ELECTROCHEMICAL AND SPECTROPHOTOMETRIC STUDY OF THE REACTIONS OF L-LEUCINE WITH PYRIDOXAL AND PYRIDOXAL PHOSPHATE

T. PINEDA[a], M. BLAZQUEZ[a,*], M. DOMINGUEZ[b] and F. GARCIA-BLANCO[c]

[a] *Departamento de Quimica Fisica y Termodinamica Aplicada, Facultad de Ciencias, Universidad de Cordoba, E-14004 Cordoba, Spain*
[b] *Departamento de Quimica Fisica, C/Prof. Garcia Gonzalez s/n, Universidad de Sevilla, E-41071 Sevilla, Spain*
[c] *Departamento de Fisicoquimica, Facultad de Farmacia, Universidad Complutense, E-28040 Madrid, Spain*

Received December 14, 1992
Accepted April 2, 1993

The reactions of L-leucine (Leu) with pyridoxal (PL) and pyridoxal-5′-phosphate (PLP) were studied over pH 6 – 13.5. Formation of the Schiff bases was indicated by UV-VIS absorption bands and reduction waves on a mercury electrode. A comparative electrochemical and spectrophotometric study on the reaction mixtures as a function of pH and the amino acid-to-aldehyde concentration ratio was carried out. Apparent formation constant, K_{pH} and acid-base constants for the Schiff bases were obtained. Reduction mechanisms of the imines are proposed in different pH zones. The formation constant, K_{So}, of an unprotonated Schiff base from unprotonated species of aldehyde and amino acid was determined by voltammetry. The results are compared to those obtained in analog Schiff bases in order to improve the quantitative description of the enzymatic models.

The reactions of pyridoxal (PL) and pyridoxal-5′-phosphate (PLP) with primary amines and amino acids have been studied by several authors in order to characterize the formation of Schiff bases as a straightforward model for the binding of the coenzyme (PLP) to a protein[1,2]. Schiff bases and aldehydes are electroactive species that yield reduction waves at different potentials. Earlier polarographic studies on Schiff bases involved a variety of aldehydes, yet none dealt with PLP[3 – 6].

In earlier work, we studied the electroreduction of this coenzyme[7 – 9] and its Schiff base with n-hexylamine and L-alanine[10 – 12]. The imine hydrolysis, aldehyde hydration and acid-base reactions of these compounds give rise to complex multi-equilibria in solution.

* The author to whom correspondence should be addressed.

This paper reports on electrochemical and spectrophotometric studies of the reactions of PL and PLP with L-leucine (Leu) focusing the effect of the amino acid residue in the stability and acid-base properties of the Schiff bases. A comparison with other related Schiff base is considered and differences in behaviour concerning the electroreduction process are also described. These studies provide a description of the equilibria involved in these reactions in order to be able to predict theirs properties in structural and functional research of proteins.

EXPERIMENTAL

Apparatus

Classical polarography (CP) and differential pulse polarography (DPP) recordings were obtained by using a Metrohm 626 polarograph. Potential step experiments were carried out on a Tacussel polarograph acting as potentiostat equipped with a Houston 2000 recorder. Voltammetric measurements were made by using a single-shot triangular wave generator and a fast potentiostat furnished with a built-in automatic compensator (from Belport) for the IR drop. Data were acquired by a Prowler digital oscilloscope.

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

Cells and Electrodes

Measurements were made in a thermostatted cell employing a saturated calomel reference electrode. The working electrode was a mercury capillary for polarographic and potential step experiments and a Metrohm EA 290 hanging mercury drop electrode for voltammetric measurements.

Solutions, Products and Measurements

PL, PLP and L-leucine were purchased from Sigma Chemical Company and were used without further purification. The Schiff bases of L-leucine with PL and PLP were obtained in solution by adding known amounts of the amino acid to PL and PLP solutions of known concentrations. These were prepared immediately prior to the electrochemical experiments and were freed from oxygen by purging with purified nitrogen. The initial concentration ratio of amine to aldehyde is stated for each experiment owing to the hydrolysis of the Schiff base – particularly in acidic and neutral media – and to the protonation reactions of the Schiff bases, PLP and PL, coupled to the formation reaction. All measurements were made at 25 ± 0.1 °C after the reaction had reached equilibrium. Buffered solutions consisting 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 ionic strength and pH were adjusted with $NaNO_3$ and NaOH, respectively.

The instantaneous currents (*i–t* curves) on single drop involved the following steps:

1. A polarogram was recorded to define potentials at the limiting zone.

2. From a starting potential where no Faradaic process occurred, a step was applied in order to find a potential on the plateau of the wave. The *i–t* curves were recorded for each first drop by manually synchronizing the potential application with the drop fall. This experiment was also performed in the absence of depolarizer to subtract residual current.

