79. Kinetic Study on Stability of *Schiff* Base of Pyridoxal 5′-Phosphate and Leucine in Water Media with Cationic Surfactants

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We studied the stability of the *Schiff* bases formed between pyridoxal 5′-phosphate (PLP) and leucine in the presence of (hexadecyl)trimethylammonium bromide (CTAB) over a wide pH range by determining the kinetic constants of formation and hydrolysis of these compounds. The results show that the stability of the *Schiff* bases is increased by the presence of CTAB as a result of increased rates of formation and decreased hydrolysis rate constants. The ionic head groups of CTAB favour the formation of the bases, while its hydrophobic rests protect the imine double bond from hydrolysis. This model system permits one to obtain partially hydrophobic media with no need for any non-aqueous solvents.

Introduction. – Pyridoxal 5′-phosphate (PLP) takes part as coenzyme in a wide variety of reactions involved in the metabolism of amino acids (*e.g.* transaminations, dealdolations, decarboxylations, *etc.*) [1] [2]. As a rule, it binds to enzymes to form *Schiff* bases (imines) by bonding to the terminal ε-amino group of lysine in the polypeptide chain [3].

The character of the environment where *Schiff* bases occur varies from enzyme to enzyme. Thus, it is highly hydrophobic in phosphorylases [4] [5] and hydrophilic or only partially hydrophobic in aminotransferases and decarboxylases [6] [7].

Recently, we studied the behaviour of *Schiff* bases of PLP and analogues in hydrophobic environments; H_2O/EtOH, H_2O/dioxane mixtures and various non-aqueous solvents [8] [9]. On the other hand, we also performed kinetic and spectroscopic studies on the *Schiff* bases of PLP and analogues with amino acids and amines in aqueous media, and the results obtained show the absence of ionic and/or polar groups in the vicinity of the imine double bond to increase the stability of the *Schiff* base concerned [10] [11].

To simulate a highly hydrophobic environment without using solvent mixtures, we formed the *Schiff* bases of PLP and analogues with dodecylamine (DOD). This amine is a cationic surfactant including a rather bulky hydrocarbon. The kinetic studies on formation and hydrolysis of these *Schiff* bases and the spectroscopic measurements indicate that the imine is placed in a hydrophobic environment similar to that of phosphorylases [12].

The *Schiff* bases formed between PLP analogues and amino acids in aqueous media have a hydrophilic environment [13] [14]. In this work, we have modified partially the environment by adding (hexadecyl)trimethylammonium bromide (CTAB) to the H_2O solution at concentrations below its critical micelle concentration (CMC) [15]. The systems thus obtained were used to carry out a kinetic study on the *Schiff* base of PLP and an amino acid, leucine (Leu). Leucine has an i-Bu residue that can interact with the

hydrophobic part of surfactant which makes it very suitable for these kind of studies. The results obtained are compared with those previously reported for the PLP-dodecylamine *Schiff* base (PLP-DOD) [12].

Experimental. – Pyridoxal 5′-phosphate and all other chemicals were reagent grade and purchased from *Merck*, Darmstadt. Chloroacetate, acetate, phosphate, and carbonate buffers were used in the appropriate range. Buffer concentrations were typically 0.02M, keeping the ionic strength constant and equal to 0.1 by adding the appropriate amount of KCl.

PLP solns. were prepared in a suitable buffer and kept in dark. Their exact concentrations was determined by diluting in 0.1M HCl and subsequent measurement of its absorbance a 295 nm ($\varepsilon = 6{,}700\ l \cdot mol^{-1} \cdot cm^{-1}$) [16]. Concentration thus found was *ca.* $3 \cdot 10^{-5}$ M.

Aq. solns. of (hexadecyl)trimethylammonium bromide (CTAB) were prepared by dissolving the required amount of reagent with the aid of an ultrasonic bath. The transparent solns. thus obtained were diluted in the reaction cuvette. The final CTAB concentration in the cuvette was $5 \cdot 10^{-4}$ or $9 \cdot 10^{-4}$ M, *i.e.* lower than the CMC [15].

