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THE SCHIFF BASE BETWEEN PYRIDOXAL-5′-PHOSPHATE (PLP) AND HEXYLAMINE. FORMATION OF THE UNPROTONATED FORM OF THE IMINE BY REACTION OF THE UNPROTONATED PLP AND FREE AMINE

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Electrochemical and spectrophotometric studies of the pyridoxal-5′-phosphate hexylamine Schiff base (PHSB) in strongly basic media were carried out. The equilibrium constant and rate constants of the formation and hydrolysis of the unprotonated imine from the unprotonated pyridoxal-5′-phosphate and free amine were determined by linear-scan cyclic voltammetry. The acid-base dissociation constant of the imine group was obtained by spectrophotometric measurements. Absorption bands for the free imine and the conjugate acid are characterized by a log-normal distribution. These studies are for general application to the quantitative characterization of non-enzymatic model compounds.

INTRODUCTION

The importance in enzymatic catalysis of pyridoxal-5′-phosphate (PLP) is well known.[1] The binding of PLP to protein is through a Schiff base with a lysine residue. Schiff bases of PLP have been studied in order to characterize their chemical behaviour and to correlate it with biological compounds.[1–3] The Schiff base of PLP with hexylamine (PHSB) has been studied as a simple model of this binding.[4] Electrochemical[5,6] and spectrophotometric results[6] allowed to some equilibria and protonation constants of the Schiff base to be characterized.

The overall reaction between PLP and hexylamine is

$$\mathrm{R{=}CHO} + \mathrm{R'NH_2} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{RCH{=}NR'} \qquad (1)$$

PHSB and PLP have at least four proton-accepting groups each and therefore show several macroscopic pK values. In addition, different tautomeric equilibria are involved in these substances and there are neutral and ionic species in solution.[2,3]

Taking into account all the protonation equilibria involved in both the coenzyme and the Schiff base, a practical equilibrium constant K_{pH} is defined in these systems[2] by

$$K_{\mathrm{pH}} = \frac{[\mathrm{SH}]_\mathrm{T}}{[\mathrm{PLP}]_\mathrm{T}[\mathrm{A}]_\mathrm{T}} \qquad (2)$$

where $[\mathrm{SH}]_\mathrm{T}$, $[\mathrm{PLP}]_\mathrm{T}$ and $[\mathrm{A}]_\mathrm{T}$ are the equilibrium concentrations of the respective species in the different protonation stages of the Schiff base, PLP and amine. K_{pH} is a function of pH and depends on the equilibrium constants of the ionic species:

$$K_{\mathrm{pH}} = \mathrm{f}([\mathrm{H}^+],\ K_{\mathrm{S}i}, K_{\mathrm{B}i}, K_{\mathrm{P}i}, K_\mathrm{A}) \qquad (3)$$

where $K_{\mathrm{B}i}, K_{\mathrm{P}i}$ and K_A are the macroscopic acid-base dissociation constants for the Schiff base, PLP and amine, respectively; $K_{\mathrm{S}i}$ represents individual formation constants of the Schiff base. The constants are defined

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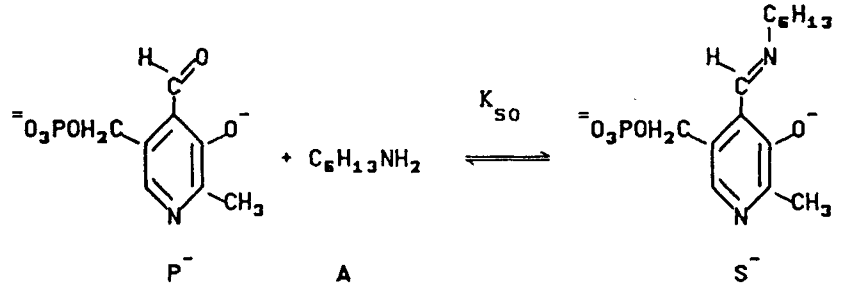


Scheme 1

between species of the Schiff base and aldehyde in such a way that proton is neither released nor taken up in the reaction (for example, $K_{SO} = [S^-]/[P^-][A]$, Scheme 1).

Equation (3) is usually expressed as a function of K_{SO} and the acid–base dissociation constants:

$$K_{pH} = g([H^+], K_{SO}, K_{Bi}, K_{Pi}, K_A) \quad (4)$$

because K_{SO} can be determined experimentally at high pH where unprotonated species exist in solution. Under different conditions the determination of other individual formation constants is complicated by the existence of multiple equilibria in solution (acid–base ionization, tautomeric equilibria and solvation effects).

