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# ENOLIMINE AND GEMINALDIAMINE FORMS IN THE REACTION OF PYRIDOXAL PHOSPHATE WITH ETHYLENEDIAMINE. AN ELECTROCHEMICAL AND SPECTROSCOPIC CONTRIBUTION

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**The enol–keto tautomerism of the Schiff bases formed by pyridoxal 5′-phosphate (PLP) with ethylenediamine (Etd) and ethylamine (Et) was studied by electrochemical and spectrophotometric methods. The spectroscopic results revealed differences between the two reactions, i.e. the enolimine/ketoenamine ratio observed in PLP–Etd mixture is higher than that of PLP–Et. The differences observed in the electroreduction mechanism and stability of the Schiff bases formed provide additional evidence for an unusually high concentration of enolimine in PLP–Etd in buffered aqueous solutions.**

**The results are consistent with a cyclic structure such as that previously proposed by Robitaille *et al.* [*J. Am. Chem. Soc.* 111, 3034–3047 (1989)] on the basis of spectroscopic data. Protonation of the terminal amino group of the Etd moiety in the Schiff base involves the formation of the cyclic species. The low basicity of ethylenediamine favours the formation of similar concentrations of the enol and keto tautomers of the Schiff base at neutral pH. This behaviour, also observed in the Schiff base formed by PLP and polylysine, may be involved in coenzyme–protein linkages.**

## INTRODUCTION

Conversion of internal to external aldimines in pyridoxal 5′-phosphate (PLP)-dependent enzymes is believed to occur through a concerted mechanism known as the transimination reaction. Elucidation of the intermediates involved has so far been focused on model reactions of PLP with diamines.

Abbot and Martell[1] reported the occurrence of a carbinolamine intermediate, whereas Tobias and Kallen[2] suggested the formation of a geminaldiamine following that of a Schiff base.

Metzler *et al.*[3] reported a geminaldiamine to occur in the reaction of pyridoxal (PL) with ethylenediamine in a basic medium. More recently, Robitaille *et al.*[4] confirmed the presence of a geminaldiamine in the reaction of PLP with diamines in a strongly basic medium on the basis of $^1$H NMR and UV–visible data, but suggested the occurrence of an enolimine form at lower pH values.

The assumption that an enol form is favoured over a geminaldiamine in buffered aqueous solutions requires checking by other methods since enolimines are generally not observed for PLP Schiff bases in polar solvents.[5,6]

Recent studies in our laboratory on the electroreduction mechanism for PLP and some of its Schiff bases with simple amines or amino acids[7–9] revealed a relationship between the stability and basicity of the amine residue.[10] Therefore, electrochemical characterization of the PLP–diamine equilibrium, yet unreported, might provide new information not available from bases formed by PLP and diamines.

This paper presents an electrochemical and spectrophotometric study of the reactions of PLP with ethylamine (Et) and ethylenediamine (Etd). Some information on the enol–keto equilibrium and stability of the Schiff bases obtained is reported.

## EXPERIMENTAL

PLP was purchased from Sigma. All other reagents

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used were of p.a. grade from Merck and used without further purification. The Schiff bases were obtained in dissolved form by adding known amounts of ethylamine or ethylenediamine to PLP solutions of known concentrations. These measurements were made after reaction equilibrium had been reached.

For the electrochemical experiments, buffered solutions of 0·02 M acetic acid and 0·02 M phosphoric acid for pH < 8·5 and 0·02 M phosphoric acid and 0·02 M $K_2CO_3$ for pH > 8·5 were used as supporting electrolytes. The pH was adjusted with KOH and the ionic strength was adjusted to 0·5 M with $KNO_3$ when necessary.

Spectrophotometric measurements were made on a Perkin-Elmer Lambda 3B UV–visible spectrophotometer furnished with 1 cm quartz cuvettes at 25 ± 0·1 °C.

DC and DP polarograms were recorded automatically on a Metrohm Model 626 polarograph. A saturated calomel electrode (SCE) was used as the reference electrode. Polarographic measurements were made at 25 ± 0·1 °C by using thermostated Amel 494 and Metrohm cells in a nitrogen atmosphere.

