Band-shape Analysis and Resolution of Electronic Spectra of Pyridoxal 5'-Phosphate with Amino Acids

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Formation equilibria of Schiff bases of pyridoxal 5'-phosphate (PLP) and different amino acids have been studied over a wide range of pH. The acid dissociation constant and absorption spectrum of every ionic species have been calculated. The latter has been resolved into components with log-normal curves, to provide a precise description of the band shapes, the peak positions and the area under the curves, which allowed the estimation of tautomerization constants and microscopic acid dissociation constants. Because of the acidity of the α-hydrogen of amino acids, α-amino acid Schiff bases are in equilibrium with quinonoid forms which are hydrolysed to yield pyridoxamine 5'-phosphate (PMP). This process could explain the bathochromic shifts of the peak position in α-amino acid Schiff bases to the corresponding band in non-α-amino acid systems.

Pyridoxal 5'-phosphate (PLP) and enzymes requiring it as a cofactor carry out a wide variety of chemical transformations of amino acids that rely on the ability of the cofactor to act as an electron sink, temporarily storing electrons that are later used for the formation of new bonds. The enzymes have several common mechanistic and stereochemical features, many of which can be duplicated in enzyme-free chemical models.[1,2]

A great deal of information about the enzyme mechanism has been obtained from the application of multifarious physico-chemical methods, particularly optical methods.[3,4] These methods have also been used for the clinical analysis of vitamin B_6 analogues.[5] Detailed studies have established that the absorption spectra of these compounds are pH dependent, and have also determined the equilibrium constants between different ionic and tautomeric forms.[6–9]

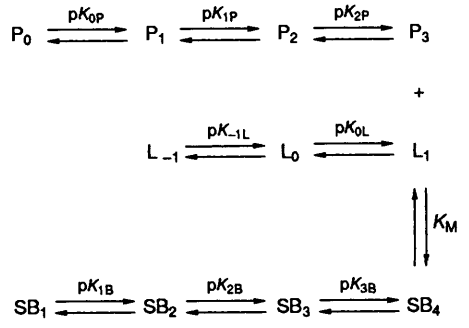
The formation of Schiff bases of PLP and related aldehydes with various amino acids and amines has been recently reported.[10–15] The resulting spectra were analysed with log-normal curves to evaluate tautomeric equilibria and to determine a precise mathematical description of the electronic absorption spectra. The results of these studies show that absorption maxima and band-widths of some tautomeric forms of Schiff bases of α-amino acids are slightly different from those obtained for the Schiff bases of amines or other (non-α) amino acids.

To explain these results the spectra of four Schiff bases of PLP with different amino acids have been analysed: three with α-amino acids, the other with an ε-amino acid. This study has allowed us to characterize the absorption bands of the different tautomeric forms of such Schiff bases. In addition, the differences between the spectra of α- and non-α-amino acid Schiff bases have been explained.

Experimental

Glycine (Gly), L-leucine (Leu), L-isoleucine (Ile) and ε-aminocaproic acid (CA) were purchased from Sigma. Pyridoxal 5'-phosphate (PLP) and all other chemicals were reagent grade and purchased from Merck. Dioxane was purified by being refluxed over sodium and distilled, such that a peroxides test (2% KI) on the freshly distilled dioxane was negative.

The ionic strength was maintained at 0.1 whenever possible using an appropriate buffer (acetate, phosphate or carbonate). Higher pH values were obtained using NaOH alone. Over a range near their pK values the amino acids themselves served as the buffer. Solutions containing a very high concentration of the amino acid component (up to 0.8 mol dm^{-3}) were sometimes needed to permit evaluation of spectra of the most protonated and completely dissociated species of the Schiff base. Although these solutions exceeded the ionic strength limits set, their inclusion in the data set caused little distortion in any of the pK values other than that in the pH 12 range.



Scheme 1 Ionic species in solution for amino acid–PLP Schiff bases. P = PLP; L = amino acid; SB = Schiff base.

The solutions were allowed to equilibrate after mixing for *ca.* 25 min. Complete spectra of the solutions were then recorded on a Zeiss DMR11 spectrophotometer. Data were collected in the range ν = 20 000–38 000 cm^{-1} (λ = 500–263 nm). Absorbances were normally recorded to the nearest 0.001 unit at intervals of 50 or 200 cm^{-1}. The reference cell was filled with a solution containing all of the components except the aldehyde.

The fluorescence spectra were recorded on an MPF 66 Perkin-Elmer spectrofluorimeter furnished with a 150 W xenon lamp. The pH measurements were carried out with a Crison pH meter, using a Metrohm EA 120 combined electrode previously calibrated with aqueous buffers at 25 °C.