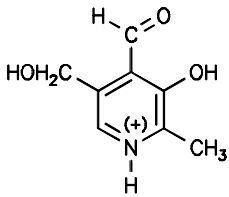
Kinetic parameters were obtained from the *i–E* curves for the Schiff base. A polarogram was recorded in order to choose an appropriate scale. The foot of the wave was recorded at full scale. The background current was eliminated by linear regression extrapolation. The Tafel slope was obtained from an *E* vs log *i* plot. Reaction orders were determined by studying the influence of pH.

RESULTS AND DISCUSSION

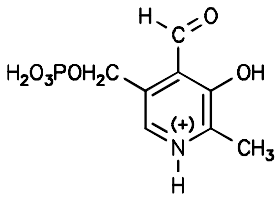
General Behavior

Protonated pyridoxal (PL) and pyridoxal-5′-phosphate (PLP) react with an amine group to yield a Schiff base according to the following reversible reaction (*A*):

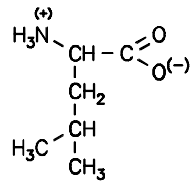
$$\text{R–CHO} + \text{R′–NH}_2 \rightleftharpoons \text{R–CH=N–R′} + \text{H}_2\text{O} \ . \qquad (A)$$



PL



PLP



L–Leu

Reaction (*A*) takes place in two steps[13]: The attack of the amine on the carbonyl group to yield a carbinolamine and the dehydration of the carbinolamine to a Schiff base. The former is the rate determining step (r.d.s.) in acid media, while the latter one is in basic media. The carbinolamine intermediate has been detected to occur in basic media by fast kinetic procedures[1,2].

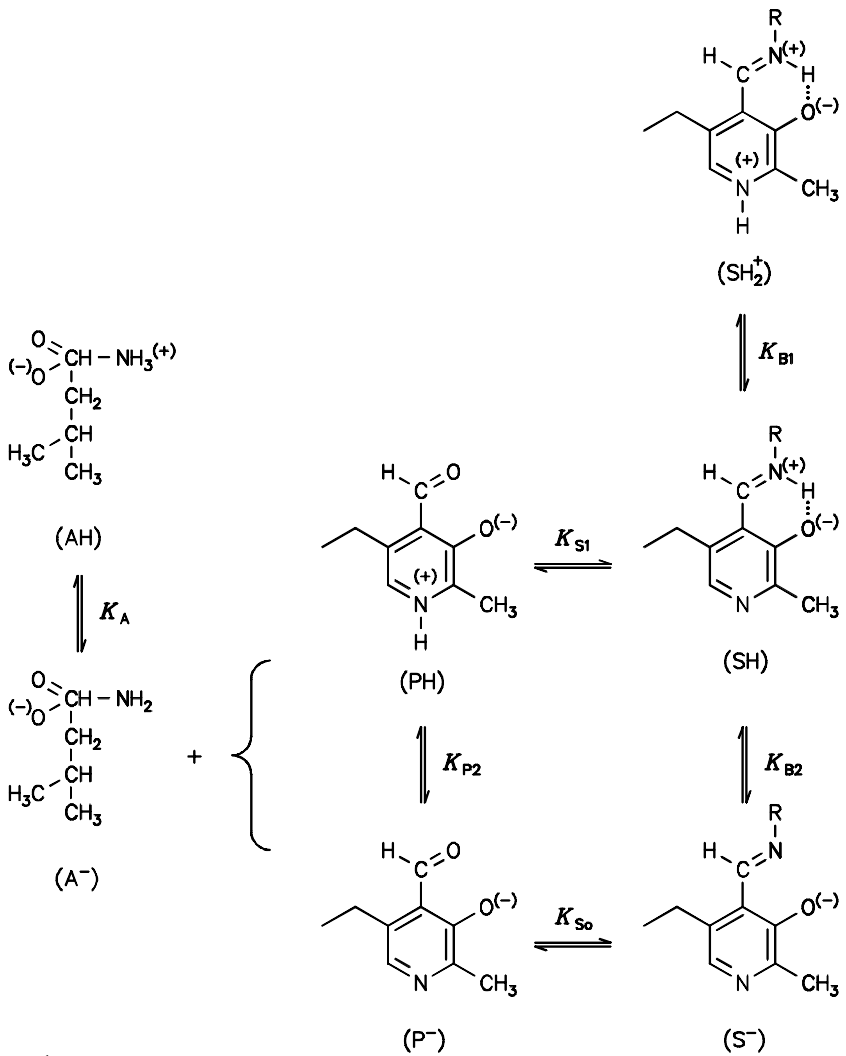
Taking into account all the protonation equilibria involving either the aldehyde or the Schiff base, a practical equilibrium constant K_{pH} can be defined for these systems[1,2] as

$$K_{pH} = \frac{[SB]}{[P]\,[A]} \ , \qquad (1)$$

where [SB], [P] and [A] are the total concentration of Schiff base, aldehyde and amino acid existing in equilibrium. The concentration [P] includes hydrated and free aldehyde (for PLP) and hemiacetal, hydrated and free aldehyde (for PL). K_{pH} is a function of pH and depends on the equilibrium constants of the ionic species (Scheme 1).