Absorption spectra were deconvoluted into individual components by using a log-normal distribution, which has been shown to ensure precise resolution of the shape of absorption bands.[11–13]

The following function describes the molar absorptivity, $\varepsilon$, in terms of four selectable parameters, viz, $\lambda_0$, $\varepsilon_0$, $w_\nu$ and $\rho$,

$$\varepsilon = \varepsilon_0 \exp\left( -\frac{\ln 2}{(\ln \rho)^2} \left\{ \ln\left[ 1 + \frac{2(\lambda - \lambda_0)}{\lambda\lambda_0} \frac{k\left(\frac{\rho^2 - 1}{\rho}\right)}{w_\nu} \right] \right\}^2 \right) \quad (1)$$

where $w_\nu = \nu_U - \nu_L$ and $k$ is a constant of proportionality between the chosen wavenumber unit, $\nu$, and the wavelength, $\lambda$, i.e. if $\nu$ and $\lambda$ are expressed in kilokayser (1 kK = $10^3$ $cm^{-1}$) and nanometers, respectively, $k$ is $10^{-4}$. The wavelengths at which $\varepsilon$ falls to $\varepsilon_0/2$ are denoted by $\lambda_U$ and $\lambda_L$ (upper and lower values). Therefore, the wavenumber and wavelength are related by $\nu_U = 10^4/\lambda_L$ and $\nu_L = 10^4/\lambda_U$, and $\rho = (\nu_U - \nu_0)/(\nu_0 - \nu_L)$ (a band asymmetry index).

Hence $\rho$ is greater than 1 for curves skewed higher wavenumbers. As $\rho$ approaches 1, the log-normal curve approaches a symmetric Gaussian shape.

Spectra were fitted to log-normal curves by using previously reported methods based on non-linear least-squares regression.[13,14]

## RESULTS

### Spectroscopic properties of PLP–amine mixtures

PLP reacts with primary amines to yield Schiff bases in a reversible reaction; on the other hand, its reaction with diamines can proceed to a geminaldiamine in a second, reversible step.[2]

The UV–visible spectrum of the Schiff base formed by PLP and ethylamine (PLP–Et) shows two bands centred at about 410 and 274 nm, the absorbance and maximum wavelength of which remained constant over the pH range 7–10 [Figure 1(a)]. Under these conditions, the Schiff base is monoprotonated at the imine group (SH).

An analysis of the whole pH range studied gave the results listed in Table 1. The unprotonated species ($S^-$) shows a single band at 345 nm and the changes observed in an acidic medium are partly due to overlap with PLP absorption bands.

These features are almost invariably observed with other PLP Schiff bases obtained from amines and amino acids and can be assigned to ketoenamine forms that prevail in polar solvents.[5,6] An analysis of the spectra for the PLP–ethylenediamine mixture revealed major differences [Figure 1(b), Table 1]. Ketoenamine bands are red shifted at pH 7 and an intermediate value of $pK_a \approx 8·2$ for the terminal amino group of the ethylenediamine moiety is obtained from the variation with pH. In addition, the spectrum shows a new band centred at about 336 nm that is also sensitive to $pK_a$ [Figure 1(b)].

These results are consistent with the terminal amino group resulting in differences in the stability of the Schiff base (the lower the pH, the lower is the concentration of the ketoenamine over the pH range 7–10).

The absorption band at 313 nm (Table 1) is assigned to a cyclic geminaldiamine in equilibrium with the unprotonated Schiff base.[2,3] This is believed to hold even under conditions where some monoprotonated Schiff base exists.[4]

The results of our experiments suggest that this assignment is correct at pH values above the $pK_a$ (8·2). Below $pK_a$, protonation of the amino group prevents a nucleophilic attack on the imine bond. The assignment is also supported by the similarity with the spectroscopic features of some vitamin $B_6$ derivatives bearing a C′-4 carbon with $sp^3$ hybridization (Table 2).

According to Table 2, it is unlikely that the 336 nm band for PLP–Etd may correspond to a geminaldiamine (a C′-4 $sp^3$ derivative) as originally assigned.[2] This absorption, which has lately been proposed for a cyclic form,[4] is consistent with absorption features of the Schiff bases of PLP in low-polarity solvents, where the single band obtained, centred at 335 nm, is ascribed to an enolimine tautomer.[5,6] Thus, the enol/keto ratio is known to be a function of the solvent polarity. This is illustrated for PLP–hexylamine in Figure 2.