The pH measurements were made with the aid of a *Crison* pH-meter, using a *Metrohm EA120* combined electrode previously calibrated with aq. buffers at 25°. As verified for each experiment, the difference between the initial and final pH of the reaction cell was never greater than ± 0.04 pH units.

Light-scattering measurements were carried out on a *Perkin-Elmer MPF 66* spectrofluorimeter provided with a thermostated cell at 25°. Measurements were made at 415 nm, the CTAB system showing no absorption at this particular wavelength.

Reaction was started by adding a few ml (0.2–1.6) of the amino-acid soln. to the cell which already contained the previously thermostated PLP and CTAB soln., prepared in the same buffer and at the same pH. Amine concentration in the measuring cell was 50–500 times as high as that of PLP. Reaction was monitored by measuring the increase in absorbance at 415 nm. A *Uvikon 940* spectrophotometer furnished with cells of 1.0-cm path length was used in every case.

The overall reaction between aldehyde an amine can be represented by:

$$R{-}CHO + R'{-}NH_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} R{-}CH{=}N{-}R' + H_2O \tag{1}$$

Methods used to determine k_1 and k_2 values are described in detail in [8] [17]. Equilibrium constant corresponding to each pH, K_{pH}, was calculated as the ratio k_1/k_2.

The overall rate constants of hydrolysis and formation of the aldimine can be described on terms of the rate constants for individual ionic species present in each case. The ionic species existing in soln. in the pH range studied are given in *Scheme 1*. This scheme has been extensively used in order to describe the kinetic behaviour of these kind of systems [17] [18]. For the hydrolysis mechanism, the OH^- attack on the ionic species B_3 has been proposed to occur.

On the base of that scheme, *Eqns. 2–5* can be obtained:

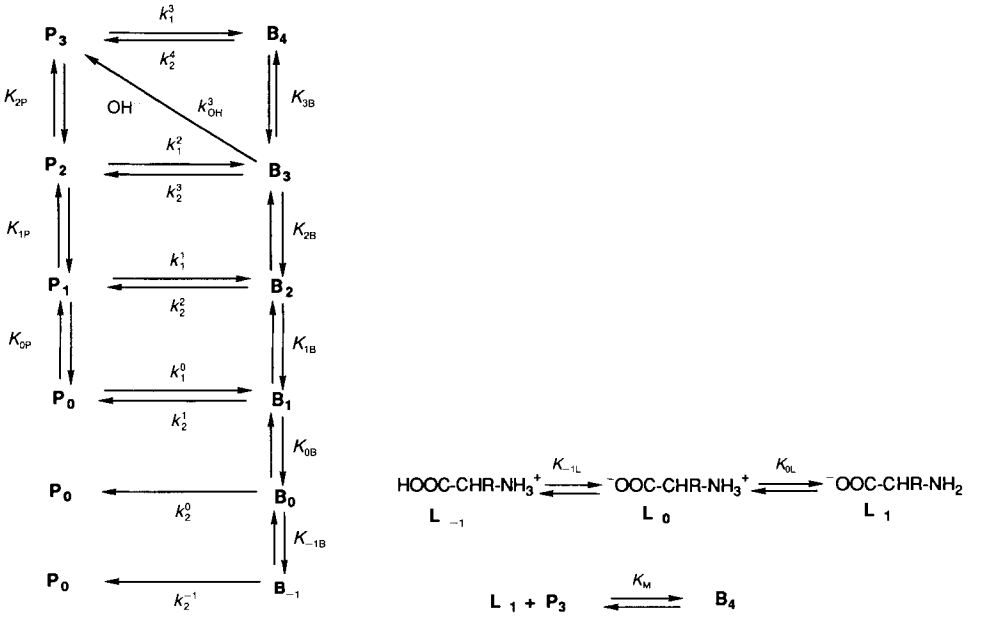
$$k_1 = \frac{k_1^3 + k_1^2 \frac{h}{K_{2P}} + k_1^1 \frac{h^2}{K_{2P}K_{1P}} + k_1^0 \frac{h^3}{K_{2P}K_{1P}K_{0P}}}{\left(1 + \frac{h}{K_{0L}} + \frac{h^2}{K_{0L}K_{-1L}}\right)\left(1 + \frac{h}{K_{2P}} + \frac{h^2}{K_{2P}K_{1P}} + \frac{h^3}{K_{2P}K_{1P}K_{0P}}\right)} \tag{2}$$