The overall reaction between PLP and amino acids can be represented by eqn. (1). The equilibrium constant corresponding

$$\mathrm{RCHO} + \mathrm{R'NH_2} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{RCH{=}NR'} + \mathrm{H_2O} \qquad (1)$$

to each pH, K_{pH}, was calculated as the ratio k_1/k_2. The methods used to determine k_1 and k_2 values are described in detail in refs. 13 and 14, and values for these rate constants are given in ref. 15.

The ionic species existing in solution in the pH range studied are shown in Scheme 1, in which P, L and SB represent PLP,

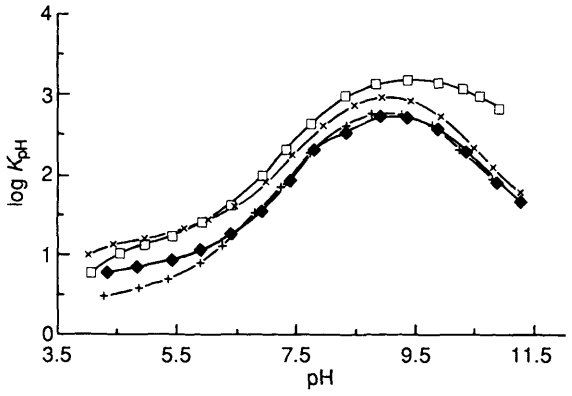


Fig. 1 Variation of log K_{pH} as a function of pH for the Schiff bases of PLP and: (□) ε-aminocaproic acid; (×) L-isoleucine; (◆) L-leucine; (+) glycine. The points are experimental values and the lines are the theoretical fitting to eqn. (2).

Table 1 Formation constants and pK values for PLP and Schiff base formed from amino acid

	pK values		
Amino acid	Amino acid	Schiff base	log K_M
Gly	9.76		1.33
	2.34		
pK_{3B}		11.35	
pK_{2B}		6.36	
pK_{1B}		5.46	
CA	10.45		2.07
	4.07		
pK_{3B}		11.70	
pK_{2B}		6.45	
pK_{1B}		5.69	
Leu	9.76		1.19
	2.31		
pK_{3B}		11.61	
pK_{2B}		6.55	
pK_{1B}		5.68	
Ile	9.77		1.31
	2.32		
pK_{3B}		11.57	
pK_{2B}		6.56	
pK_{1B}		5.67	

amino acid and aldimine, respectively, and the subscripts (0–4) indicate the number of net negative charges on the molecules. K_{-1L} and K_{0L} are the ionization constants of the amino acid. On the basis of this scheme, eqn. (2) can be obtained, where C stands for the proton concentration.

$$K_{pH} = \frac{\left(1 + \frac{C}{K_{3B}} + \frac{C^2}{K_{3B}K_{2B}} + \frac{C^3}{K_{3B}K_{2B}K_{1B}}\right) K_M}{\left(1 + \frac{C}{K_{0L}} + \frac{C^2}{K_{0L}K_{-1L}}\right)\left(1 + \frac{C}{K_{2P}} + \frac{C^2}{K_{2P}K_{1P}} + \frac{C^3}{K_{2P}K_{1P}K_{0P}}\right)} \quad (2)$$

The equilibrium constant can be also written as the ratio shown in eqn. (3), where $[SB]_e$, $[P]_e$ and $[L]_e$ are the total

$$K_{pH} = \frac{[SB]_e}{[P]_e \times [L]_e} \quad (3)$$

concentrations of Schiff base, PLP and amino acid at the equilibrium.

In the wavelength region of interest, the absorption of a mixture of PLP and its aldimine is due solely to the PLP and the Schiff base absorptions. At equilibrium the absorption at a particular wavelength is given by eqn. (4), where $\varepsilon_P(\lambda)$ and $\varepsilon_{SB}(\lambda)$

$$A(\lambda) = [P]_e\varepsilon_P(\lambda) + [SB]_e\varepsilon_{SB}(\lambda) \quad (4)$$

are the molar absorptivities of PLP and Schiff base, respectively.

Spectra of the Schiff bases were obtained by a computer-assisted method, fitting experimental results to eqn. (4). $[P]_e$ and $[SB]_e$ were calculated from eqn. (3) and $\varepsilon_P(\lambda)$ values were taken from Ref. 8.