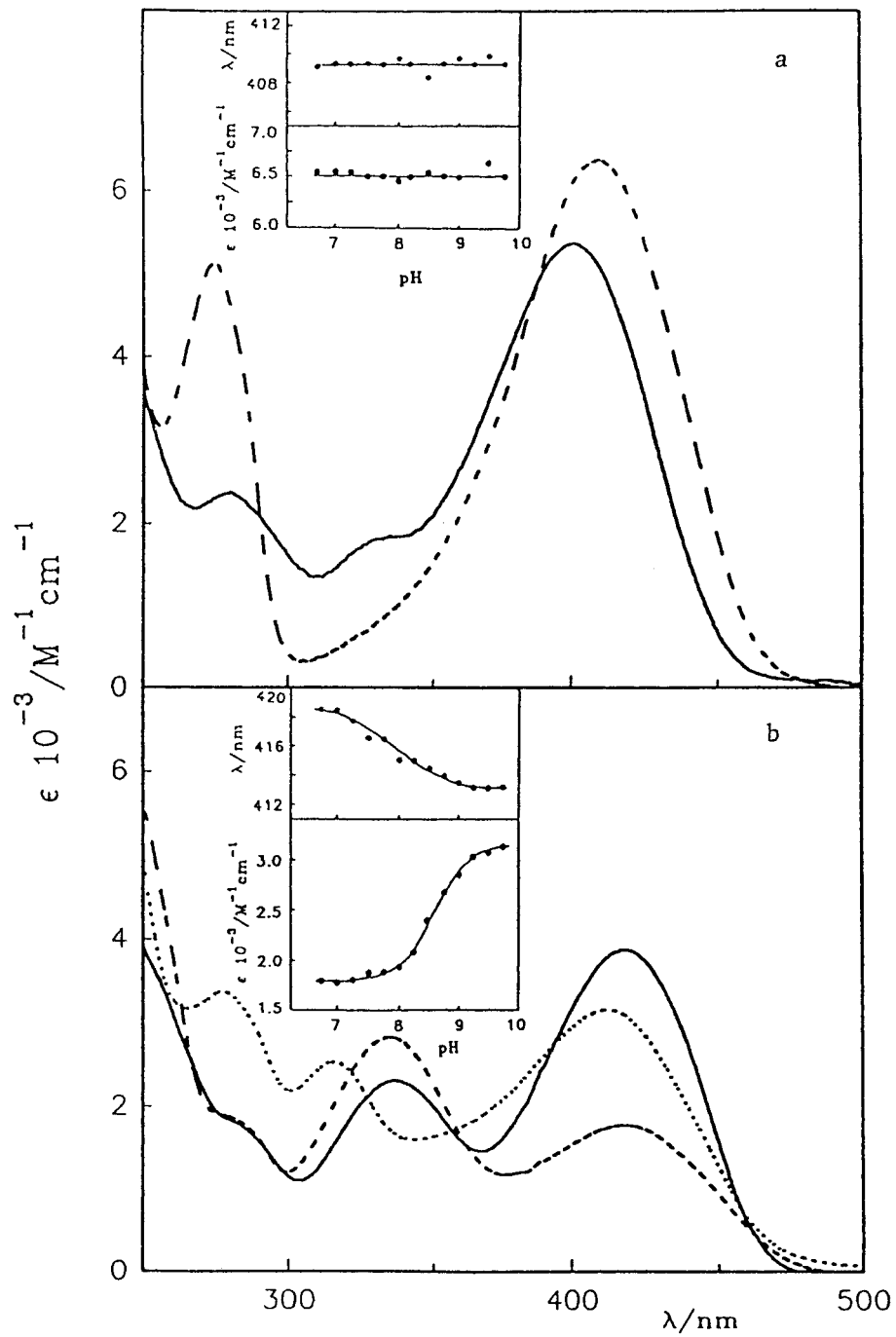



Figure 1. Absorption spectra for the PLP–Schiff bases studied. (a) PLP–Et, (— — —) pH 8·5 and (———) pH 4; (b) PLP–Etd, (···) pH 10, (— — —) pH 7 and (———) pH 4. Inset: variation of the maximum wavelength and molar absorptivity with pH. (a) PLP–Et; (b) PLP–Etd