$$k_2 = \frac{k_{OH} + k_2^3 \frac{h}{K_{3B}} + k_2^2 \frac{h^2}{K_{3B}K_{2B}} + k_2^1 \frac{h^3}{K_{3B}K_{2B}K_{1B}} + k_2^0 \frac{h^4}{K_{3B}K_{2B}K_{1B}K_{0B}} + k_2^{-1} \frac{h^5}{K_{3B}K_{2B}K_{1B}K_{0B}K_{-1B}}}{\frac{h}{K_{3B}} + \frac{h^2}{K_{3B}K_{2B}} + \frac{h^3}{K_{3B}K_{2B}K_{1B}} + \frac{h^4}{K_{3B}K_{2B}K_{1B}K_{0B}} + \frac{h^5}{K_{3B}K_{2B}K_{1B}K_{0B}K_{-1B}}} \tag{3}$$

$$k_{OH} = k_2^4 + k_{OH}{}^3P_w/K_{3B} \tag{4}$$

$$K_{pH} = \frac{\left(1 + \frac{h}{K_{3B}} + \frac{h^2}{K_{3B}K_{2B}} + \frac{h^3}{K_{3B}K_{2B}K_{1B}} + \frac{h^4}{K_{3B}K_{2B}K_{1B}K_{0B}} + \frac{h^5}{K_{3B}K_{2B}K_{1B}K_{0B}K_{-1B}}\right) K_M}{\left(1 + \frac{h}{K_{0L}} + \frac{h_2}{K_{0L}K_{-1L}}\right)\left(1 + \frac{h}{K_{2P}} + \frac{h^2}{K_{2P}K_{1P}} + \frac{h^3}{K_{2P}K_{1P}K_{0P}}\right)} \tag{5}$$

Scheme 1



Experimental data for k_1, k_2, and K_{pH} were fitted simultaneously to *Eqns. 2–5* by means a nonlinear regression method and minimizing the functions U_i

$$U_i = \Sigma(\log k_{i,e} - \log k_{i,t})^2 \tag{6}$$

where $i = 1$, 2 or pH and subscripts e and t refer to experimental and theoretical data.

To determine the critical micelle concentration of the CTAB in our experimental conditions, we recorded the light-scattering at different CTAB concentrations. As can be seen in *Fig. 1*, the CMC of CTAB in our system is *ca.* $9 \cdot 10^{-4}$ M. In H_2O, the CMC increases until a value of $1.0 \cdot 10^{-3}$ M. These values agree with those in [15]. In addition, dramatic changes of light-scattering with pH are not observed. It is clear from *Fig. 1* that, in our experimental conditions, the solns. of CTAB are true solns.

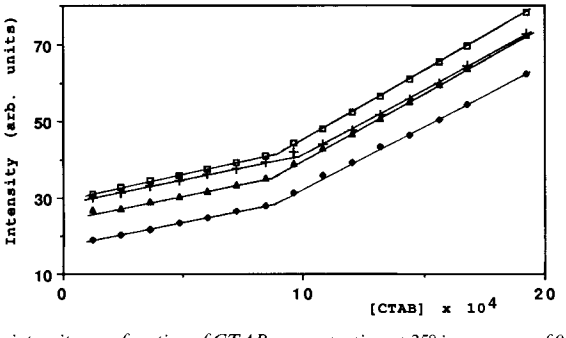


Fig. 1. *Light-scattering intensity as a function of CTAB concentration at 25° in presence of 0.01* M L-leucine, I = *0.1* M. ◆: pH = 6.9 (phosphate buffer); ▲: pH = 10.5 (carbonate buffer); □: pH = 4.4 (acetate buffer); +: H_2O (in absence of both leucine and inert salt).