Spectra were deconvoluted to log-normal curves using a method previously described by Metzler and co-workers.[7,8] Input data comprised four parameters: absorption maximum wavenumber, molar absorptivity maximum, width of the band and the skewness of the band. The program minimizes the sum of squares of deviation, and from the best fit the output parameters are obtained. From this fitting procedure the areas (integrated intensities) of the absorption bands of tautomeric forms could be estimated. Once the areas of the absorption bands (a_i) and the molar areas of each tautomer are known, the fraction of Schiff base present as each of the tautomeric forms can be easily calculated using the relationship $x_i = a_i/a_i^o$.

Results and Discussion

Formation Equilibrium Constants.—The formation equilibrium constants of Schiff bases of PLP and glycine, L-leucine, L-isoleucine and ε-aminocaproic acid were determined over a wide pH range. Fig. 1 shows the variation of these equilibrium constants with pH. The points represent the experimental values and the lines are theoretical functions obtained from the best fitting of experimental values to eqn. (2), using the constant values given in Table 1.

Functions plotted in Fig. 1 show the same qualitative behaviour for each of the four Schiff bases studied here. They show a maximum value lying between the pK value of the amine and the highest pK of the aldehyde, as in similar cases previously described.[10,13,14]

Quantitatively, the highest stability constants found are for the ε-aminocaproic Schiff base. The major structural difference between ε-aminocaproic acid and leucine or isoleucine is the carboxy group position. The α-position of the amino group seems to yield Schiff bases less stable than those obtained with amino acids in which the amine group is further from the carboxy group. Stability data obtained from PLP–n-hexylamine Schiff bases support this view.[13,14]

Factors other than the carboxy group position must also affect the relative stability of the Schiff bases of PLP and amino acids, because slight differences can be observed among the values of the equilibrium constants in Fig. 1. The explanation of these differences must come from kinetic studies on the formation and hydrolysis of the Schiff base, since the equilibrium constant can be described as the ratio between the formation and hydrolysis kinetic constants. Our kinetic studies on such systems showed that solvation effects on the imine bond affected its stability and that the presence of bulky non-polar groups surrounding the imine group stabilizes the linkage.[15]

In Table 1 the pK values of Schiff bases obtained from the best fitting of experimental data to eqn. (2) are given. The values obtained for the different Schiff bases are very close to each other and are not influenced by the side group in the amino acid moiety. pK_{3B} is due to protonation of the imine nitrogen.[16] pK_{2B} and pK_{1B} must correspond to ionization of pyridine

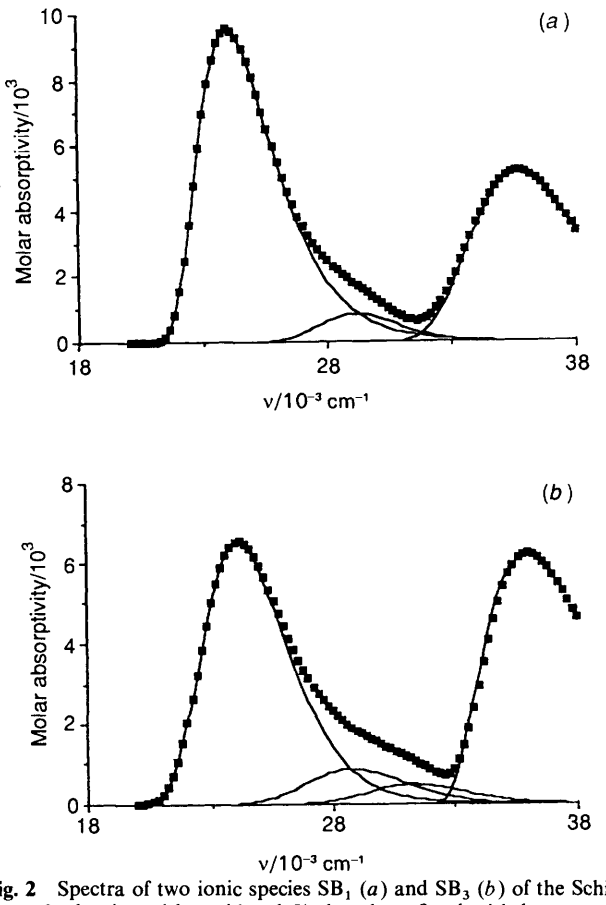


Fig. 2 Spectra of two ionic species SB_1 (*a*) and SB_3 (*b*) of the Schiff base of L-leucine with pyridoxal 5′-phosphate fitted with log-normal distribution curves

nitrogen and phosphate, respectively, as has already been pointed out elsewhere.[17]

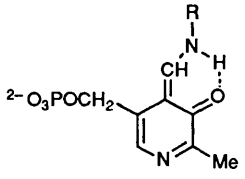
Band-shape Analysis.—Fig. 2 shows the calculated spectra of the different species of PLP–Leu Schiff base, as well as the log-normal curves obtained as described in the experimental section. The same procedure has been followed for the other Schiff bases, and in Table 2 the typical log-normal parameters of peak position, height, width, skewness and area are displayed. Concordances between data shown here and results for the glycine–PLP Schiff base previously published by Mitra and Metzler[11] are obvious, although these workers just considered three different protonated species.