Table 1. Spectroscopic properties of the mixtures of PLP (20 $\mu$M) with ethylamine (200 mM) and PLP (100 $\mu$M) with ethylenediamine (500 mM) in buffered aqueous solutions at 25 °C, and acid–base constants for the Schiff bases formed

| Sample | Species | $\lambda_a$/nm | | | group | p$K_a$ |
|---|---|---|---|---|---|---|
| PLP–Et | $SH_2^+$ | 279 | 402 | | Ring-$^+$NH | 6·2 |
| | SH | 275 | 412 | | Imine-$^+$NH | 11·7 |
| | $S^-$ | 345 | | | | |
| PLP–Etd | $SH_3^{2+}$ | 279 | 418 | 336 | Ring-$^+$NH | 5·3 |
| | $SH_2^+$ | 279 | 418 | 334 | End-$^+NH_3$ | 8·2 |
| | SH | 276 | 413 | 313 | Imine-$^+$NH | 11·7 |
| | $S^-$ | 344 | | 312 | | |
| Ethylamine (Et) | | | | | | 10·5 |
| Ethylenediamine (Etd) | | | | | | 7·5 |
| | | | | | | 10·5 |

Table 2. Absorption wavelengths (nm) for some vitamin $B_6$ derivatives

| Compound | Dipolar species | Anion | Ref. |
|---|---|---|---|
| Pyridoxal (hemiacetal) | 316 | 301 | 11 |
| Deoxypyridoxal (hydrate) | 320 | 300 | 11 |
| Pyridoxamine | 326 | 308 | 12 |
| Pyridoxamine 5′-phosphate | 327 | 308 | 12, 15 |

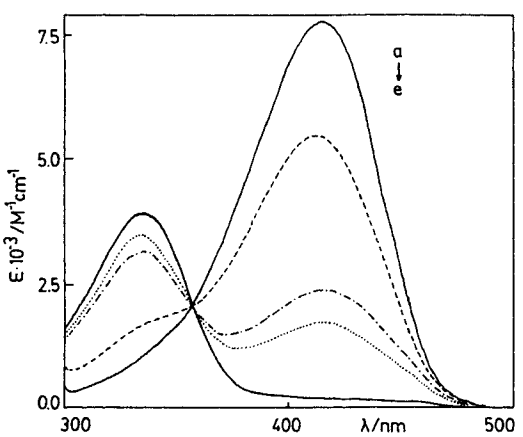



Figure 2. Variation of absorption spectrum for the PLP–hexylamine Schiff base as a function of the solvent composition: (a) water; (b) 60:40 (v/v) water–dioxane; (c) methanol; (d) ethanol; (e) methyl acetate. A decrease in the solvent polarity led to an increase in the enolimine tautomer fraction (absorption band at 335 nm)

Based on the above results, a significant concentration of an enolimine-like form should occur in buffered aqueous solutions of the PLP–Etd mixture (compare the absorption at 335 nm in Figure 1).

The protonation of the ring nitrogen in PLP Schiff bases (the keto tautomer) occurs at *ca* pH 6·0. This causes a decrease in the Schiff base concentration. At pH 4·0, for instance, a shift from 410 to 390 nm (the absorption wavelength for PLP) and a simultaneous decrease in the 274 nm band are observed [Figure 1(a)]. However, the bands at 335 and 420 nm for the PLP–Etd mixture are preserved at this pH. Accordingly, appreciable concentrations of the enol and keto tautomer must be present [Figure 1(b)].

These results can be explained by comparing the basicity of ethylenediamine and ethylamine (Table 1). The lower is the basicity of the amine the lower the pH at which the Schiff base is formed.[10]

## Electrochemical properties of PLP–amine mixtures

The Schiff bases of PLP have been shown to be reduced at a mercury electrode[8,9] via a two-electron, two- or three-proton transfer. Because the limiting current is diffusion controlled,[8] information on the PLP–Schiff base equilibrium can readily be obtained by using polarographic methods and the equation.

$$K_{pH} = \frac{1}{c_A} \frac{I_L}{1 - I_L} \qquad (2)$$

where $c_A$ is the initial amine concentration (provided that the amine is in excess over PLP), $I_L$ ($=i_L/i_D$) is the normalized limiting current and $i_L$ and $i_D$ are the limiting currents corresponding for the first (Schiff base formation) and overall process, respectively. The apparent formation constants for the adducts obtained from these reaction mixtures are shown in Figure 3.