$$K_{pH} = f([H^+], K_{Si}, K_{Bi}, K_{Pi}, K_A) \ , \qquad (2)$$

where K_{Bi}, K_{Pi} and K_A are the respective macroscopic acid-base dissociation constants for the Schiff base, aldehyde and amino acid; and K_{Si} is the individual formation constant of the Schiff base. These constants are defined between species of the Schiff base and aldehyde in such a way that no proton is released nor taken up in the reaction (e.g. $K_{So} = [S^-]/[P^-][A^-]$, in Scheme 1).



SCHEME 1

Formation of the Schiff Base. Polarographic Study

The reaction mixture yields two reduction waves at a given initial amino acid/aldehyde concentration ratio. The first wave corresponds to the reduction of the Schiff base, while the second corresponds to the electroreduction of the aldehyde (PLP or PL). This was checked by studying the reduction of the aldehydes in the absence of the amino acid. In strong basic media, PLP yields three reduction waves, the limiting currents of which are functions of pH. In the presence of L-leucine, the first wave of the PLP is masked by the wave of the Schiff base. At high initial amino acid to PLP concentration ratio, the first wave clearly differentiated from second and third, corresponds to the electroreduction of the Schiff base. The amino acid yields no reduction wave in the absence of the aldehyde. Figures 1 and 2 show the variation of the limiting current in classical polarography with pH.

The formation of the Schiff base (first wave) from the PLP–Leu mixture is particularly favorable in the pH range 8 – 10. The total limiting current for the PL–Leu mixture increases over the pH range 6.5 – 8 and is pH-independent above pH 9. The formation of the PL hemiacetal[14] is responsible for this variation.

The instantaneous current (i–t curves) on single drop suggest diffusion control on the plateau of the first wave and the overall process over a wide pH range for both reaction mixtures. The slope of the log i_L vs log t is close to 0.19, the theoretical value for a diffusion process with spherical correction[15]. However, in strong basic media, mixed diffusion-kinetic control ($0.2 < \partial \log i_L / \partial \log t < 0.6$) and diffusion control ($\partial \log i_L / \partial \log t = 0.19$) are obtained for the top of the first wave (Schiff base) and the overall process, respectively, for the PLP–Leu mixture. The slope for a purely kinetic[15] process is 0.67. Slight kinetic control is observed for the first wave of the PL–Leu mixture.

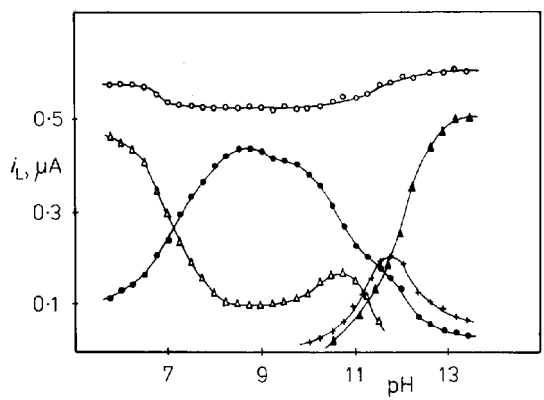
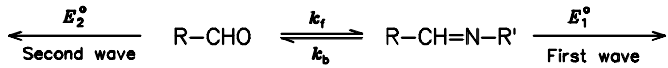


FIG. 1
Classical polarography. Variation of the limiting current with pH. Initial concentrations: $1 \cdot 10^{-4}$ M PLP, 0.01 M Leu. ● First wave (Schiff base); Δ second wave (PLP); + third wave (PLP); ▲ fourth wave (PLP); ❍ overall process

Under the experimental conditions used in this work, the electrode process can be represented by two electroactive species coupled to a pseudo first-order chemical reaction (Scheme 2).



SCHEME 2

Neither the hydration, nor the hemiacetal formation are considered in this scheme. The approach is experimentally supported for PLP in neutral and basic media, and for PL above pH 8.5 (Figs 1, 2).