The PLP Schiff bases exist as several tautomeric forms whose relative concentrations depend on pH and the medium polarity. Scheme 2 shows the structural formulae of these forms.[10]

At low pH in aqueous media the major tautomers are those which have their imine nitrogen protonated: **1a** and **2a** forms. Raman resonance spectroscopy experiments[18,19] have shown that the correct structure of **1a** and **2a** forms is the zwitterionic form. These tautomers have two absorption bands at 415–417 and 279–280 nm, the higher energy band having the greater area (see Table 2).

Bathochromic shifts of these bands with respect to the free aldehyde bands (λ390 and 250 nm) show the conjugation between the protonated imine nitrogen and the PLP ring. The shifting towards higher wavelength, as well as the high basicity of the imine nitrogen, have been attributed to the quinonoid resonance structure, which is usually referred to as the ketoamine.

The **1b** and **2b** tautomer bands present maxima at λ = 335–343 nm, close to the maximum wavelength of the free PLP enolic



Quinonoid form

form.[8] In aqueous media the area of this band is smaller than the area of the **1a** and **2a** bands, proving that in these conditions **1a** and **2a** are the major tautomers.

In the resolution of the spectrum of the SB_3 species, two minor bands at λ = 347 and 319 nm must be considered. Tautomer **3b** must be responsible for the absorption maximum at λ = 347 nm, since the corresponding species of the *N*-methyl-5′-deoxypyridoxal–valine Schiff base has its absorption maximum at a very similar wavelength. The **3c** form absorbs at λ = 319 nm, in agreement with the value of λ = 324 nm assigned to the similar tautomer of the salicylaldehyde–valine system.[21]

The least protonated species, SB_4, can exist as only one tautomer with an absorption maximum at λ = 345 nm, in agreement with a previous report.[10]

From the comparison of spectra of Schiff bases presented here it follows that the peak positions coincide in the four systems except when the imine nitrogen is not protonated. All the tautomers of α-amino acid Schiff bases, which have the imine nitrogen deprotonated, show bathochromic shifts in their bands with respect to the corresponding band of the PLP–CA system. Mitra and Metzler[11] have already reported a similar behaviour in PLP–TRIS [TRIS = tris(hydroxymethyl)aminomethane] and PLP–Gly Schiff bases. These authors suggested that the Schiff bases of TRIS form some kind of adduct at high pH, but they did not explain what happens at lower pH.

Because of the structure of α-amino acid Schiff bases, it is believed that this bathochromic shift could be due to three different causes: (i) formation of intramolecular hydrogen bonding between the phenolic group, the imine nitrogen and the carboxylate group; (ii) the presence of quinonoid forms and their hydrolysis to yield PMP; and (iii) the presence of some kind of adduct, especially at high pH.

The **4a** species, which is stable at high pH, cannot have intramolecular hydrogen bonding since neither the imine nitrogen nor the phenolic group is protonated. Furthermore, this species is not hydrolysed to yield PMP because this process is extremely slow at high pH.[22] On the other hand, because of the electronic spectra of the PMP trianion (bands at λ = 307 and 245 nm), the presence of this compound *should* produce a hypsochromic shift (from λ = 330 nm to lower wavelength), but we have obtained the opposite effect. For all these reasons, therefore, the shift in **4a** must be due to the presence of some kind of adduct whose formation is favoured at high pH, as Mitra and Metzler claimed.[11]

At lower pH (species **2** and **3**), the presence of such an adduct is ruled out, and hence the bathochromic shift must be due to intramolecular hydrogen bonding and/or quinonoid forms and their hydrolysis to yield PMP.