The curve for PLP–Et exhibits the typical variation

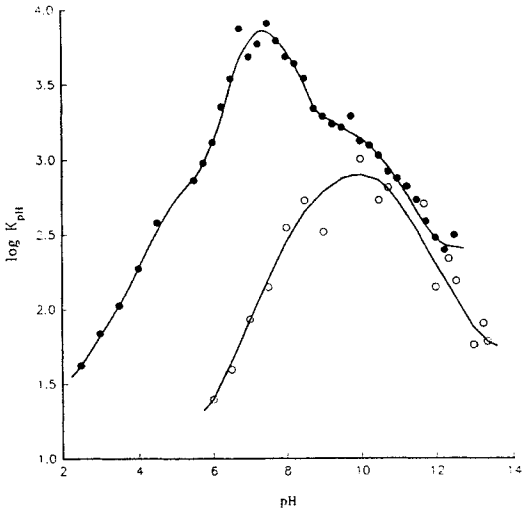



Figure 3. Apparent formation constant ($K_{pH}$) for the PLP–Schiff bases: (○) PLP–Et; (●) PLP–Etd. $K_{pH}$ was calculated from the limiting current of the polarographic waves using equation (2)

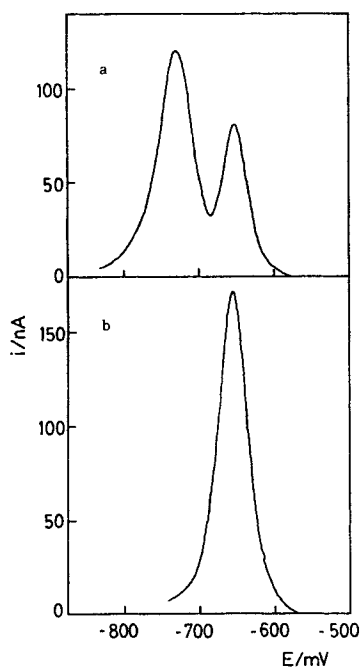
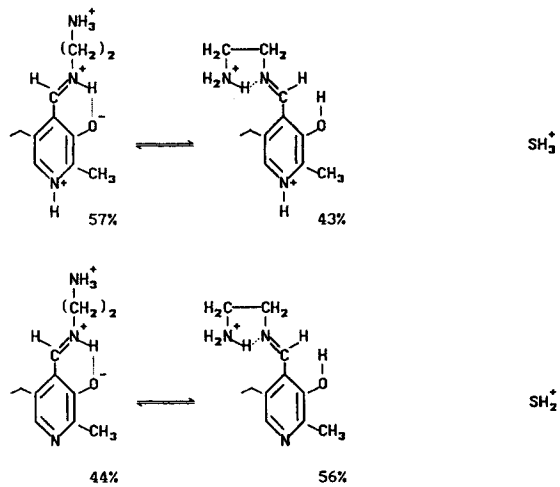



Figure 4. Differential-pulse polarograms for (a) PLP–Et and (b) PLP–Etd at identical concentrations of amine (10 mM) and PLP (0·1 mM) in buffered aqueous solutions at pH 7

for the Schiff bases of PLP. A stability maximum is observed near the p$K_a$ for ethylamine, as is usually the case with amino acids and amines.[10] It should be noted that the shape is an indication of the apparent stability of the ketoenamine tautomer.

PLP–Etd gives rise to a dramatic shift of the overall curve relative to the PLP–Et curve and a small, bell-shaped curve on the top of the first one.

Judging from these variations, the stability of PLP–Etd is mostly determined by the first p$K_a$ for ethylenediamine. Also, $K_{pH}$ increases between the first and second p$K_a$ for PLP–Etd. The polarograms for the PLP–Et and PLP–Etd reaction mixtures clearly show PLP–Etd to be the more stable (Figure 4). These results strongly suggest that $K_{pH}$ at neutral pH is determined by the formation of a cyclic enolimine (Scheme 1).




Scheme 1

In view of the spectroscopic results, the cyclic geminaldiamine appears at pH > p$K_{a2}$ for PLP–Etd. However, the PLP–Etd curve is similar to that for PLP–Et within experimental errors. Since conversion of the geminaldiamine into a Schiff base is very fast, it furnishes the electrode with Schiff base to be reduced.