Assuming that the chemical reaction is slower than the electrode process (*i–t* curves) the Eqs (*3*) and (*4*) can be applied in the limiting zone of the first wave[10]

$$\frac{1}{i_{L1}} = \frac{1}{i_D} + \frac{1}{i_D K_{pH}[A]} \qquad (3)$$

$$K_{pH} = \frac{1}{[A]} \frac{I_{L1}}{1 - I_{L1}} \quad , \qquad (4)$$

where i_{L1} and i_D are the limiting current of the first wave and the diffusion current of the overall process, respectively, and $I_{L1} = i_{L1}/i_D$, the normalized limiting current for the first wave. Other symbols were previously defined. Under high experimental ratios of amino acid to aldehyde, [A] can be taken as initial concentration of amino acid.

At a constant pH, linear plots according to Eq (*3*), $1/i_{L1}$ vs 1/[A], were obtained for both reaction mixtures and i_D calculated from the intercept agrees well with the experimental value. This fact confirms the validity of the approach and from the slope,

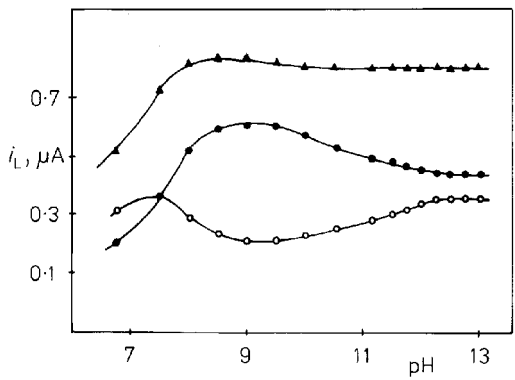


FIG. 2
Classical polarography. Variation of the limiting current with pH. Initial concentration: 1 . 10^{-4} M PL, 0.05 M Leu. ● First wave (Schiff base); ❍ second wave (PL); ▲ overall process

K_{pH} can be obtained. On the other hand, from the normalized limiting current of the first wave, K_{pH} as a function of the pH is obtained from Eq. (*4*) for both reaction mixtures.

A strong variation is observed for the Schiff base of PLP in the whole pH interval. The apparent stability is higher for the Schiff base of PLP than for that of PL (Fig. 3). These variations are due to protonation, hydration and hemiacetal reactions coupled to the formation of the Schiff base. The ratio K_{pH} is an uncorrected formation constant involving hydrated and hemiacetal species but it can be regarded as a parameter indicating the availability of free aldehyde that reacts with amino acid. This features possible differences in the reactions of PLP and PL with amino groups of proteins.

Other case regarding the chemical reaction involved in Scheme 2 corresponds to a fast chemical reaction, namely CE type process. These processes were studied theoretically by Koutecky[16] and Matsuda and Ayabe[16]. More recently, a modified expression was reported[17]

$$\frac{i_{L1}}{i_D - i_{L1}} = 1.386\ (K^2kt)^{0.545}\ , \qquad (5)$$

where $K = k_f/k_b = [SB]/[P]$, $k = k_f + k_b$ and t is the drop time. A convenient form to rearrange this equation is given by Eq. (6)

$$\frac{i_D}{i_{L1}} = [1.386\ (K^2k)^{0.545}]^{-1}\ t^{-0.545} + 1\ . \qquad (6)$$

The kinetic effect observed in the limiting zone in strong basic media have been checked by using Eq. (*6*). At pH 10 a horizontal line is obtained that indicated no kinetic control. This behaviour is also observed at pH < 10. In strong basic media, a

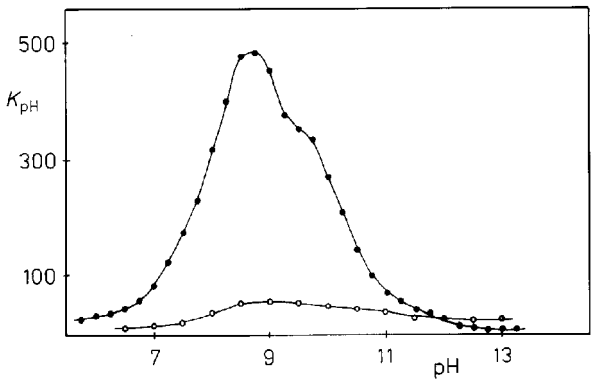


FIG. 3
Variation of K_{pH} with pH. ● PLP–Leu Schiff base; ❍ PL–Leu Schiff base

linear variation is obtained in the i_D/i_{L1} vs $t^{-0.545}$ plot with an unity intercept and a slope proportional to $(K^2\,k)^{-0.545}$ that are consistent with Eq. (*6*) and indicative of a kinetic contribution to the limiting current. Therefore, the K_{pH} value, calculated from Eq. (*4*) for strongly basic media has some contribution of the Schiff base formed at the electrode on the time scale of the polarographic method.

