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BINDING OF PYRIDOXAL-5′-PHOSPHATE AND PYRIDOXAMINE-5′-PHOSPHATE: ELECTROCHEMICAL CHARACTERIZATION

F. ERASO, T. PINEDA AND M. BLAZQUEZ*
Departamento de Química Física y Termodinámica Aplicada, Facultad de Ciencias, Universidad de Córdoba, 14004 Córdoba, Spain

F. GARCIA-BLANCO
Instituto Rocasolano, CSIC, 28006 Madrid, Spain

AND

M. DOMINGUEZ
Departamento de Química Física, Universidad de Sevilla, C/Prof. Garcia Gonzalez s/n, 41071 Seville, Spain

ABSTRACT

The interaction between pyridoxal-5′-phosphate (PLP) and pyridoxamine-5′-phosphate (PMP) was studied by d.c. and differential pulse polarography. Two reduction waves (or two peaks) were observed. The first wave corresponds to the reduction of the adduct and the second to the reduction of free PLP. The behaviour was similar in analogous Schiff bases. The effects of pH, PMP concentration and the capillary characteristics were studied. The apparent formation constant as a function of the pH was calculated. Thermodynamic parameters were estimated at pH 7 and 0·1 M ionic strength. The polarographic and kinetic results show that the overall electrode process appears to be irreversible in a basic medium.

INTRODUCTION

It is well known that enzymes containing pyridoxal-5′-phosphate (PLP) play important roles in amino acid metabolism,[1] including transamination, racemization and decarboxylation. PLP and pyridoxamine-5′-phosphate (PMP) are coenzymes for enzymatic transaminations. Most of these enzymatic reactions have been simulated by non-enzymatic model reactions.[2–11]

In these non-enzymatic reactions, analytical methods have been proposed for measurements of pyridoxal (PL) and pyridoxamine (PM) mixtures.[4,5] The absorption spectra of these substances have been studied.[3–9,11,12] Some absorption bands were used to obtain the total vitamin B_6 content.

Possible interactions between PL and PM or between PLP and PMP in these non-enzymatic

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* Author to whom correspondence should be addressed.

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studies were not considered. Recently, the interaction between PL and PLP with PM in the presence of metal ions has been described.[13–15]

The aim of this work was to study the interaction between PLP and PMP, which may help in the quantitative interpretation of these non-enzymatic models. In addition, the study of the Schiff bases formed between PLP and different amines is useful in order to simulate the properties of the active site of PLP[16–24]. In this paper the electrochemical characterization of the PLP–PMP adduct is described.

EXPERIMENTAL

PLP and PMP were purchased from Sigma. All chemicals were of analytical-reagent grade. The supporting electrolyte was a buffered solution 0·02 M acetic acid and 0·02 M phosphoric acid for $pH < 8{\cdot}5$ and 0·02 M phosphoric acid and 0·02 M $NaHCO_3$ for $pH > 8{\cdot}5$. The pH was adjusted with NaOH and the ionic strength to 0·5 M with $NaNO_3$. All potentials were measured against a saturated calomel electrode. All solutions were purged with purified nitrogen prior to the electrochemical experiments. The temperature was kept at 25 ± 0·1 °C.

Polarographic measurements were carried out on a thermostated AMEL 494 cell equipped with a saturated calomel electrode, a platinum auxiliary electrode and a dropping mercury electrode, with the following characteristics: $m = 0{\cdot}946$ mg s^{-1}, $t = 5$ s and open circuit with the buffer at pH 1·94 and $h = 40$ cm. In differential pulse polarography (DPP) the drop time was 2·0 s and the pulse amplitude 20 mV.

D.c. polarograms and DPP curves were recorded automatically by means of a Metrohm 626 polarograph. A Beckman 3500 pH meter was used to measure the pH. The solution temperature was kept constant with the aid of a Selecta Frigiterm thermostat.

The PLP–PMP adduct was obtained by adding known amounts of PMP to PLP solutions of known concentration. Thus, solutions with different molar ratios were prepared. The mixtures were kept in the dark to avoid photolysis reactions.