Different studies have focused on the experimental determination of K_{SO} in model compounds.[2,3] In spectrophotometric studies, the absorption band of PLP partly overlaps either the band of the conjugate acid of the imine or the band of the free imine in strongly basic media. This is due to the low stability of the free imine species (low K_{SO}). The overlap is observed even at a high concentration ratio of the amine to the aldehyde. Some kinetic studies on the formation and hydrolysis of the Schiff base at lower pH have been reported.[7–10] From the analysis of the curves of apparent rate constant vs pH an estimate of K_{SO} was obtained.[7–9] In a few cases, fast kinetic methods have been used in the determination of individual rate constants of both the formation step and the further dehydration of carbinolamine.[3]

This paper describes a study of the reaction of free hexylamine with the unprotonated PLP to give the completely unprotonated form of the Schiff base. We analysed electrochemical and spectrophotometric data in order to determine K_{SO} and the rate constants of formation and hydrolysis. From an electrochemical point of view the different reduction potentials of the imine and PLP in strongly basic media are an advantage. The kinetics of the reaction were followed by applying a fast technique such as linear-scan cyclic voltammetry. In addition, kinetic parameters of the reaction were checked by a chronoamperometric method on a dropping mercury electrode. A general knowledge of the electrochemical behaviour of the PLP Schiff base reaction is useful. We have recently contributed to a general study of different Schiff bases of PLP and related compounds and some general conclusions were drawn.[5,11,12] Finally, by fitting UV–visible spectra with functions of four parameters (position, height, width and skewness), a log-normal[13] characterization of the absorption bands of the free imine and the conjugate acid was obtained. These studies are for general application to the quantitative characterization of nonenzymatic model compounds.

EXPERIMENTAL

Pyridoxal-5′-phosphate was purchased from Sigma. All other chemical were supplied by Merck and were of reagent grade. Phosphate and carbonate buffers were used. Ionic strength was adjusted with potassium chloride. All measurements were made at 25 ± 0·1 °C.

D.c. polarograms were recorded by means of an Amel polarograph. A saturated calomel electrode (SCE) was used as a reference electrode. The working electrode was a mercury capillary. All measurements were made in a nitrogen atmosphere.

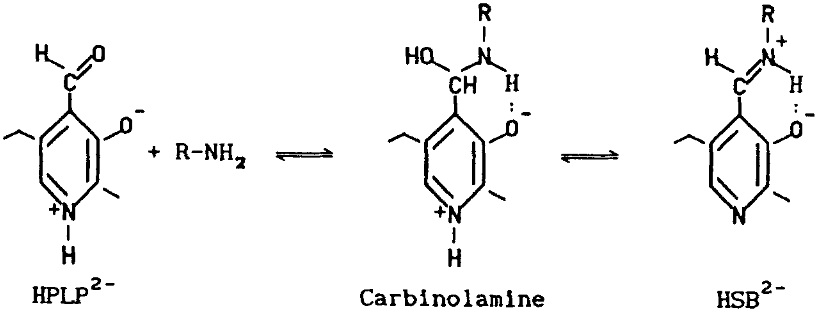
Linear-scan cyclic voltammetry (LSCV) measurements were carried out with a Belport 305 wave generator and a Belport 105 fast potentiostat compensating for *IR* drop. Data were collected with a Norland Prowler digital oscilloscope. The working electrode was a Metrohm EA 290 hanging mercury drop electrode.

Spectrophotometric measurements were performed on a Varian Cary 219 spectrophotometer with 1-cm quartz cuvettes.

The Schiff base was obtained by adding known amounts of hexylamine to PLP solutions of known concentrations. In all cases the electrochemical and spectrophotometric studies were carried out after the reaction reached equilibrium.

RESULTS AND DISCUSSION

The general study of the Schiff bases indicated that the formation occurs in two steps.[14] The first reaction is the attack of the free amine on the carbonyl group to yield a carbinolamine and the second is the dehydration of



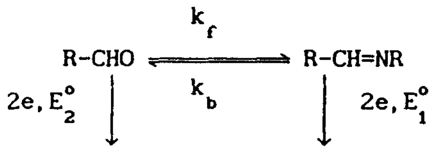
Scheme 2

this carbinolamine intermediate. The first reaction is the rate-determining step in an acidic medium and the second reaction in a basic medium.

In the Schiff bases of PLP a rate-limiting dehydration of the carbinolamine following a fast pre-equilibrium was observed[2,3] (Scheme 2). The equilibrium concentration of the carbinolamine is usually very low and its presence can be detected only by indirect methods. This low concentration of carbinolamine can be neglected in the evaluation of the equilibrium constant for the overall reaction. This approach has been used in the analysis of the kinetic profiles of the formation and hydrolysis of the Schiff base and global constants k_1 and k_{-1} in different Schiff bases of PLP were estimated.[7-10] In these studies, the formation equilibrium constant, $K_{\mathrm{pH}} = k_1/k_{-1}$ [reaction (1)], showed a pH dependence according to the predictions of equation (3). From the kinetic analysis, evidence of hydroxide catalysis[10] in the hydrolysis of the Schiff base and intramolecular general acid–base catalysis for the formation of the Schiff base was obtained and microscopic equilibrium and rate constants were evaluated.[7-9]

General electrochemical behaviour

In a basic medium the electrochemical results agree with these conclusions. Under the experimental conditions, reaction (1) can be simplified by two pseudo-first-order chemical reactions with $k_f = k_1 c_A$ (Scheme 3). The rate constants are pH dependent owing to protonation equilibria and the acid–base catalysis involved. The electrochemical results account for the reduction of both electroactive species, Schiff base and PLP. At pH < 11 two waves are obtained in d.c. polarography for the PLP–Schiff base equilibrium (Figure 1).