UV-VIS Spectrophotometric Study

UV-VIS absorption spectra for the reaction mixture were studied as a function of pH. The formation of the Schiff base of PLP is indicated by absorption bands at 415 and 280 nm (refs[1,2]). In strongly basic media, stability decreases as indicated by the variation of K_{pH} vs pH (Fig. 3). The shift of the main band is from 415 to 385 nm. This reflects the influence of the free coenzyme (390 nm) and free base of the imine, S^- ($\approx$ 370 nm). The variations of the absorbances at 415, 385 and 280 nm with pH show inflections at pH 11.2 ± 0.1, that corresponds to pK_{B2} (Fig. 4*a*, Scheme 1, Table I). In neutral and weakly acidic media the hydrolysis of the Schiff base occurs (Fig. 3) and the influence of the free coenzyme appears at 390 nm. The difference between inflections in the absorbance vs pH curve at 415 and 280 nm reflected this effect. From the variation of the absorbance at 280 nm with pH an estimate of pK_{B1} 6.2 is obtained (Fig. 4, Scheme 1, Table I).

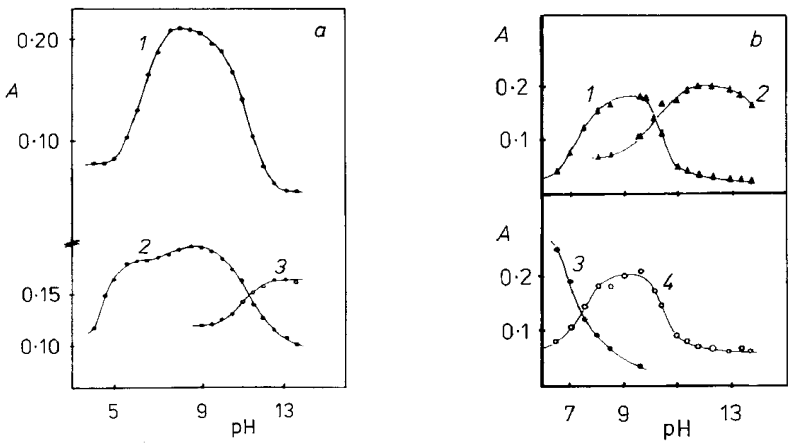


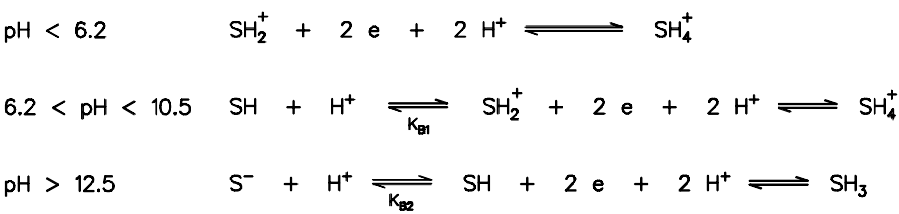
FIG. 4

Spectrophotometric results of the reaction mixtures. Initial concentrations: 2 . 10^{-5} M aldehyde; 0.11 M amino acid. Variation of the absorbance with pH: *a* PLP–Leu mixture, λ: *1* 280 nm, *2* 415 nm, *3* 385 nm. *b* PL–Leu mixture, λ: *1* 414 nm, *2* 367 nm, *3* 320 nm, *4* 282 nm.

The formation of the Schiff base of PL is indicated by absorption bands at 414 and 282 nm. In strongly basic media, the main band shifts from 414 to 367 nm, which indicates the formation of the free imine, S^-. From the variation of the absorbance at 414, 367 and 282 nm an inflection at pH 10.4 ± 0.1 is obtained, that corresponds to pK_{B2} (Fig. 4, Scheme 1). The concentration of the Schiff base decreases strongly in weakly basic media by the formation of the hemiacetal (maximum wavelength 320 nm). Estimate of pK_{B1} is not reached in this case.

Electrode Process

Polarographic and kinetic parameters for the reduction wave of the PLP–Leu Schiff base were obtained over a wide pH range. The plot of $E_{1/2}$ (or the peak potential, E_p) against pH shows, several linear segments from which slopes of –61, –89, –50 and –80 mV/dec were estimated for pH < 6.5, 6.5 < pH < 11.6, 11.6 < pH < 12.6 and pH > 12.6. Partial overlap of the first and second wave occurs in the pH range 11 – 12. The logarithmic analysis of the first wave as E vs log $i/(i_L - i)$ yielded a linear segment with a slope close to –29 mV/dec at pH < 9.5 and pH > 11.0. These results are consistent with a two-electron transfer coupled to fast protonation reactions, which is typical of Schiff bases[5,6]. The plot of E vs log $i/(i_L - i)$ varies with pH in the form of an S-shaped curve over the pH range 9.5 – 11. This variation corresponds to a polarographic $pK' = 10.5$. In Scheme 1 are shown the main species involved in the electroreduction of the Schiff base.