Results and Discussion. – The mechanisms of formation and hydrolysis of *Schiff* bases are of great biological significance and have been studied in detail by several authors [18–21]. As a rule, the formation process takes place throughout the formation of a carbinolamine and releasing of a H_2O molecule or OH^- ion to yield the corresponding *Schiff* base. The carbinolamine dehydration was found to be the rate-determining step throughout the wide pH range studied. The hydrolysis reaction involve the addition of a H_2O molecule or OH^- ion to the C=N bond, whether protonated or unprotonated.

We recorded the UV absorption spectra of PLP in $5 \cdot 10^{-4}$ and $9 \cdot 10^{-4}$ M solutions of CTAB over the pH range 2.5–13. The spectra obtained were quite consistent with those reported by *Metzler et al.* [22] for aqueous media. According to these results, the PLP molecule slightly interacts with the hydrophobic part of the CTAB and is placed in a hydrophilic media due to its high polarity which includes numerous polar or ionic groups (carbonyl, phosphate, phenoxide, and pyridine N-atom), depending on the pH of the medium. This behaviour allows one to process the kinetic data obtained from solutions containing CTAB in the same way as those found for purely aqueous solutions.

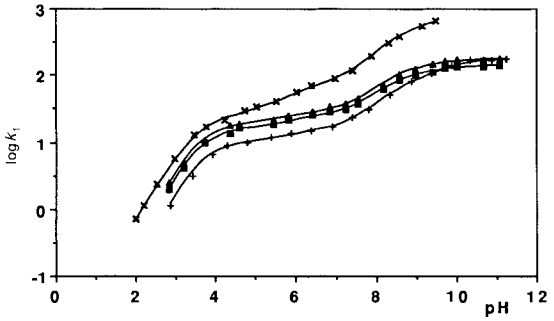


Fig. 2. *Variation of* log k_1 *as a function of pH for the* Schiff *bases of PLP and:* ×: *dodecylamine;* +: *leucine;* ■: *leucine.* [CTAB] = $5 \cdot 10^{-4}$ M. ▲: Leucine, [CTAB] = $9 \cdot 10^{-4}$ M. Points are experimental values, and continous lines are the theoretical fitting calculated from *Eqn. 2* and parameters given in the *Table.*

Fig. 2 shows the logarithms of the kinetic constants of formation of the *Schiff* bases of PLP with Leu and dodecylamine (DOD). Dots represent experimental values and the theoretical lines were obtained by applying data listed in the *Table* to *Eqn. 2.* As can be seen, $\log k_1$ increases with pH up to a value roughly equal to the pK of the amino acid or amine. Adding CTAB to the reaction medium used for the formation of the imine of PLP and Leu results in a slight increase in the rate constant except a very high pH value. This is a result of the amphiphilic character of the CTAB system, which directs the hydrocarbon chain of the carbinolamine towards its hydrophobic part and the rest of the molecule towards the ionic head groups. In acid and neutral media, the carbinolamine releases a H_2O molecule *via* a very polar transition state that is stabilized by the ionic groups of the surfactant. In basic media, mainly at pH values higher than the amine pK of amino acid, the rate constant values of the system with and without CTAB are quite similar each other which may be indicative of a similar micro-environment in both carbinolamines.