Scheme 3

The first wave corresponds to a two-electron reduction of the imine group of the PHSB and the second wave to a two-electron reduction of the carbonyl group of the PLP.

The total limiting current is independent of pH. Diffusion control in the limiting current of the first and overall process is observed ($\mathrm{d}\log i_L/\mathrm{d}\log t \approx 0{\cdot}2$, Figure 2).[15,16] This allows the calculation of the equilibrium concentrations of the electroactive species in solution[6] by using

$$\frac{i_{L1}}{i_D} = \frac{K}{K+1} \qquad (5)$$

where i_{L1} and i_D are the limiting current of the first wave and the overall process, respectively, and $K = k_f/k_b = K_{\mathrm{pH}} c_A$. This behaviour indicates that under polarographic conditions, the parameter kt (with

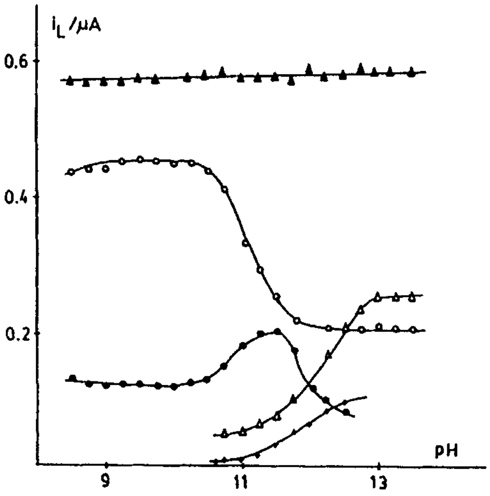


Figure 1. D.c. polarography: variation of the limiting current with pH. $c_{\mathrm{PLP}} = 1 \times 10^{-4}$ M; $c_{\mathrm{hex}} = 4 \times 10^{-3}$ M. (○) Schiff base; (●) PLP1; (+) PLP2; (△) PLP3; (▲) overall process

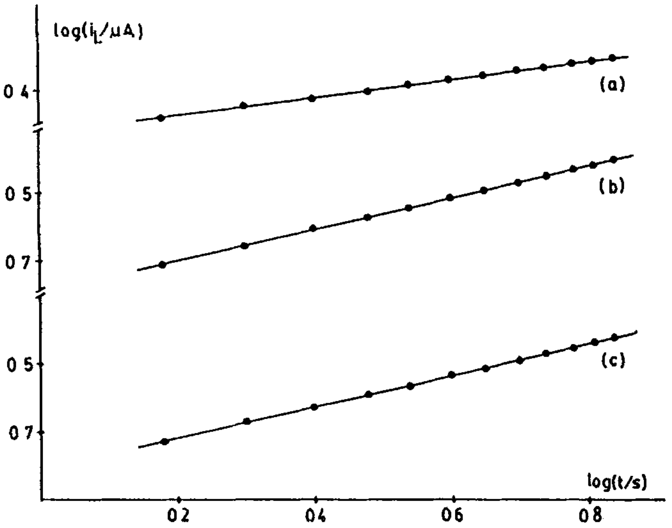


Figure 2. Log i_L vs log t. $c_{PLP} = 1 \times 10^{-4}$ M; $c_{hex} = 4 \times 10^{-3}$ M. (a) pH = 10; (b) pH = 12; (c) pH = 13·2

$k = k_f + k_b$ and t is the drop time) is low enough and the perturbation of the equilibrium concentrations due to the reduction of the Schiff base is not compensated for by the chemical reaction at the electrode (Scheme 3). This agrees with the slow dehydration of carbinolamine. However, owing to its low concentration, the evaluation of K_{pH} from equation (5) is not significantly affected.[5,6]

At pH > 10·5 two new waves are observed. This is due to a decrease in the stability of the Schiff base and indicates the presence of the PLP in solution. The limiting current of the first wave decreases with increase in pH, resembling an acid–base dissociation curve with an inflection close to pH 11. Earlier studies on the electroreduction of PLP over a wide pH range showed one, two or three waves for which limiting current depends on the pH and the buffer composition.[17,18] In strongly basic media these waves decrease with increase in pH and a new wave at a more negative potential is observed.[18]

In the presence of an amino acid or amine, the formation of the Schiff base is indicated by the first wave. The second and third waves are minor and correspond to electroreduction of the protonated species of PLP. They appear as residual waves depending on the pH and the experimental conditions. The more cathodic wave corresponds to a two-electron reduction of the unprotonated PLP.