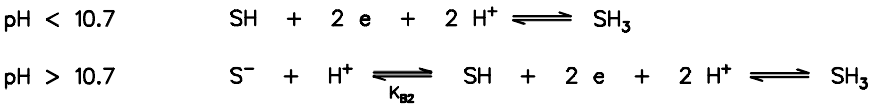


SCHEME 3

The electrode process taking place depending on pH is shown in Scheme 3. As a rule, the protonated imine is electroactive over a wide pH range[5,6]. Below pH 6.5, the dissolved species, SH_2^+ (also protonated at the ring nitrogen), is electroactive according to our results. Above pH 6.5, the dissolved species in solution, SH, recombines with an H^+ ion to yield SH_2^+. The rate of this reaction decreases as the pH increases. The polarographic $pK' = 10.5$ is related to this reaction. Under our experimental conditions (drop time), and taking into account the value of $pK_{B1} = 6.2$, one can estimate the rate constant of this reaction to be $\rho = 3.9 \cdot 10^{-3}\ s^{-1}$ according to Mairanovskii[18]. The pK_A of the amino acid is 9.74 (ref.[19]). Therefore, the zwitterionic amino acid can take part

in the reaction as a proton donor. Above pH 10.5, our results show electroreduction of species SH^+. At pH 12.6, two linear segments in the $E_{1/2}$ vs pH plot intersect, which may be related to pK_{B2}. The difference between polarographic and spectrophotometric values can be ascribed to the kinetic contribution at the top of the first wave in strong basic media. Above pH 12.6, the species present in the solution is S^-. The polarographic results suggest recombination with a H^+ ion to yield SH. Scheme 3 should have included the formation of the Schiff base. However, this reaction is slow enough over a wide pH range to result in diffusion control on the plateau of the first wave. Therefore, the behavior is similar to those observed for a pH-dependent analytical concentration of the Schiff base in the bulk solution in the absence of a prior formation reaction.

Only two linear segments are obtained in the $E_{1/2}$ vs pH plot for the reduction wave of the PL–Leu Schiff base, the slopes of which are –60 and –94 mV/dec below and above pH 10.7, respectively. These segments intercept near the pK_{B2} value obtained from absorption spectra. The logarithmic analysis of the wave as E vs log $i/(i_L - i)$ yields a linear segment with a slope close to –30 mV/dec throughout pH range. Our results are consistent with a reversible two-electron reduction process coupled to fast protonation reactions (Scheme 4). The reduction of species SH_2^+ is not observed owing to its low concentration. In a weakly basic medium, the hydrolysis of the imine and the competitive formation of the hemiacetal (maximum wavelength, 320 nm) do not favor formation of this species. Below pH 10.7, the reduction of the protonated imine, SH, is indeed observed.



SCHEME 4

TABLE I
Electrochemical and spectrophotometric results for the Schiff bases of PLP and PL with L-leucine

Schiff base	pK_{B1}		pK_{B2}		K_{So}, l/mol
	(s)	(e)	(s)	(e)	
PLP	6.2	6.5[a]	11.2	11.6 – 12.6	2.4
PL	–	–	10.4	10.7	15.8

(s) Spectrophotometric; (e) electrochemical. [a] $pK' = 10.5$ for the recombination of SH with H^+.

The difference between pK_{B2} and the second polarographic pK (Table I) is lower for the Schiff base of PL than for that of PLP. This is a result of the small kinetic contribution at the top of the first wave in the Schiff base of PL. The kinetic effect may be related to an increase in the carbinolamine concentration[20]. In both Schiff bases, the dissociation pK_{B2} of the conjugate acid of the imine is higher than the pK_A of the zwitterionic amino acid. As a rule, the dissociation pK_{B2} of a Schiff base lies 2 to 5 pH units below the dissociation pK of the conjugate acid of the amino group reactant[13]. The results obtained at the foot of the first wave confirm the above electrode process. Thus, for PLP Schiff base the Tafel slope is independent of the pH, having an average value of −30 mV at 25 °C. The reaction order with respect to the H^+ ion concentration is 3 in the pH range of 6.5 – 10.5. For PL Schiff base, Tafel slope has also an average of −30 mV/dec at 25 °C. The reaction order with respect to the H^+ ion concentration are 2 below pH 10.7 and 3.1 at above pH 10.7, respectively. The orders are independent of the potential in the zone where Tafel's law is obeyed.