The log k_1 values obtained for the formation of the *Schiff* base of PLP and DOD [12] are much larger than those reported for any other amine or amino acid [8–10] [17] for

Table. *Best Kinetic Constants, pK and K_M, Obtained from Fitting of Experimental Values of* k_I [$l \cdot mol^{-1} \cdot min^{-1}$], k_2 [min^{-1}], *and* K_{pH} [$l \cdot mol^{-1}$] *to* Eqns. 2–5 *for PLP-Leu in H_2O and Different Concentrations of the Cationic Surfactant*

[CTAB]/mol·dm^{-3}	0	$5 \cdot 10^{-4}$	$9 \cdot 10^{-4}$
pK_{2P}	8.34	8.33	8.33
pK_{1P}	5.90	5.90	5.88
pK_{0P}	3.61	3.58	3.56
pK_{0L}	9.77	9.77	9.77
pK_{-1L}	2.31	2.31	2.31
pK_{3B}	11.61	11.60	11.52
pK_{2B}	6.65	6.46	6.38
pK_{1B}	5.68	5.60	5.55
pK_{0B}	2.83	2.75	2.69
pK_{-1B}	2.25	2.10	2.07
$\log k_1^3$	2.25	2.15	2.25
$\log k_1^2$	3.36	3.51	3.61
$\log k_1^1$	5.05	5.27	5.35
$\log k_1^0$	7.16	7.41	7.50
$\delta(k_1^i)$	0.007	0.011	0.009
$\log k_2^4$	1.08	0.61	0.49
$\log k_2^3$	− 0.90	− 1.29	− 1.36
$\log k_2^2$	0.08	− 0.10	− 0.14
$\log k_2^1$	0.17	− 0.02	− 0.09
$\log k_2^0$	− 0.06	− 0.54	− 0.59
$\log k_2^{-1}$	− 0.58	− 0.82	− 0.88
$\delta(k_2^i)$	0.004	0.006	0.006
$\log K_M$	1.19	1.54	1.76
$\delta(K_M)$	0.015	0.019	0.017

several reasons. First of all, DOD is a cationic surfactant and a powerful amphiphilic agent. The carbinolamine of the PLP-DOD system will always lie close to the ionic head groups of the surfactant, whereas that of the PLP-Leu in CTAB system may lie in the vicinity of the ionic head groups or in the bulk solvent. Finally, the geometric shape of the DOD molecules results in larger molecular aggregates than with CTAB molecules and, hence, in an even more marked dual hydrophobic-hydrophilic character [15].

In the partially hydrophobic media provided by scarcely polar solvents, the rate constants of dehydration of the carbinolamine are smaller than those obtained in aqueous media [8] [9]. This can be attributed to the less polar environment where the carbinolamine lies, which instabilizes the corresponding transition state. However, the reaction rate is increased by amphiphilic systems (*e.g.* enzyme systems), which thus make more suitable models.

Fig. 3 shows the variations with pH of the logarithms of the rate constants of hydrolysis for the *Schiff* bases of PLP-Leu at different CTAB concentrations, and those of the PLP-DOD system. As in *Fig. 2*, dots denote experimental curves and the lines are the theoretical curves obtained from *Eqn. 3* by using data listed in the *Table*. As can be seen, CTAB decreases the hydrolysis constants, though not so much as to make them close to those of the PLP-DOD system [12]. This behaviour can be accounted for by interpreting

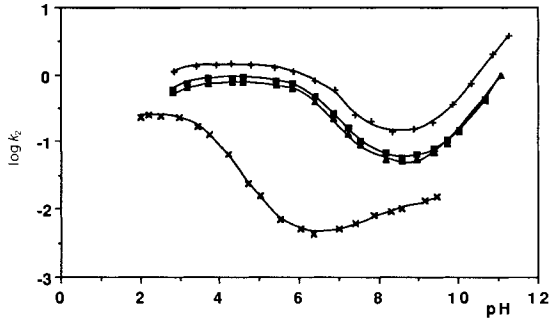


Fig. 3. *Variation of log* k_2 *as a function of pH for the* Schiff *bases of PLP and:* ×: *dodecylamine;* +: *leucine;* ■: *leucine,* [CTAB] = $5 \cdot 10^{-4}$ M. ▲: Leucine, [CTAB] = $9 \cdot 10^{-4}$ M. Points are experimental values, and continous lines are the theoretical fitting calculated from *Eqn. 3* and parameters given in the *Table*.