The possible intramolecular hydrogen bondings present in α-amino acid Schiff bases are shown in Scheme 3. There are three principal reasons why these interactions are considered not to be the cause of the observed bathochromic shifts. Firstly, these interactions should not be very important, owing to the free rotation of the carboxylate group. Secondly, if hydrogen bonding were the cause, the **1a**, **2a** and **3a** tautomers should also show this shift, and they do not. Thirdly, theoretical calculations[22] show that bathochromic shifts due to the formation of intramolecular hydrogen bonding should be *ca.* 0.15 eV (10 nm): such a shift has not been detected in the

Table 2 Positions and shapes of absorption bands of four PLP Schiff bases (SB) resolved with log-normal distribution curves

Schiff base	Band maximum nm	Band maximum $cm^{-1} \times 10^{-3}$	Height ($dm^3\ mol^{-1}\ cm^{-1}$)	Width ($cm^{-1} \times 10^{-3}$)	Skewness	Area ($mmol^{-1} \times 10^{-6}$)
Gly–PLP						
SB_1	417	24.00	9.23	3.71	1.60	380.6
	343	29.18	0.63	3.66	1.16	24.6
	280	35.76	4.88	5.13	1.36	271.4
SB_2	416	24.01	7.52	3.73	1.60	311.7
	343	29.12	0.41	3.63	1.19	15.9
	279	35.90	4.59	5.26	1.39	262.5
SB_3	415	24.10	6.30	4.11	1.39	281.5
	347	28.78	0.55	4.75	1.18	28.0
	319	31.32	0.50	4.60	1.16	24.6
	278	36.03	5.87	5.26	1.76	349.9
SB_4	345	28.94	4.66	5.95	1.30	299.1
CA–PLP						
SB_1	415	24.08	11.03	3.71	1.54	452.0
	334	29.96	2.24	3.92	1.16	102.3
	279	35.83	5.41	5.15	1.43	304.1
SB_2	417	24.00	9.03	3.71	1.40	364.6
	335	29.85	1.92	4.42	1.14	90.6
	279	35.86	6.81	5.15	1.45	383.5
SB_3	414	24.16	7.39	4.28	1.47	346.6
	338	29.60	1.11	4.75	1.19	56.5
	305	32.82	0.74	4.26	1.15	33.7
	277	36.09	6.94	5.39	1.89	383.5
SB_4	330	30.24	6.80	4.11	1.41	304.4
Leu–PLP						
SB_1	416	24.02	9.55	3.65	1.58	386.5
	343	29.14	0.83	3.72	1.16	33.0
	280	35.71	5.18	5.13	1.36	288.1
SB_2	417	24.00	8.12	3.69	1.57	331.9
	343	29.13	0.93	3.63	1.19	36.1
	279	35.83	6.23	5.20	1.37	351.6
SB_3	414	24.13	6.53	4.11	1.39	291.8
	347	28.78	0.87	4.75	1.18	44.3
	319	31.32	0.50	4.60	1.16	24.6
	277	36.05	6.15	5.26	1.76	366.6
SB_4	343	29.17	6.20	4.37	1.42	295.4
Ile–PLP						
SB_1	416	24.02	10.55	3.62	1.55	422.1
	344	29.07	1.07	3.72	1.16	42.6
	280	35.78	5.83	5.10	1.38	323.3
SB_2	416	24.02	8.62	3.71	1.60	355.4
	344	29.05	1.22	3.62	1.16	47.2
	280	35.76	6.61	5.25	1.35	375.9
SB_3	415	24.10	6.85	4.10	1.43	306.5
	347	28.82	0.91	4.72	1.19	46.0
	319	31.36	0.64	4.63	1.15	31.7
	278	36.03	6.45	5.28	1.79	387.4
SB_4	342	29.21	6.60	4.34	1.44	312.9