The electroreduction mechanism for most PLP Schiff bases at pH > p$K_{a1}$ involves ring nitrogen protonation (see Table 1). Since the rate constant for this reaction is a function of the $H^+$ concentration, a polarographic p$K'$ is reached at *ca* 2–4 pH units above p$K_{a_1}$ which depends on the proton donors present in solution. Usually, the conjugate acid of the amine acts as the main donor.[8,9]

In fact, PLP–Et behaves in this way (Table 3). At p$K_{a_1}$ < pH < p$K'$, SH species in solution undergo fast protonation to $SH_2^+$, which is readily reduced at the electrode. However, at pH > p$K'$ (p$K'$ = 9·2), direct

Table 3. Reaction order with respect to $H^+$ as calculated by polarographic methods

| Sample | Range | $H^+$ order | Species | |
|---|---|---|---|---|
| | | | Bulk solution | Electroactive |
| PLP–Et | $pK_{a_1} < pH < pK' = 9{\cdot}2$ | 2·9 | SH | $SH_2^+$ |
| | $pH > pK'$ | 2·1 | SH | SH |
| PLP–Etd | $pK_{a_1} < pH < pK' = 9$ | 2·2 | $SH_2^+$ | $SH_2^+$ |
| | $pH > pK'$ | 2·9 | SH | $SH_2^+$ |

electroreduction of SH is feasible. This is reflected in a change in the $H^+$ reaction order from 3 to 2.

In clear contrast, PLP–Etd exhibits a change in the reaction order from 2 to 3 (Table 3) that is consistent with enolimine reduction.

The cyclic enolimine in Scheme 1 seemingly meets the requirements. The $pK_a$ for the ring nitrogen in enolimine forms[16] is *ca* 3, and such a low basicity, together with the charge on the amine group, prevents protonation of the heterocycle. In addition, the cyclic structure provides the conjugate acid (at the electroactive group),

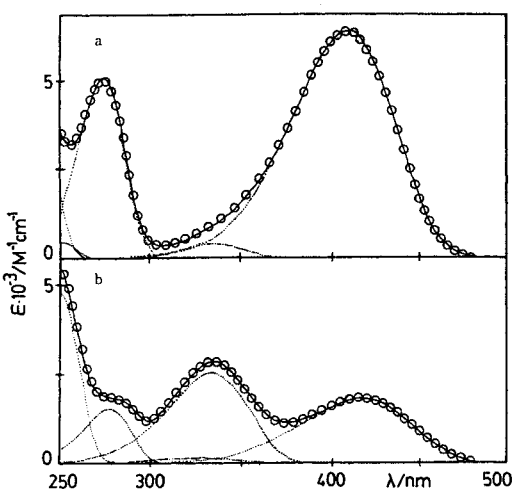



Figure 5. Log-normal deconvolution of the absorption spectra for the (a) PLP–Et and (b) PLP–Etd Schiff base at pH 7 in buffered aqueous solutions. Major individual contributions of the ketoenamine and enolimine tautomers are denoted by dotted curves. Circles represent experimental points and the solid lines correspond to theoretical profiles calculated from equation (1). The spectrum for PLP–Et was fitted to two major bands at 410 nm and 274 nm (ketoenamine) of width 4·1 and 4·2 kK and skewness 1·46 and 1·31, respectively. The spectrum for PLP–Etd was fitted to three major bands at 418, 278 (ketoenamine) and 335 nm (enolimine), of width 4·2, 4·1 and 4·9 kK and skewness 1·52, 1·49 and 1·49, respectively. From deconvolution of the spectra, estimates of 13% and 56% for the enolimine tautomer of PLP–Et and PLP–Etd, respectively, are obtained.

which is more readily reduced under most experimental conditions.

The change in the reaction order above the $pK_{a_2}$ for PLP–Etd suggests that the conjugate acid of ethylenediamine acting as the proton donor yields the $SH_2^+$ species. This is logical in view of the higher basicity of the amino group relative to the ring nitrogen.

### Deconvolution of spectra

Log-normal fitting has proved useful for analysing complex absorption spectra.[11–13] A combination of spectroscopic and electrochemical approaches has been applied to the Schiff base of PLP and *n*-hexylamine.[14,18]

In view of the preceding results, it was thought of interest to resolve the absorption spectra for the PLP–Et and PLP–Etd mixtures. Figure 5 shows typical deconvoluted spectra for the Schiff bases. As expected, the results shows that the ketoenamine (*ca* 90%) predominates over the enolimine in PLP–Et at pH 7. However, the proportion of both tautomers of PLP–Etd remains fairly constant and similar over the pH range 4–7, where a high concentration of Schiff base is still present. On the other hand, the enolimine predominates over the ketoenamine at pH 7 (Scheme 1).