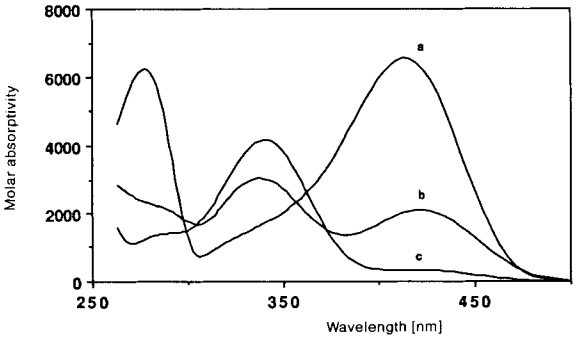


Fig. 4. *Absorption spectra of the equivalent ionic species of* Schiff *bases. a*) PLP-Leu in water (B_3); *b*) PLP-Leu in H_2O and [CTAB] = $9 \cdot 10^{-4}$ M (B_3); *c*) PLP-DOD in H_2O (B_0).

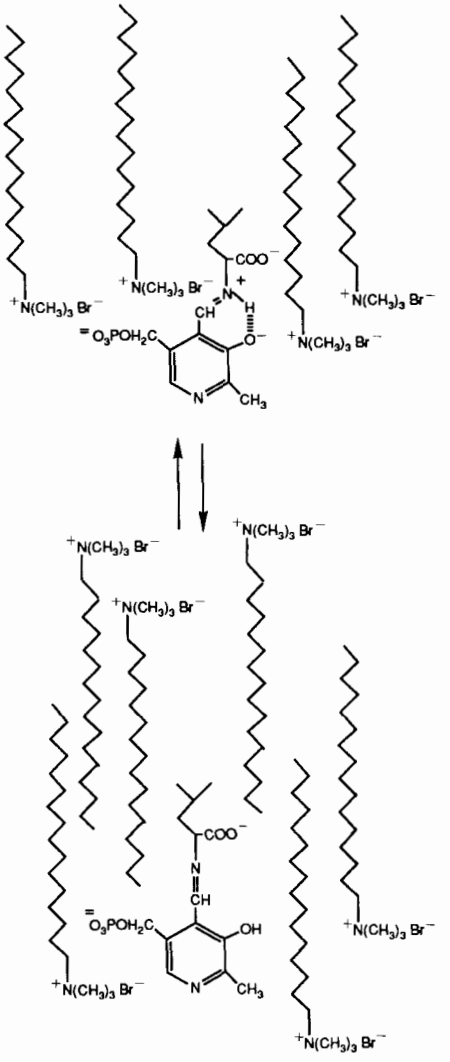
the UV spectrum of the *Schiff* bases. As can be seen in *Fig. 4*, the absorption spectrum of PLP-DOD presents maximum at 339 nm, whereas PLP-Leu in H_2O presents it at 417 nm. That is, because, in these conditions, the predominant tautomer of PLP-DOD is a enol form, and for PLP-Leu the predominant is a zwitterion [12] [22] [23].

In the absorption spectrum of PLP-Leu in H_2O with CTAB, both maxima are present, showing that the tautomeric equilibrium is not totally shifted either to zwitterion or to enol. Moreover, band analysis shows that there is a bathochromic shift of the maximum wavelength of zwitterion of PLP-Leu in CTAB with respect to that obtained for PLP-Leu in H_2O, which means the imine bond in the former system lies in a much more hydrophobic microenvironment than in H_2O [24].