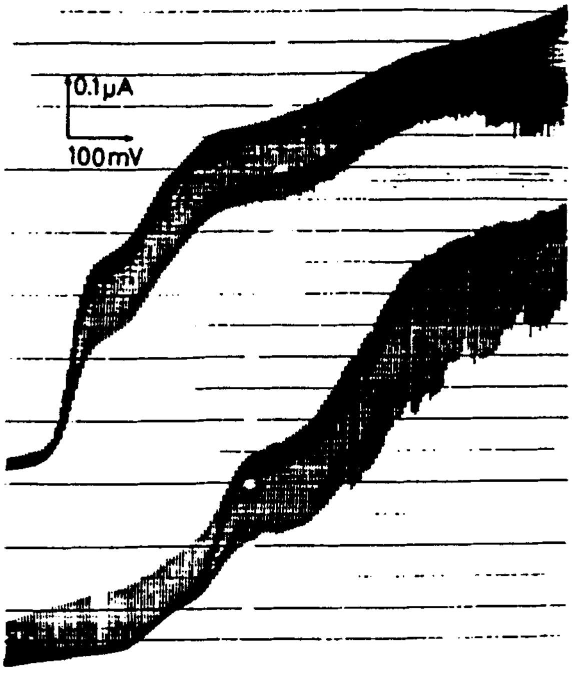


Figure 3. D.c. polarography: initial potential −900 mV; pH = 12. Lower i–E curve $c_{PLP} = 1 \times 10^{-4}$ M; upper i–E curve, $c_{PLP} = 1 \times 10^{-4}$ M, $c_{hex} = 4 \times 10^{-3}$ M

This behaviour is shown in Figure 3. Note that in Ref. 5, although the presence of these waves was indicated in the text, the limiting current of first (Schiff base) and second (PLP) is shown only in Figure 2 in that paper, owing to poor resolution. In this work, the ionic strength was adjusted to 0·5 M with potassium chloride, to keep the appropriate conditions for LSCV, and an approximate estimate of these waves was achieved at pH < 12·5. The total limiting current of the process is independent of pH. In this pH range, the electrochemical results show that the limiting current of the first wave has contributions from kinetic and diffusion control[15,16] ($0{\cdot}2 < \mathrm{d}\log i_L/\mathrm{d}\log t < 0{\cdot}67$, Figure 2) and in the limiting zone of the overall process (top of the more cathodic wave) diffusion control ($\mathrm{d}\log i_L/\mathrm{d}\log t \approx 0{\cdot}2$) is obtained. The kinetic contribution on the top of the first wave indicates that the formation of the Schiff base by fast dehydration of the carbinolamine is detected at the electrode (Scheme 3).

An $E_{1/2}$ vs pH plot is shown in Figure 4. For the first wave, two linear segments are obtained with a break at pH 11·5. The slopes of the plots at pH < 11·5 and pH > 11·5 are −63·8 and −90·8 mV per decade, respectively. The slope of the logarithmic plot of E vs $\log i/(i_L - 1)$ is −30 mV per decade. These results indicated a reversible two-electron transfer coupled with two or three protons, the electroactive species being monoprotonated in the —C=N— group. This agrees with the general behaviour in the electroreduction of imines.[19,20] In strongly basic media, the last wave is independent of pH and for the logarithmic analysis the electroreduction of the unprotonated PLP appears irreversible.

Schiff bases of PLP with different amino acids and amines show protonation of the imine ($\mathrm{p}K_{\mathrm{B1}}$) at pH > $\mathrm{p}K_{\mathrm{A}}$ ($\mathrm{p}K_{\mathrm{A}}$ being for protonation of the amino group), owing to the formation of hydrogen bonds.[2,3,14] Thus, the break in Figure 4 should be related to $\mathrm{p}K_{\mathrm{B1}}$. The increase in the absolute value of the slope of $E_{1/2}$ vs pH indicates a recombination of the free imine with H^+ ion, at the electrode, in strongly basic media.[5,12]

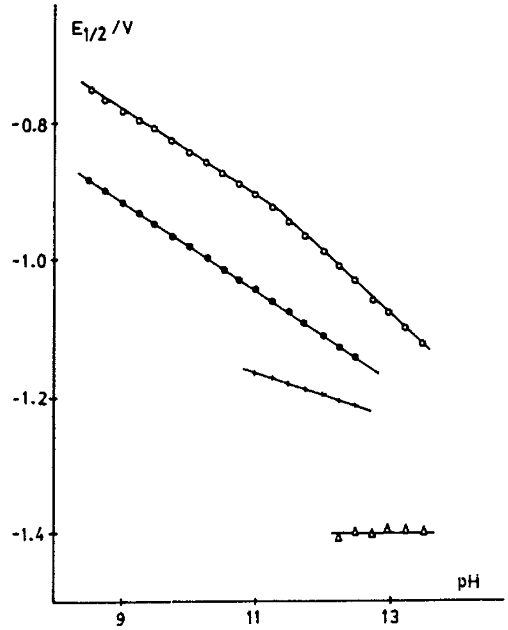


Figure 4. D.c. polarography: variation of $E_{1/2}$ with pH. $c_{\mathrm{PLP}} = 1\times10^{-4}$ M; $c_{\mathrm{hex}} = 4\times10^{-3}$ M. (○) Schiff base; (●) PLP1; (+) PLP2; (△) PLP3