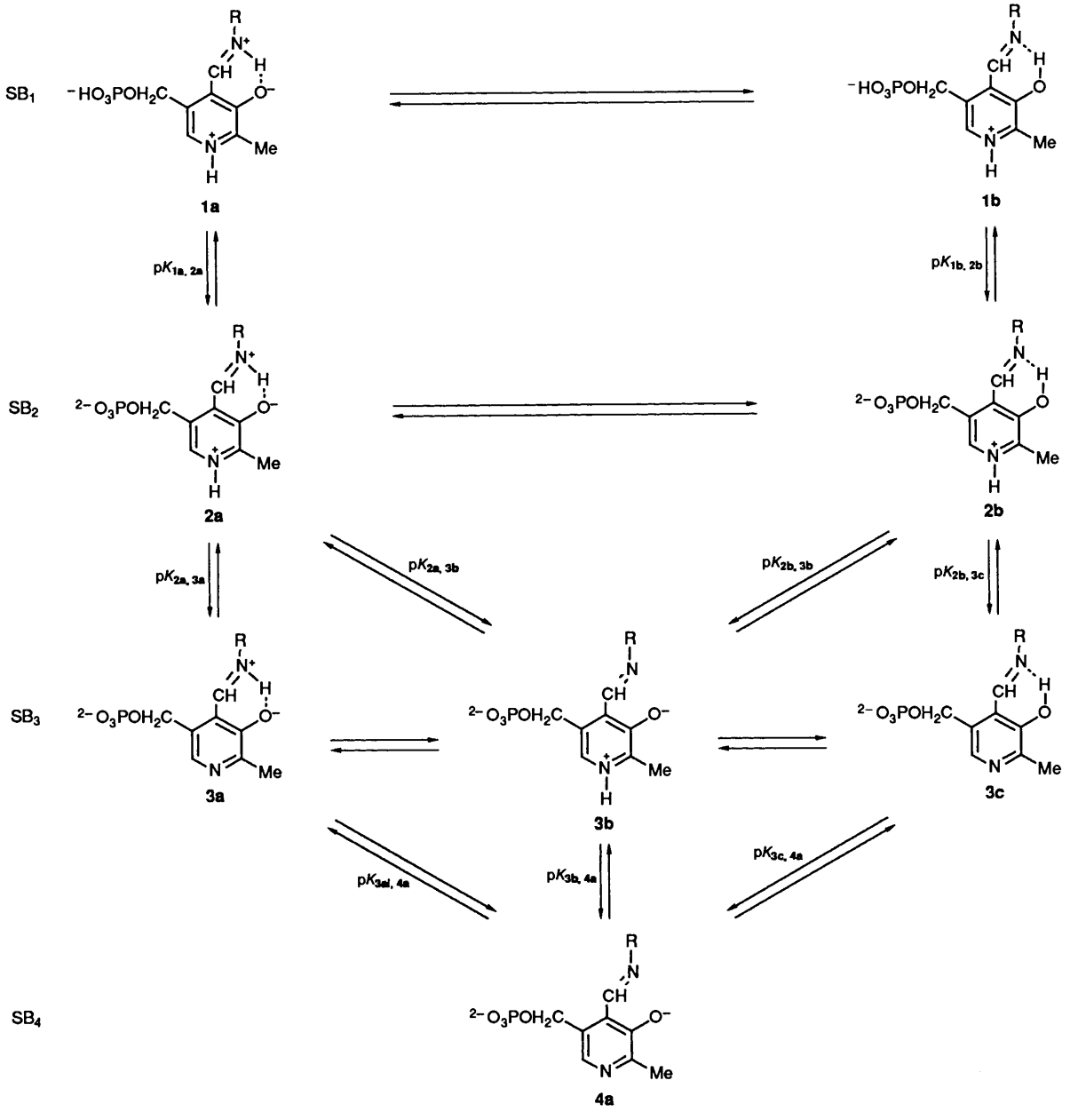
protonated imine nitrogen bands. Hence, the importance of the intramolecular hydrogen bonding on this shift must be ruled out.

Metzler and co-workers,[23] studying PLP–diethyl aminomalonate Schiff bases, postulated the existence of quinonoid species, which are favoured by the acidity of the α-proton of the carboxylate group. Such species have also been detected in the presence of metal ions.[24] It is well known that these species are quite unstable, undergoing hydrolysis to yield the ketoacidate ion and PMP. The mechanism of this reaction is shown in Scheme 4. We have observed that, once the α-amino acid Schiff bases have been formed, the intensity of the band at λ = 320–330 nm increases by *ca.* 20%. This is evidence for the formation of PMP, which has a band at λ = 328 nm with a high molar absorptivity. This effect has not been observed in PLP–CA Schiff bases.

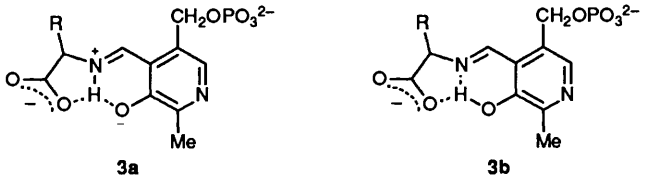
The existence of PMP has been corroborated by spectrofluorimetric measurements (Fig. 3). The emission spectrum of the PLP–CA Schiff base is similar to data obtained by Arrio-Dupont[25] for the n-butylamine–PLP system, which has a maximum at λ = 425 nm. The emission spectra of α-amino acid Schiff bases show a shift of the maximum, in the range λ = 425–410 nm. We can assume that these last spectra are the resultants of two different bands, one of them having its maximum at λ = 425 nm and being attributable to the Schiff base, the other one being due to PMP and having a maximum at 400 nm (see Fig. 3).

In summary, the bathochromic shifts of the bands of the α-amino acid Schiff bases are due to the formation of quinonoid forms and their subsequent hydrolysis to yield PMP at pH < 10, and the presence of adducts at higher pH.

