frac{[H^+]}{K_{B_i}} + q_5 \qquad (16)$$

c_P is the concentration of PLP and other symbols used have the aforementioned meaning. Equation (15) is valid assuming that there is no substance with emission by excitation at 415 nm, other than the Schiff base, and that there is not pH-dependent buffer quenching. These statements were verified for our experimental conditions.

Equation (15) gives a relationship between the fluorescence intensity and equilibria involved in the formation of the Schiff base (**1**). This equation was fitted to the experimental data of Fluorescence *vs.* pH. Input parameters were the experimental conditions, c_P and c_A, and the pK values of PLP and hexylamine (pK_{P_i}, pK_N)[33,34]. In both media good agreement is observed between the theoretical and experimental fluorescence (Figure 5). The best fits were obtained using the parameters given in Table 5.

The values of q_i obtained indicate that Schiff base species (**1**)–(**3**), *i.e.*, species bearing a protonated ring nitrogen, are the most fluorescent (Scheme 2). A similar conclusion was obtained in other studies of the vitamin B_6 group.[42] These results show some differences between second and third pK values (Table 5) and macroscopic pK (Table 1). However, these pK values are very similar to some microscopic pK values given in Table 4 ($pK_{2A/3A} = 5.30 \pm 0.1$, $pK_{3C/4A} = 6.9 \pm 0.1$). Species 1A and 2A are responsible for fluorescence in an acid medium. However, low fluorescence is observed due to the low stability of the Schiff base in this medium as is shown by the electrochemical study (Figure 2). In the pH range 5–7 a fluorescence intensity maximum is observed. This variation is the result of an increase in the stability of the Schiff base as the pH increases. In weak basic media the concentration of species 2A is negligible. Therefore, the observed fluorescence is due to the multipolar species (*i*C) for which the absorption maximum is *ca.* 400 nm. In basic media the concentration of these species decreases as the pH increases, as indicated by the absence of the corresponding band in the absorption spectra. Schiff base stability increases as the ethanol content increases in the solution.[27,43] However, a shift towards enolimine species occurs as the polarity of the solvent decreases[8,10,12] (*i.e.* from *i*A to *i*B species in Scheme 2). The experimental behaviour shows a decrease in the fluorescence intensity as compared with aqueous media. A fluorescence maximum appears at pH *ca.* 5, where a compensation between the increase in its stability and the decrease of the concentration of the fluorescent species 2A is obtained (Scheme 2). In less polar media the multipolar species is not detected in the pH range studied and this explains the decrease in fluorescence, even in a weakly acidic medium.

On the other hand, fluorescence results agree with the u.v. study. At pH 6, excitation fluorescence spectra in ethanol solution (60%, v/v), show a band at 415 nm due to the ketoenamine species (*i*A). However, in aqueous solution the maximum wavelength appears at 408 nm, showing the contribution of both ketoenamine (415 nm) and multipolar (404 nm) fluorescent species.

In conclusion, the quantitative characterization of the adduct PLP–hexylamine was obtained. Data for the species in solution are given in the Tables. In all cases the standard deviation of the parameters obtained from the fitting of the experimental data to theoretical models are indicated. In general the standard deviations in the spectra deconvolution were <0.01 when *ca.* 100 values of wavelength were fitted. Deviations in the parameters of the Schiff base species were determined mainly by experimental error. Data corresponding to the species present to a lesser extent show an appreciable deviation due to the inaccuracy of the parameters obtained. In these cases the error can be minimized in part by an adequate selection of experimental conditions.

The combined use of electrochemical and spectroscopic techniques is shown as a valuable alternative to the study of binding models. The quantitative characterization is useful in explaining the shift observed in the environments in which this coenzyme acts. Although the Schiff base studied here is the simplest model of PLP binding, the method developed is of general application. Our future goal is to apply it in models of increasing complexity, approaching physiological environ-

ments. In this sense, some studies of PLP to poly-L-lysine binding seem to confirm the potential of the method.

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