Spectrophotometric study

A spectrophotometric study of the PLP–Schiff base equilibrium was carried out. Absorption spectra in the range 500–220 nm were recorded. In Figure 5 the absorbance is shown as a function of pH at different wavelengths. The variation obtained indicates that species are related via a protonation equilibrium (Scheme 4). The maxima at 415 and 278 nm correspond to the ketoenamine species, SH, predominantly in aqueous solution.[2,3,6] However, a shift from 415 to 360 nm is observed at pH > 12. The absorption is due to the free imine species, S^-.

The stability of the Schiff base decreases owing to the cleavage of the hydrogen bond. Thus, PLP in strongly

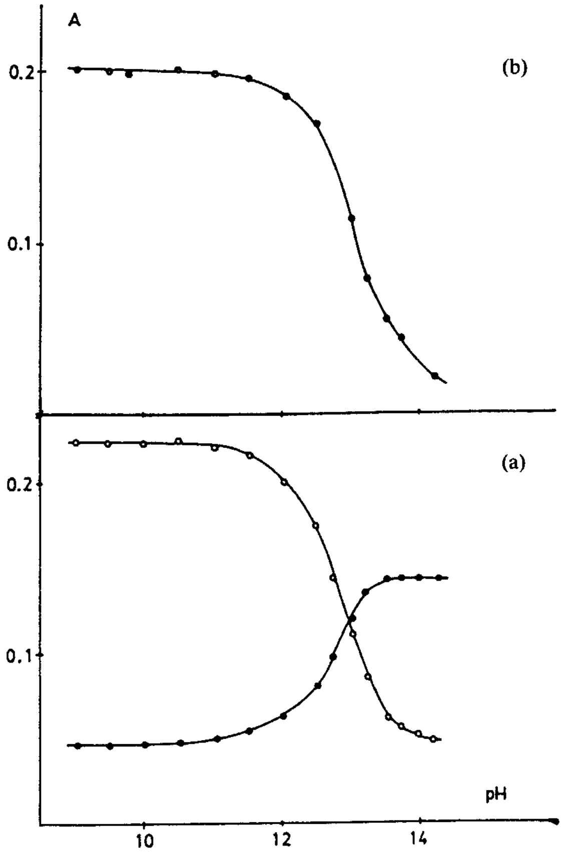
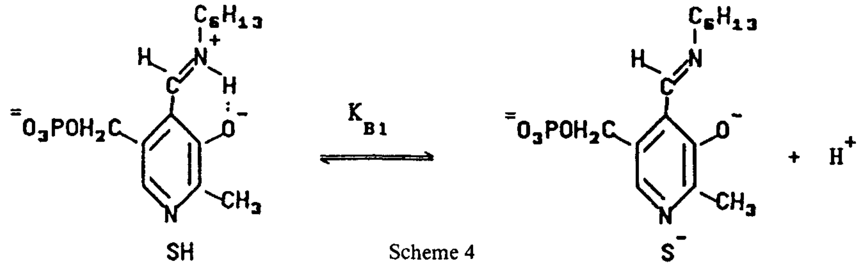


Figure 5. Absorption spectra: variation of absorbance with pH. $c_{\mathrm{PLP}} = 2\cdot10^{-5}$ M; $c_{\mathrm{hex}} = 0{\cdot}1$ M. Wavelength: (a) (○) 415; (●) 360; (b) (●) 280 nm



SH Scheme 4 S^-

basic medium can make some contribution to the absorbance at the above-mentioned wavelength (wavelength maximum at 389 nm). The absorption at 280 nm is not present with PLP and its variation with pH allows an estimation of pK_{B1}. Absorbance data obtained from extrapolation of the kinetic data of the hydrolysis reaction gives a value of 12·5 for this *pK*.[21] Our results yield a value of $pK_{B1} = 13 \pm 0\cdot1$, which is higher than that obtained by fitting kinetic[7] and electrochemical[6] profiles (*ca* 11·5). A value close to 11·5 is also obtained from the break in the $E_{1/2}$vs pH plot (Figure 4). In this pH zone, according to the above conclusions, kinetic control in the limiting zone of the reduction wave is observed. This confirms that the value is affected by the kinetics of the formation reaction. In addition, a hydrophobic environment is produced at the interface owing to the adsorption of the hexylamine in basic media. the effective H^+ concentration is lowered[22] and the free imine species could be obtained at lower pH than in solution, yielding an apparent variation of $E_{1/2}$ with pH. Therefore, the above value can only be taken as a rough estimate of the acid-dissociation constant. This explains the difference from the spectrophotometric result.