Tautomerization Ratios and Microscopic Dissociation



Scheme 2 Structures of Schiff base tautomers



3a **3b**

Scheme 3 Possible intramolecular hydrogen bondings in α-amino acid–PLP Schiff bases

Constants.—To measure the equilibrium constants of tautomerization and microscopic pK values, it is necessary to know the molar area of Schiff-base tautomers. The band area of an ionic form is not dependent on changes in the solvent composition unless tautomeric equilibria are present in the middle. In this case, the molar area of an individual species is defined by eqn. (5), where a_i and x_i are, respectively, the band

$$a_i^0 = a_i/x_i \qquad (5)$$

area and the molar fraction of the i tautomer in the solvent used.

Taking into account the fact that $\Sigma x_i = 1$, eqn. (5) can be rewritten as eqn. (6).

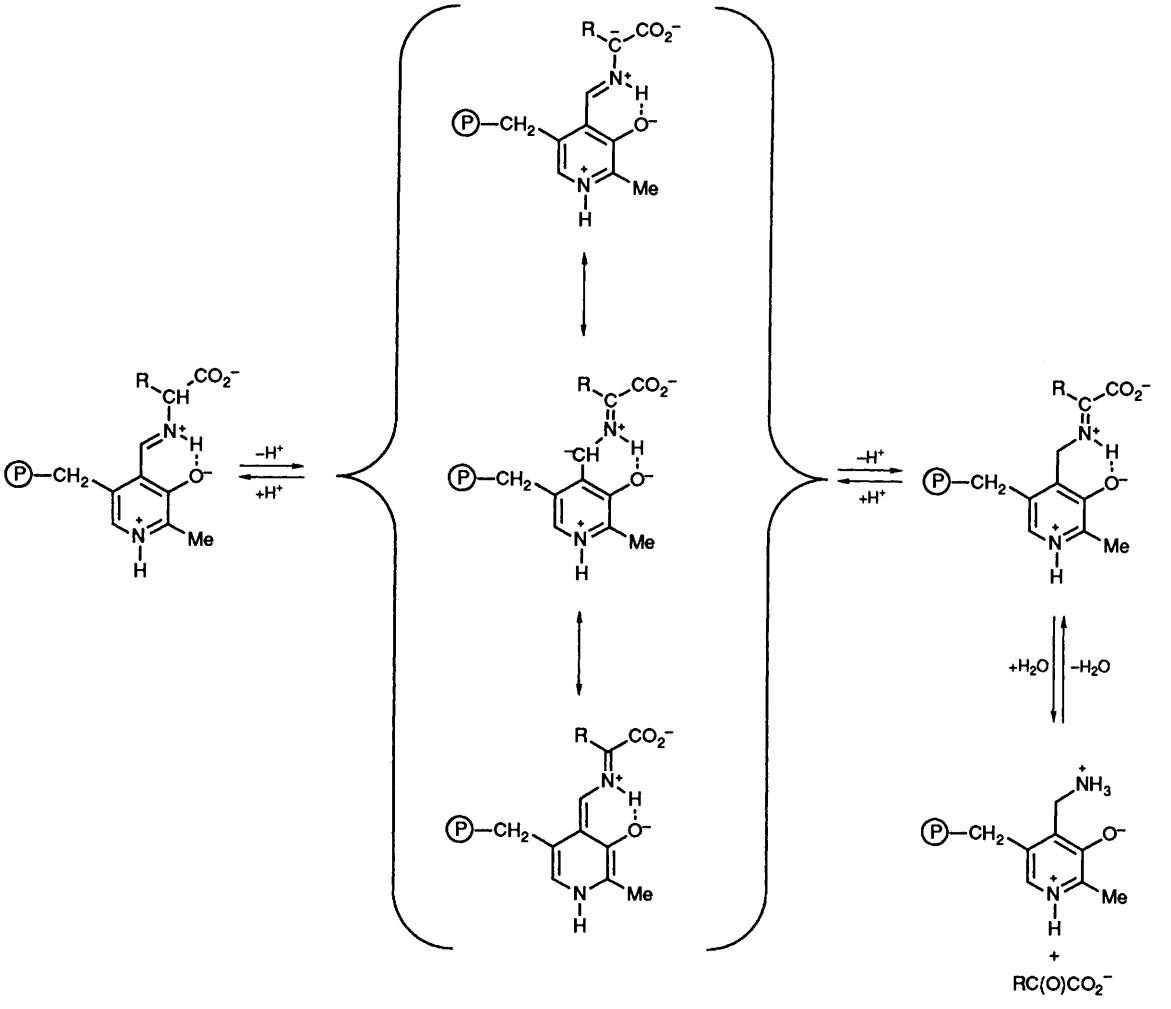
$$a_{3a}/a_{3a}^0 + a_{3b}/a_{3b}^0 + a_{3c}/a_{3c}^0 = 1 \qquad (6)$$