The overall reaction between PLP and PMP can be represented by

$$RCHO + R'NH_2 \rightleftharpoons RCH{=}NR' + H_2O \tag{1}$$

The apparent formation constant for this reaction was calculated from the expression

$$K = \frac{C_{SB}}{C_{PLP}C_{PMP}} \tag{2}$$

where C_{SB}, C_{PLP} and C_{PMP} represent the equilibrium concentrations of the Schiff base, PLP and PMP, respectively.

RESULTS AND DISCUSSION

The interaction between the two coenzymes gives rise to a Schiff base according to reaction (1). In the pH range 5–10, the reaction mixture yields two waves (or peaks) in d.c. polaragraphy (or DPP) at a PMP : PLP molar ratio of 20 : 1 (Figure 1).

Earlier studies[19,23] of similar reactions ascribed the first wave to a two-electron reduction of the imine bond and the second wave to a two-electron reduction of the free PLP carbonyl group. We checked this assignment by comparing the reduction potential of the second wave with that obtained in the reduction of PLP[25,26] in the absence of PMP. The limiting currents were independent of time in all the experiments. The occurrence of two reduction waves indicates that the reaction mixture involves significant amounts of the PLP–PMP adduct, free

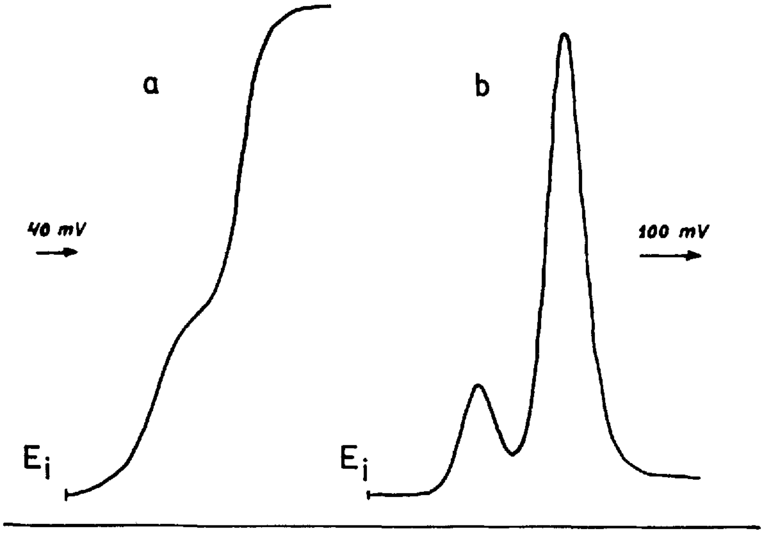

Figure 1. (a) D.c. polarography at pH 9·0. First wave, PLP–PMP adduct; second wave, free PLP $E_i = -680$ mV. (b) DPP at pH 7·5. First peak, PLP–PMP adduct; second wave, free PLP $E_i = -500$ mV

PLP and free PMP. The stability of both limiting currents with time indicates that reaction (1) reaches equilibrium under the experimental conditions used. The aldehyde group of PLP is hydrated to some extent in solution.[27,28] Hence the reduction of PLP involves a dehydration reaction prior to the electrode process. However, the reduction of PLP in the pH range 2–10 shows diffusion control in the limiting zone.[25,26]

We studied the influence of pH on the reduction waves. The overall limiting current is independent of pH in the pH range studied (5–10). The limiting current of the first wave shows a bell-shaped variation, reaching a plateau at pH 8.5. The limiting current of the second wave also varies with pH (Figure 2).

The influence of capillary characteristics on the electrode process was studied. Current–time curves using a dropping mercury electrode were recorded on the top of the waves in the pH range 5–10. Our results show that the log i_L vs log t plot corresponding to the first and overall waves were linear with slopes close to 0·2 in both cases.

These results indicate that both reduction processes are diffusion controlled. The variation of the limiting current of the waves (Figure 2) indicates that the equilibrium concentrations in reaction (1) are a function of pH. This effect is due to protonation equilibria of PLP, PMP and the Schiff base. The reduction of the Schiff base yields a single wave throughout the pH range 5–10. Hence fast tautomeric equilibria involved with these compounds are not detected at the electrode. Therefore, the Schiff base equilibrium concentration can be obtained from the limiting current of the first wave.