Determination of the kinetic parameters from instantaneous currents on the limiting zone

The results for the current–time curves in strongly basic media indicated that the electrode process is of the CE type. In this case, assuming pseudo-first-order chemical reactions, the following equation is fulfilled for an instantaneous limiting current:[23,24]

$$\frac{i_K}{i_D - i_K} = 1\cdot386\ [(k_f + k_b)t]^{0\cdot545}\ K^{1\cdot091} \qquad (6)$$

where i_K and i_D are the kinetic and diffusion current, respectively. Equation (6) can be transformed into:

$$\frac{i_D}{i_K} = \{1\cdot386[(K^2k)^{0\cdot545}]\}^{-1}\ t^{-0\cdot545} + 1 \qquad (7)$$

Equation (7) was checked at pH < 11. Under these conditions, where the current is controlled by diffusion, a *t*-independent plot was observed. At pH > 11, where kinetic control is observed, linear plots according to the theoretical predictions of this equation were obtained (Figure 6). The intercept is close to unity and from the slope the kinetic parameter $K^2k = 0\cdot118\ s^{-1}$ is obtained at pH 13·2. The calculation of the rate constants is not possible because the pseudo-equilibrium constant *K* is unknown.

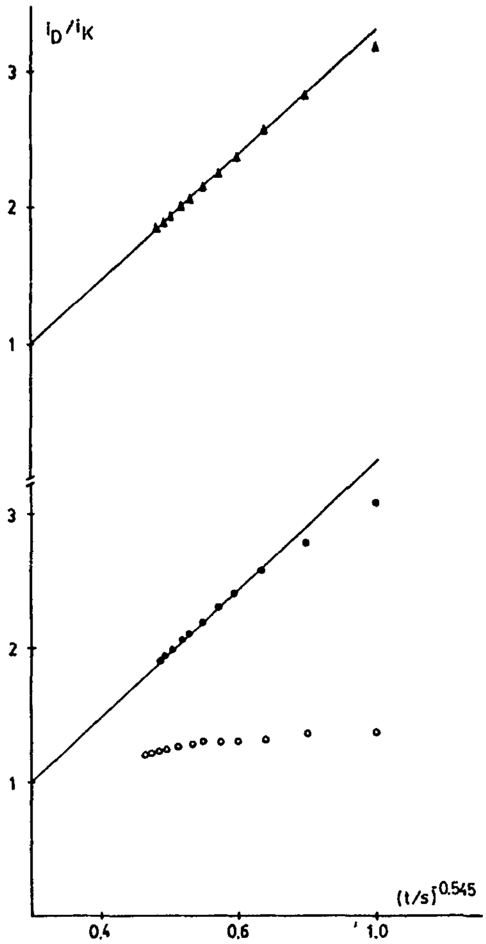


Figure 6. i_D/i_K vs $t^{-0\cdot545}$ plot. $c_{PLP} = 1 \times 10^{-4}$ M; $c_{hex} = 4 \times 10^{-3}$ M, (○) pH = 10, (●) pH = 12; $c_{hex} = 7 \times 10^{-4}$ M, (▲) pH = 13·2

Linear-scan cyclic voltammetry

The above limitation was resolved by application of a faster electrochemical technique. We studied the electroreduction of the reaction mixture in strongly basic media by linear-scan cyclic voltametry (LSCV). At pH > 13, two main peaks were observed (Figure 7). Under most of the experimental conditions the difference in potential between the two peaks is greater than 220 mV. The first peak corresponds to the Schiff base and second to the unprotonated PLP. Two residual peaks were observed, depending on the experimental conditions, between the others. The peaks can be observed in the electroreduction of PLP alone. In addition, they disappear as the scan rate increases. No anodic peaks were observed in the scan interval investigated. Figure 8 shows the influence of the scan rate, v, on the term $i_p/v.^{1/2}$ The ratio $i_p/v^{1/2}$ for the first peak decreases as the scan rate increases, which confirms that the electrode process is a CE type, the chemical reaction being the formation of the Schiff base. At higher scan rates, this term is independent of $v^{1/2}$, indicating that the PLP–Schiff base equilibrium is not disturbed during the measurement period[25] at high enough scan rates. The second peak is independent of v, corresponding to a diffusion-controlled process in the limiting zone. Under these conditions, the equilibrium concentrations of the Schiff base and PLP are proportional to the diffusion current. The analysis of the peak shape using $E_p - E_{p/2}$ as a function of the scan rate[26] confirms the reversible and irreversible character of the electroreduction of the Schiff base and PLP, respectively.