In order to calculate the molar area of the **3a**, **3b** and **3c** forms, spectra of the Schiff base in different anhydrous ethylene glycol–dioxane mixtures (100, 85, 60, 45 and 30% ethylene glycol) have been obtained, since in this kind of non-protic solvent only the monoprotonated species can exist. The resolution of these spectra into bands allowed the calculation of the a_{3a}, a_{3b} and a_{3c} values in each mixture. Fitting the results to eqn. (6) gives a_{3a}^0, a_{3b}^0 and a_{3c}^0. These values are presented in Table 3.

From the value of the tautomeric equilibrium constant between **2a** and **2b** species taken from the literature [10] and the molar areas, microscopic pK and tautomeric equilibria values can be obtained (see Table 3).

In summary, four features of these constants need to be addressed.

(i) Microscopic pK and tautomeric equilibria values are



Scheme 4 Hydrolysis of α-amino acid–PLP Schiff bases to ketoacidate and PMP

Table 3 Macroscopic and microscopic protonation constants, molar areas ($mmol^{-1} \times 10^{-6}$)[a] and tautomerization constants of PLP Schiff bases

	Gly	CA	Leu	Ile
pK_{2b}	6.44	6.53	6.59	6.60
pK_{3b}	11.40	11.76	11.65	11.62
$pK_{2a,3a}$	6.41	6.49	6.55	6.56
$pK_{2a,3b}$	7.42	7.52	7.55	7.56
$pK_{2b,3c}$	6.46	6.54	6.60	6.61
$pK_{2b,3b}$	6.42	6.50	6.57	6.58
$pK_{3a,4a}$	11.32	11.67	11.57	11.55
$pK_{3b,4a}$	10.31	10.65	10.57	10.55
$pK_{3c,4a}$	10.36	10.70	10.62	10.60
$R_{3a,3b}$	0.15	0.14	0.16	0.16
$R_{3b,3c}$	1.10	1.18	1.09	1.09
a^g_{3a}	385.6	420.1	389.1	408.6
a^g_{3b}	283.2	352.1	335.2	341.6
a^g_{3c}	198.3	262.3	223.5	236.4

[a] $R_{2a,2b}$ = 0.011 taken from Ref. 10.

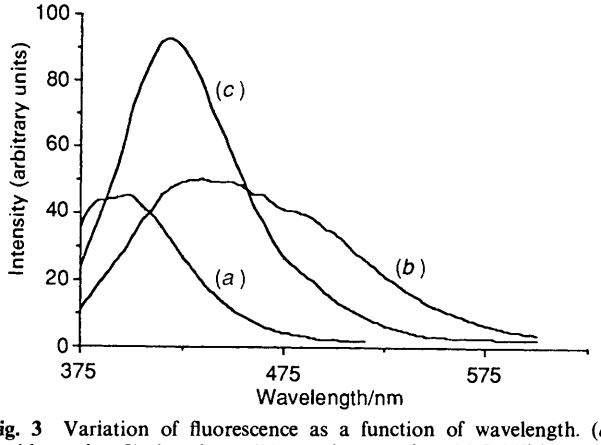


Fig. 3 Variation of fluorescence as a function of wavelength. (*a*) Pyridoxamine 5′-phosphate; (*b*) ε-aminocaproic acid Schiff base; (*c*) glycine Schiff base. Excitation wavelength 328 nm, pH = 6.

almost independent of the amino acid used to form the Schiff base.

(ii) Macroscopic pK_{3b} is similar to the microscopic $pK_{3a,4a}$ value of the **3a** tautomer, owing to the high percentage of that species existing in aqueous solution (> 77%).

(iii) Ionic species SB_2 has four different microscopic p*K*s whose values (6.5 ± 0.1) are quite similar to each other; the exception is $pK_{2a,3b}$ corresponding to imine nitrogen ionization, which could be explained on the basis of the high basicity of nitrogen.

(iv) Microscopic p*K*s of **2a**, **3a** and **4a** forms ($pK_{2a,3a}$,$pK_{3a,4a}$) have values practically equal to those p*K* values obtained *via* eqn. (2). This is because the kinetic data used to determine the equilibrium constants have been obtained at the wavelength corresponding to the absorption maxima of the **2a** and **3a** tautomeric forms.

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