These results may account for the difference in the ring nitrogen $pK_a$ between the two mixtures (Table 1). Whereas in PLP–Etd the observed value represents almost an average of both forms, in PLP–Et it is mainly determined by the ketoenamine tautomer.

## DISCUSSION

This work was aimed at determining whether any enolimine is formed from the PLP–Etd Schiff base in buffered aqueous solutions.

A comparative study of the PLP–Et and PLP–Etd Schiff bases revealed that (a) protonation of the amino group of the ethylenediamine moiety of PLP–Etd modifies the tautomer concentration ratio and (b) the low basicity of ethylenediamine favours formation of the Schiff base in neutral and acidic media.

The enolimine concentration in aqueous solutions is low for most PLP Schiff bases,[5–6] their concentration

ranges from 5 to 15% at pH 7. In fact, the PLP–Et enolimine concentration lies in this range.

In clear contrast, a high enolimine concentration was observed for PLP–Etd in aqueous buffered solutions at this pH. Therefore, one interesting finding of this study is that the enolimine and ketoenamine tautomers occur at similar concentrations at a neutral pH.

The effect of the amine basicity on the formation of the Schiff base can be quantitatively analysed at a neutral pH.

At a pH below the p$K_a$ for the amine, the reaction proceeds according to

$$\text{PLP} + \text{HAm} \rightleftharpoons \text{SB} + \text{H}^+ \quad (3)$$

The apparent formation constant, $K_{pH}$, is given by the equations

$$K_{pH} = K'_{pH}K_a \quad (4)$$

$$K'_{pH} = [\text{SB}]/([\text{PLP}][\text{Am}]) \quad (5)$$

If the tautomer ratio, $K_T$, and the ketoenamine formation constant, $K_{pH}^{keto}$, are defined by the equations

$$K_T = [\text{enol}]/[\text{keto}] \quad (6)$$

$$K_{pH}^{keto} = [\text{keto}]/([\text{PLP}][\text{Am}]) \quad (7)$$

$K'_{pH}$ can be expressed by

$$K'_{pH} = K_{pH}^{keto}(1 + K_T) \quad (8)$$

For two PLP Schiff bases derived from two amines having acid–base constants $K_{a_1}$ and $K_{a_2}$. The relationship between their stability constants is expressed by

$$\log\left[\frac{K_{pH}(1)}{K_{pH}(2)}\right] = \log\left\{\frac{K_{pH}^{keto(1)}[1 + K_T(1)]}{K_{pH}^{keto(2)}[1 + K_T(2)]}\right\} + \log\left[\frac{K_{a_1}}{K_{a_2}}\right] \quad (9)$$

By using $K_{pH}^{Etd} = 6200\,l\,mol^{-1}$ (the PLP–Etd formation constant), $K_{pH}^{Et} = 60{\cdot}7\,l\,mol^{-1}$ (the PLP–Et formation constant), $K_T(\text{Etd}) = 2{\cdot}1$, $K_T(\text{Et}) = 0{\cdot}15$, p$K_a$(Etd) = 8·2 and p$K_a$(Et) = 10·8, and pH = 7, a logarithmic ratio $\log[K_{pH}(\text{Etd})/K_{pH}(\text{Et})] = 3$ is obtained.

Since only the first acid–base dissociation of ethylenediamine is considered in this approach, this is consistent with the experimental results. Within experimental error, $K_{pH}^{Etd}$ is 100 times higher than $K_{pH}^{Et}$. These results suggest that the low basicity of the reactive amine stabilizes PLP–Etd at low pH values.

One interesting finding of this work is the existence of different electroreduction mechanisms for the tautomers that are consistent with the presence of significant concentrations of enolimine and the structure proposed in Scheme 1.

The conclusions arrived at in this work are of interest for understanding protein properties. Enolimine formation is widely accepted to indicate that the coenzyme PLP is embedded in a hydrophobic pocket.[5,6]

The above results show that the tautomer is actually formed in aqueous solution of model Schiff bases. A similar behaviour was observed in the reaction of PLP with polylysine.[19]

Even though the situation may be different in protein matrix environments, the presence of a lysine residue acting as a proton donor at the catalytic site should reasonably favour the formation of the enolimine tautomer in proteins.

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