Assuming that the diffusion coefficients of the electroactive species (Schiff base and PLP) are similar, equation (2) can be transformed into

$$\frac{i_{\mathrm{L,SB}}}{i_{\mathrm{L,PLP}}} = Kc_{\mathrm{PMP}} \qquad (3)$$

where $i_{\mathrm{L,SB}}$ and $i_{\mathrm{L,PLP}}$ are the limiting currents of the first and second waves, respectively.

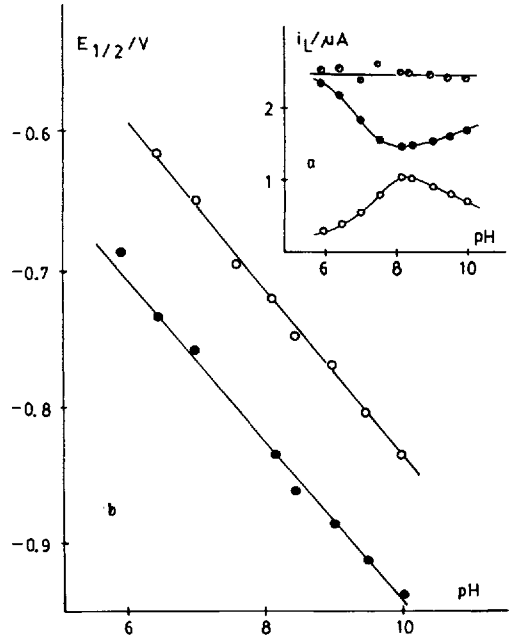

Figure 2. D.c. polarography. $c_{PLP} = 5 \times 10^{-4}$ M; $c_{PMP} = 1 \times 10^{-2}$ M. (a) Variation of the limiting current with pH. ○, First wave; •, second wave; ◐, overall process. (b) Variation of $E_{1/2}$ with pH. ○, First wave, •, second wave

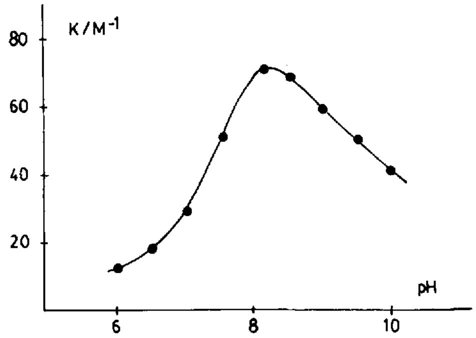

Figure 3. Variation of K with pH

Equation (3) shows that an apparent constant can be obtained from a study of the influence of the amine concentration. At pH 8·5 a plot of $i_{L,SB}/i_{L,PLP}$ vs PMP concentration yields a linear segment with a slope of 71 $l\,mol^{-1}$, the total limiting current being independent of PMP concentration.

The above results indicate that the polarographic method allows reaction (1) to be characterized. Hence from equation (3) and taking into account the limiting current at each pH the apparent constant was calculated. The plot of K_{obs} vs pH is shown in Figure 3.

The variation observed confirms that the stability of the Schiff base is related to the