From the theoretical peak current for reversible and irreversible processes in voltammetry,[27] and assuming similar diffusion coefficients, for both electroactive species, the concentration of the Schiff base, S^-, can be

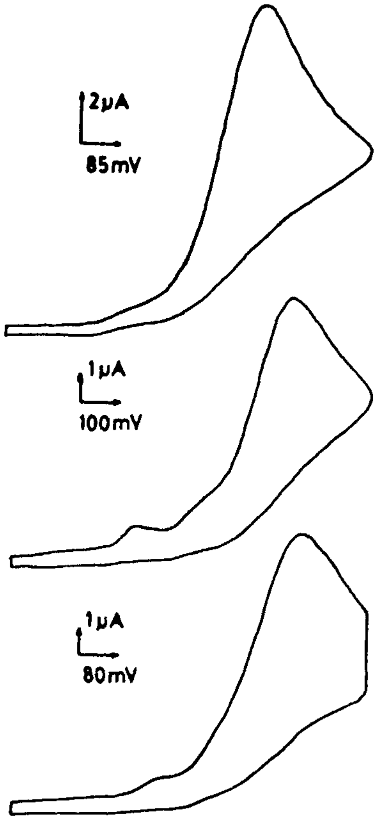


Figure 7. Linear-scan cyclic voltametry. $v = 0{\cdot}5$ V s^{-1}. Upper i–E curve, $c_{\text{PLP}} = 1 \times 10^{-4}$ M, pH = 13, initial potential −1000 mV; middle i–E curve, $c_{\text{PLP}} = 5{\cdot}5 \times 10^{-4}$ M, $c_{\text{hex}} = 1 \times 10^{-3}$ M, pH = 13·2, initial potential −800 mV; lower i–E curve, $c_{\text{PLP}} = 5{\cdot}3 \times 10^{-4}$ M, $c_{\text{hex}} = 1 \times 10^{-3}$ M, ph = 13·7, initial potential −900 mV

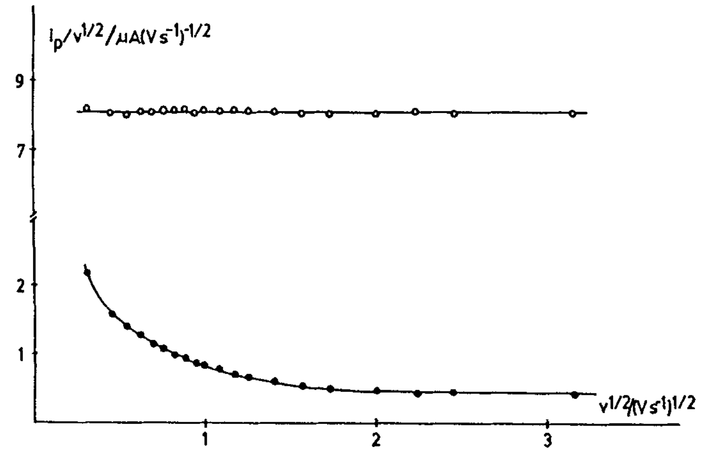


Figure 8. $i_p/v^{1/2}$ vs $v^{1/2}$ plot. $c_{\text{PLP}} = 5{\cdot}5 \times 10^{-4}$ M; $c_{\text{hex}} = 1 \times 10^{-3}$ M; pH = 13·2. (●) Schiff base; (○) PLP

expressed by

$$c_S = 0{\cdot}786\,(\alpha n_a)^{1/2}\,\frac{S_S}{S_P}\,C_P \qquad (8)$$

where s_S and s_P are the slopes of the i_p vs $v^{1/2}$ plot at high scan rates for the Schiff base and PLP, respectively. The value of αn_a was determined from a logarithmic analysis at the foot of the voltammetric curves for PLP and was checked with the value obtained from variation of $E_p - E_{p/2}$ with the scan rate.[24] The value of s_P was calculated in the absence of the amine at a known concentration of PLP. The value of s_p was also determined in the presence of the amine as the first voltammetric curves reached a plateau under experimental conditions which favour the second wave. The values of s_p were in good agreement. In general, a low concentration of the Schiff base was employed, provided that the current of the first peak was adequate, because at high hexylamine concentrations some contribution of adsorption was detected in the first peak. This was indicated by a small increase in $i_p/v^{1/2}$ at high scan rates where this effect predominates over diffusion.[28] In addition, with a low concentration of imine the extrapolation of the voltammetric tail of the first peak can be neglected.

Taking into account c_p, s_p and s_s, the value of c_s is calculated. *From* c_p *and* c_s, *the pseudo-constant* $K = c_s/c_p$ is obtained.