The decrease in $\log k_2$ caused by addition of CTAB must arise from the fact that the *Schiff* base is solvated by CTAB molecules that protect the imine C=N bond from the attack of H_2O molecules (*Scheme 2*).

The $\log k_2$ values obtained for PLP-DOD are much smaller than those of the PLP-Leu system (*Fig. 3*). This was attributed to the fact that the enol form, in which the imine bond lies in a highly hydrophobic medium that shelters it from H_2O molecules, prevails over its zwitterionic tautomer in the *Schiff* base of the PLP-DOD system (*Fig. 4*) [12]. Below

Scheme 2



pH 7, the different protonations of the *Schiff* base restore the prevalence of the zwitterionic form, which now lies in a less hydrophobic medium and gives rise to hydrolysis constants closer to those obtained for the PLP-Leu and PLP-CA systems [10].

Fig. 5 shows the variation of the equilibrium constants of formation of the *Schiff* bases studied with pH. As can be seen, the $\log K_{pH}$ values, obtained as k_1/k_2 ratios, increase with the pH up to a maximum lying between the pK of the amine and the greatest pK of the aldehyde. Addition of CTAB increases $\log K_{pH}$ as a result of the decrease in $\log k_2$ and the increase in $\log k_1$. The same facts account for the high stability of the *Schiff* bases of PLP

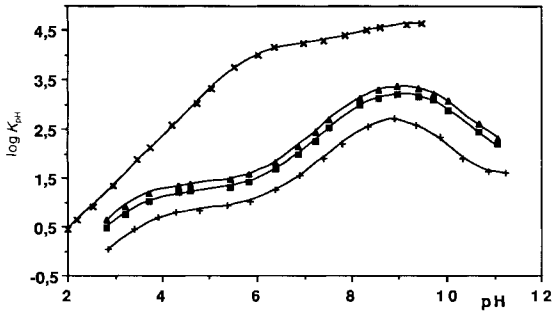


Fig. 5. *Variation of log* K_{pH} *as a function of pH for the* Schiff *bases of PLP and:* ×: *dodecylamine;* +: *leucine;* ■: *leucine,* [CTAB] = $5 \cdot 10^{-4}$ M. ▲: leucine, [CTAB] = $9 \cdot 10^{-4}$ M. Points are experimental values, and continous lines are the theoretical fitting calculated from *Eqn. 5* and parameters given in the *Table.*

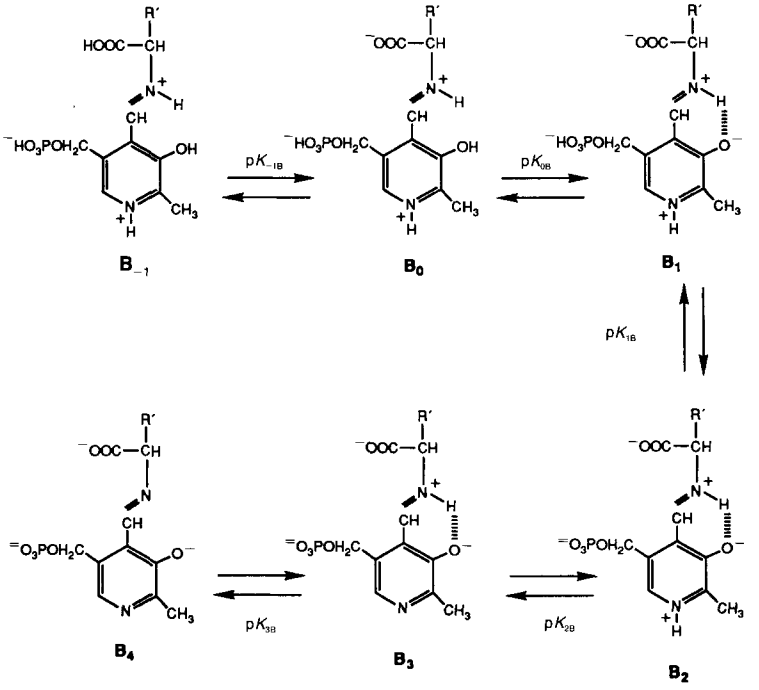
with DOD. In any case, these last log K_{pH} values are much larger than those reported for any other related system [8–14] [17] [25] [26].