Voltammetric Behavior

The kinetic effect at the top of the first wave in strong basic media was analyzed by linear scan cyclic voltammetry. Two main peaks were observed above pH 13 for the reaction mixture PLP–Leu. First peak corresponds to the Schiff base and second one to the electroreduction of PLP. The second peak corresponds to the more cathodic peak of PLP observed in polarography. However, one or two smaller peaks were also observed at intermediate potential between above two, which were negligible at $v > 1$ V/s. No anodic peak was observed in the scan interval investigated.

The $i_P/v^{1/2}$ ratio decreased as the scan rate increased for the first peak, which confirms that the electrode process is of CE-type, the chemical reaction involved being the formation of the Schiff base. At high scan rates, this ratio is scan rate independent, so the aldehyde–Schiff base equilibrium is not altered during the measurement period. The second peak shows a similar variation. However, the decrease in $i_P/v^{1/2}$ is less marked than for the first peak. Under these conditions, the equilibrium concentrations of the Schiff base and PLP are proportional to the diffusion current.

The limiting diffusion current for PLP at the same initial concentration, but in absence of the amino acid, was also determined. By comparing this ratio under diffusion control at a high scan rate with the extrapolated value at $v \rightarrow 0$, an estimation of the free aldehyde concentration was obtained. In this pH zone, PLP occurs mainly as free aldehyde, even though a low concentration of hydrate is also present in solution. The cathodic peak of PLP corresponds to an irreversible process.

The concentration of PLP as free aldehyde in equilibrium with the Schiff base was obtained by comparing results obtained in the presence and absence of amino acid at a scan rate where diffusion control was prevalent.

From the theoretical peak current for reversible and irreversible processes in voltammetry[21] and, assuming similar diffusion coefficients for both electroactive species, the concentration of the Schiff base (S^-) can be expressed as

$$c_S = 0.786\,(\alpha n_a)^{1/2}\,\frac{s_S}{s_P}\,c_P \quad , \qquad (7)$$

where s_S and s_P are the slopes of the linear plot of i_P vs $v^{1/2}$ at high scan rates for the Schiff base and PLP, respectively. The value of αn_a for PLP was determined from $E_P - E_{p/2}$. From these results and, taking into account the concentration of leucine, the formation constant was obtained. The method was also applied to PL and its Schiff base. The calculated values are estimates of K_{So} for these Schiff bases since unprotonated species are present in solution at this pH (Table I). Under different conditions, the experimental determination of other individual formation constants is complicated by the occurrence of acid-base and tautomeric equilibria.

Schiff Bases of PLP and PL

By comparing with other Schiff bases of PLP the maximum value of K_{pH} of PLP–Leu (500 l/mol) is higher than for PLP–Ala (120 l/mol) and the results confirm the lower stability of the Schiff bases derived from amino acid in comparison to n-hexylamine (1 500 l/mol) (ref.[12]). The formation constant, K_{So}, for unprotonated Schiff base is very similar for both amino acids (PLP–Ala, 3.4 l/mol; PLP–Leu, 2.4 l/mol) but lower than for PLP–hex (33.0 l/mol^{-1}). The Schiff base PLP–hex has been studied as a simple model of the binding of PLP to protein simulating a terminal lysine of the enzyme. This behavior shows the influence of the amino residue in the stability of the Schiff base.

On the other hand in these Schiff bases, $\Delta pK = pK_{B2} - pK_A$ is greater than one unity of pH, namely 2.4, 2.0 and 1.5 for PLP–hex, PLP–Ala and PLP–Leu, respectively. This finding is in agreement with a high stabilization of the conjugate acid of the imine by a hydrogen-bonding with the *o*-hydroxyl group[1,2].

The electrode process is similar for the Schiff bases derived from amino acid showing reduction of species SH_2^+ and SH. However, in PLP–hex reduction of species SH_2^+ is not reached at moderate concentration of amine due to the adsorption of hexylamine that inhibited the recombination reaction.

For PL–Leu, the K_{pH} vs pH plot shows a slight bell shaped variation. However, in the Schiff base of PL–hex a plateau is observed in basic medium[11]. The apparent formation constant reached a maximum value (620 l/mol) higher than in PL–Leu (55 l/mol). The value of ΔpK is 0.2 and 0.6 for PL–hex and PL–Leu, respectively.

The electrode process only shows the electroreduction of species SH. The influence of the hemiacetal formation produce a low concentration of SH_2^+ species.