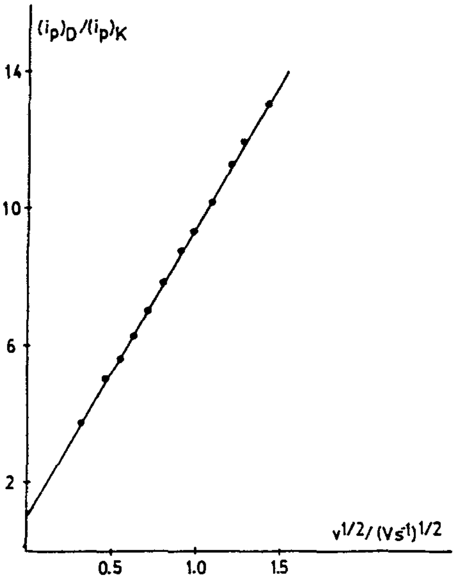


Figure 9. i_{pD}/i_{pK} vs $v^{1/2}$ plot. $c_{PLP} = 5{\cdot}5 \times 10^{-4}$ M; $c_{hex} = 1 \times 10^{-3}$ M; pH = 13·2

At low scan rates, the following equation is valid in voltammetry:[26]

$$\frac{i_{P,D}}{i_{P,K}} = 1{\cdot}02 + \frac{0{\cdot}531}{Kl^{1/2}}\,b^{1/2} \qquad (9)$$

where $b = (\alpha nF/RT)v$ and $l = k$. Equation (9) is equiva-

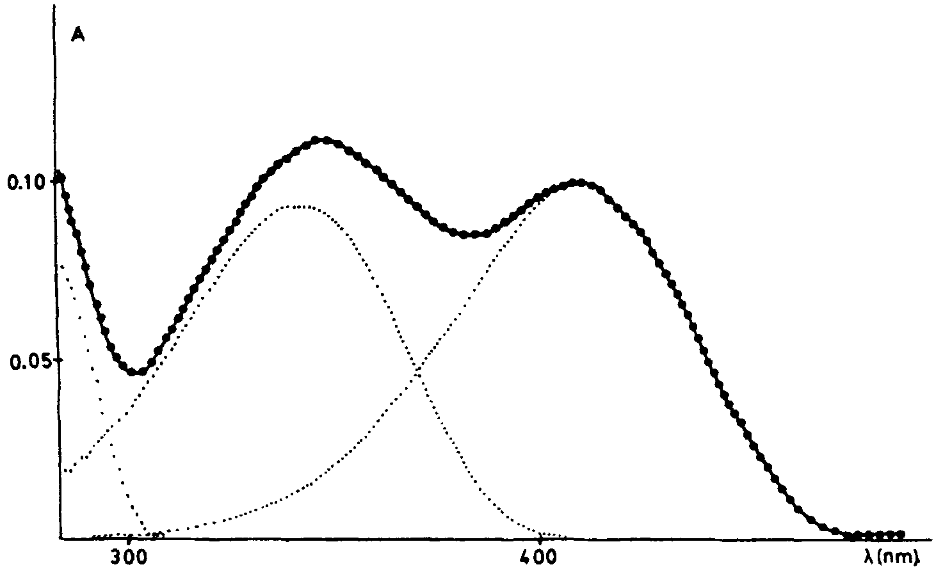


Figure 10. Absorption spectrum. $c_{PHSB} = 1{\cdot}53 \times 10^{-5}$ M; pH = 13. (•) Experimental spectrum. Theoretical profiles calculated by log-normal function: (———) total absorbance; (...) individual absorbance

lent to equation (7) used for an instantaneous current. The plot of $i_{P,D}/i_{P,K}$ vs $b^{1/2}$ is linear (Figure 9). The intercept is close to unity and from the slope, the term $K^2 l$ is obtained. The value of $K^2 l = 0{\cdot}295\ \mathrm{s}^{-1}$ agrees fairly well with the results of the instantaneous current, taking into account the differences in the experimental conditions.

From the above results $k_f = 8{\cdot}7 \pm 0{\cdot}5\ \mathrm{s}^{-1}$ and $k_b = 262 \pm 15\ \mathrm{s}^{-1}$ were calculated. Under the experimental conditions of the reaction, the values of $k_1 = (8{\cdot}7 \pm 0{\cdot}4) \times 10^3\ \mathrm{l\ mol^{-1}\ s^{-1}}$ and $k_{-1} = 262 \pm 15\ \mathrm{s}^{-1}$ were finally obtained. From the above results, $K_{SO} = 33 \pm 2.\ \mathrm{l\ mol^{-1}}$. This value is lower than the approximated constant of *ca* 250–600 $\mathrm{l\ mol^{-1}}$ previously reported.[6,7] The value obtained here fits better with the K_{SO} values collected for different Schiff bases of PLP and primary amines or amino acids[2].

Finally, Figure 10 shows the absorption spectrum of the PLP–hexylamine Schiff base corrected for free PLP in solution according to the calculated K_{SO}. The fitting by a log-normal distribution[13,29] allow the spectrum to be resolved into two bands, assuming that under the conditions used the ketoenomine species are predominant. Parameters of the band corresponding to the free imine and conjugate acid are given in Table 1.

Table 1 Molar absorptivity and molar area of the species of the Schiff base

Species	λ (nm)	ε (dm^3 mol^{-1} cm^{-1})	a^0 (km mol^{-1})
SH	411	12418 ± 171	587 ± 24
S$^-$	340	12549 ± 176	802 ± 33

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