All kinetic and equilibrium constants are the result of the different microscopic kinetic constants (k_1^i, k_2^i) and the different ionization constants of Leu, PLP, and their *Schiff* bases, according to *Scheme 1* and *Eqns. 2–5*. The kinetic microscopic constants and ionization constants obtained in the best fitting of the experimental data to *Eqns. 2–5* are listed in the *Table*. As can be seen, k_1^i increases with decreasing pH of the medium, which has been described as a result of the intramolecular acid catalysis on the carbinolamine dehydration, which involves every protonable group in the PLP molecule [6–9] [12] [17] [23]. By plotting $\log k_1^i$ *vs.* the corresponding pK of PLP, one obtains very good linear correlations with similar slopes (0.8) that increase on addition of CTAB to the reaction medium, hence the surfactant increases the efficiency of the intramolecular catalysis.

The $\log k_2^i$ values obtained for the PLP-Leu system are decreased by the presence of CTAB in the reaction medium. As stated above, this can be attributed to the sheltering effect of the C_{16} chain of the CTAB molecule, which hinders the attack by H_2O molecules and OH^- ions. Moreover, the Br^- ions of CTAB replace OH^- ions in the vicinity of the imine bond, thereby exerting an inhibitory effect on the hydrolysis [15]. The decrease in k_2^i is more marked in basic media, because deprotonation of the pyridine N-atom decreases the polarity of the molecule and, hence, increases the hydrophobic character of the environment.

Quantitative differences between k_2^i values must be explained taking into account both enolic and zwitterionic forms, but qualitative ones can be rationalized attending only to zwitterions. So, the chemical species with the smallest hydrolysis constant ($\log k_2^3$) is B_3 (*Scheme 3*) on account of the intramolecular H-bond established between the imine N-atom and the pyridinio-oxide group. The second protonation corresponding to the pyridine N-atom instabilizes the *Schiff* base as a result of the deactivating effect of the protonated heterocycle. The subsequent protonation of the phosphate group has a slight instabilizing effect on the *Schiff* base, while that of the pyridinio-oxide group again increases its stability, particularly in the presence of CTAB, as a result of the loss of negative charge close to the imine C=N bond, which places it in a more hydrophobic medium and, hence, more strongly protected from hydrolysis. The last protonation, *viz.*

Scheme 3



that of the COO^- group, has a smaller specific hydrolysis constant, which supports the hypothesis that the smaller the charge on the *Schiff* base in the vicinity of the imine C=N bond is, the more it will penetrate into the nonpolar region, and the more protected from the attack by the H_2O molecules it will be.

Overall, the ionization constants of the *Schiff* base (pK_{iB}) decrease on addition of CTAB to the reaction medium as a result of electrostatic unstabilization of the protonated *Schiff* base caused mainly by the drop in proton concentration in the vicinity of the head ionic groups of the cationic surfactant.

pK_{3B} of the imine N-atom is the less affected by presence of CTAB, because, in very basic media, leucine is completely deprotonated and thus deprived of its role as co-surfactant of the amphiphilic system [15] [28]. However, the deprotonation of the pyridine N-atom is the most sensitive to the presence of the surfactant, as its pK decreases from 6.65 in its absence to 6.38 in its presence. This arises from the contribution of leucine as co-surfactant and from the fact that the deprotonation involves the loss of a positive charge.

In summary, the *Schiff* bases of PLP in the presence of cationic surfactants are suitable models for enzyme systems in partially or completely hydrophobic environments. By modifying the nature of the cationic surfactent and its concentration, we should be able to simulate PLP-dependent enzyme environments.

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