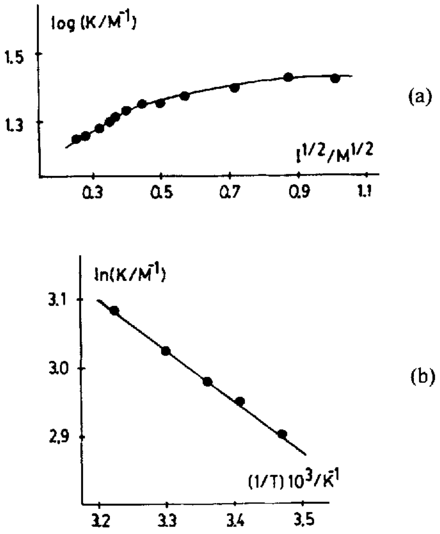

Figure 4. (a) Influence of ionic strength. pH 7·0. Plot of log K vs $I^{1/2}$. $c_{\mathrm{PLP}} = 1 \times 10^{-4}$ M; $c_{\mathrm{PMP}} = 3 \times 10^{-2}$ M; 0·02 M phosphate buffer; $T = 25\,^{\circ}\mathrm{C}$. (b) Influence of temperature. pH 7·0. Plot of ln K vs $(1/T) \times 10^3$. $c_{\mathrm{PLP}} = 1 \times 10^{-4}$ M; $c_{\mathrm{PMP}} = 3 \times 10^{-2}$ M; 0·02 M phosphate buffer; ionic strength, 0·1 M

protonation equilibria of the adduct and the coenzymes.[7,29] Similar variations have been reported.[19,23] However, a lower stability is observed in this case.

A study of the influence of the ionic strength at pH 7 and 25 °C was carried out. The values of the apparent constant obtained from equation (3) are shown in Figure 4a. The constant decreases as the ionic strength decreases. A minimum amount of salt was necessary to avoid a migration current under the polarographic conditions. Therefore, it was not possible to extrapolate the values to zero ionic strength in order to obtain the thermodynamic constants. However, from the value of the constant at 0·1 M ionic strength an approximate value of the standard free enthalpy change of $-1{\cdot}8 \pm 0{\cdot}1$ kcal mol^{-1} was obtained, showing that the formation of the Schiff base is a spontaneous process. A study of the influence of temperature was carried out at 0·1 M ionic strength. The plot of ln K vs $1/T$ was linear in the temperature range 15–37 °C (Figure 4b). From the slope a ΔH value of $+1{\cdot}5 \pm 0{\cdot}2$ kcal mol^{-1} was obtained. This method assumes that ΔH is constant in the temperature range studied. From the positive value of the enthalpy change it can be concluded that the binding process is endothermic. An estimation of the entropy change gives a value of $+11 \pm 1$ cal mol^{-1} K^{-1}.

Finally, we determined some polarographic and kinetic parameters of the electrode process. The $E_{1/2}$ vs pH plot displays linear segments in the pH range 6–10, with slopes of -63 mV decade^{-1} (first wave) and -66 mV decade^{-1} (second wave) (Figure 2).

A logarithmic analysis of the first wave was carried out. In a weakly acidic medium the plot of E vs log $i/(i_{\mathrm{L}} - i)$ was linear with a slope of -30 mV decade^{-1}. In a weakly basic medium this plot shows two linear segments. The slope of the linear segment at potentials more positive than $E_{1/2}$ was close to -40 mV decade^{-1}. This result suggests that the electrode process is irreversible in basic medium.

The polarographic parameters at the foot of the wave were also analysed. In basic medium an average value of -39 mV decade^{-1} for the Tafel slope was obtained. In addition, a study of the i–t curves was also carried out. The plot of log i vs log t at the foot of the first wave was linear with a slope close to 0·5. These values confirm the irreversible character of the electrode process. The slope of the plot of $E_{1/2}$ vs pH indicates that two protons are involved in the electrode process.

DPP confirmed these results. In the pH range 5–10 two peaks were obtained (Figure 1). The peak potentials shift to more cathodic values as the pH increases. The E_P vs pH plot of the first peak displays a linear segment with a slope of -63 mV decade^{-1}. The variation of the peak current was similar to that obtained by d.c. polarography. Under some experimental conditions the waves in d.c. polarography are very close. Under these conditions the peaks obtained in DPP show a better resolution. The limiting currents corresponding to the processes in d.c. polarography were also obtained from the characteristic parameters of these peaks.[30] In this case we checked that the calculated values of the apparent constant agree well with the K_{obs} values in Figure 3.

CONCLUSION

This study has demonstrated the interaction between PLP and PMP. The electrochemical characterization of the adduct was carried out by d.c. polarography and DPP. The formation constant was calculated as a function of the pH, showing a lower stability than for other Schiff base of PLP. An estimation of thermodynamic parameters at pH 7 was carried out. These results should be of interest in non-enzymatic model reactions. The electrode process involves two protons and appears to be irreversible in basic medium.

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