By comparing PL and PLP Schiff bases, the formation constant is higher in the Schiff base of PLP and the stability decreases from amine to amino acid residue in both cases. There is also a lower stabilization of the hydrogen bonding in the PL Schiff bases as it is indicated by the difference between p*K* of the conjugate imine and amine.

This work was supported by DGICYT (Project PB88-0284-C03-01) and the Junta de Andalucia. One of us (T. P.) is grateful to the Junta de Andalucia for award of a pre-doctoral fellowship.

REFERENCES

1. Leussing D. L. in: *Vitamin B_6 Pyridoxal Phosphate: Chemical, Biochemical and Medical Aspects*, Part A (D. Dolphin, R. Poulson and O. Avramovic, Eds), Chap. 4, p. 69. Wiley, New York 1986.
2. Kallen R. G., Korpela T., Martell A. E., Matsushima Y., Metzler C. M., Metzler D. E., Morozov Y. V., Ralston I. M., Savin F. A., Torchinsky Y. M., Ueno H. in: *Transaminases* (P. Christen and D. E. Metzler, Eds), p. 19. Wiley, New York 1985.
3. Zuman P.: Nature *165*, 485 (1950).
4. Zuman P.: Collect. Czech. Chem. Commun. *15*, 839 (1950).
5. Kitaev Yu. P., Troepolskaya T. V. in: *Progress in Electrochemistry of Organic Compounds* (A. N. Frumkin and A. B. Ershler, Eds), p. 43. Plenum Press, London 1971.
6. Lund H. in: *The Chemistry of Functional Groups. The Chemistry of the Carbon–Nitrogen Double Bond* (S. Patai, Ed.), Ch. 11, p. 533. Interscience, London 1970.
7. Izquierdo R., Blazquez M., Dominguez M., Garcia-Blanco F.: Bioelectrochem. Bioenerg. *12*, 25 (1984).
8. Blazquez M., Izquierdo R., Garcia-Blanco F., Dominguez M.: Bioelectrochem. Bioenerg. *16*, 325 (1986).
9. Izquierdo R., Dominguez M., Garcia-Blanco F., Blazquez M.: J. Electroanal. Chem. *266*, 357 (1989).
10. Pineda T., Blazquez M., Dominguez M., Garcia-Blanco F.: J. Electroanal. Chem. *294*, 179 (1990).
11. Pineda T., Sevilla J. M., Blazquez M., Garcia-Blanco F., Dominguez M.: J. Electroanal. Chem. *304*, 53 (1991).
12. Dominguez M., Sevilla J. M., Garcia-Blanco F., Blazquez M.: Bioelectrochem. Bioenerg. *16*, 317 (1986).
13. Jencks W. P. in: *Catalysis in Chemistry and Enzymology*, p. 490. McGraw-Hill, New York 1969.
14. a) Matsushima Y., Martell A. E.: J. Am. Chem. Soc. *89*, 1322 (1967); b) Heyl D., Luz E., Harris S. A., Folkers K.: J. Am. Chem. Soc. *73*, 3430 (1951); c) Metzler D. E., Snell E. E.: J. Am. Chem. Soc. *77*, 2431 (1955).
15. a) Guidelli R. in: *Electroanalytical Chemistry* (A. J. Bard, Ed.), Vol. 5, p. 281. Dekker, New York 1971; b) Kuta J., Smoler I. in: *Progress in Polarography* (P. Zuman and I. M. Kolthoff, Eds), Vol. 1, p. 43. Interscience, New York 1962.
16. a) Koutecky K.: Collect. Czech. Chem. Commun. *18*, 597 (1953); *20*, 116 (1955); *21*, 1056 (1956); b) Matsuda H., Ayabe Y.: Bull. Chem. Soc. Jpn. *28*, 422 (1955); c) Munoz E., Camacho L., Avila J. L., Heras A. M., Ruiz J. J.: Bull. Soc. Chim. Belg. *96*, 255 (1987); d) Migchielsen P. W. C., Sluyters-Rehbach M., Sluyters J. H.: J. Electroanal. Chem. *44*, 301 (1973).
17. Rodriguez J. M., Camacho L., Ruiz J. J.: Electrochim. Acta *29*, 1493 (1984).

18. Mairanovskii S. G.: *Catalytic and Kinetic Waves in Polarography*, Chap. 4. Plenum Press, New York 1968.
19. Dawson R. M. C., Elliott D. C., Elliott W. H., Jones K. M.: *Data for Biochemical Research*, 3rd ed.. Clarendon Press, Oxford 1986.
20. Metzler C. M., Cahill A., Metzler D. E.: J. Am. Chem. Soc. *102*, 6075 (1980).
21. Bard A. J., Faulkner L. R.: *Electrochemical Methods, Fundamentals and Applications*, p. 213. Wiley, New York 1980.