

## Polarographic Behaviour of Pyridoxal 5'-Phosphate

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The polarographic reduction of pyridoxal 5'-phosphate in several *N,N*-dimethylformamide-water mixtures has been investigated. It has been found that one, two, or three waves appear depending on the experimental conditions. The dependence of their half-wave potentials and the variation of their intensities with pH for each solvent were studied. The rate of interconversion between the free and hydrated form of the carbonyl group is very fast. Indirect evidence for rapid intramolecular proton transfer was obtained. Tetramethylammonium cation and *N,N*-dimethylformamide have a large influence on the relative intensities of the first wave. This can be explained by the formation of a complex between those species and the radical formed in the environment of the dropping mercury electrode.

THE electrochemistry of biological compounds has been the subject of increasing interest in recent years.<sup>1-5</sup> In the study of organic reversible redox systems in solution, polarography, like potentiometry, serves for the determination of two important thermodynamic values. Thus, the half-wave potentials  $E_{1/2}$ , and from their dependence on pH, the acid dissociation constants  $K$  of the oxidized and/or reduced forms can be obtained. In the case of irreversible electrode reactions the processes are determined by the substance undergoing the electrode reaction and it is not possible to obtain information on the reduced forms.<sup>3,6,7</sup> Nevertheless information can be obtained about the equilibrium and kinetic parameters of the oxidized forms, and, in many cases, the pathway of the electrode reaction, intermediate reactions, proton transfers, and so on. This may allow an extension of knowledge of the biological role of these molecules.

Several compounds of the vitamin B<sub>6</sub> group are important cofactors in a large number of enzymic reactions.<sup>8-11</sup> In many of them pyridoxal 5'-phosphate (PLP) is bound to the enzymes through a Schiff's base with the terminal amino-group of a lysine residue of the polypeptide chain.<sup>8-11</sup> Several papers concerning the polarographic reduction of vitamin B<sub>6</sub> compounds in

water have been published.<sup>12-16</sup> However, some results appear to indicate that the hydrophobicity of the PLP sites could be high in some cases.<sup>17-21</sup> Therefore, we have surveyed the influence of solvent polarity on the electrochemical behaviour of PLP.

### EXPERIMENTAL

Polarographic current-voltage curves were performed at 22 °C in water-dimethylformamide (DMF). A saturated calomel electrode (s.c.e.) was always used as reference. A salt bridge was only inserted into the cell while the polarograms were recorded (usually 5–10 min) in order to obtain the least possible variation in the water content of the solutions. The dropping mercury electrode (d.m.e.) used had the following properties:  $m$  1.8 mg s<sup>-1</sup> and  $t$  2.4 s, determined in aqueous solution at zero potential ( $E$  0).

Unless otherwise specified the buffers used were sodium acetate-acetic acid, monosodium phosphate-disodium phosphate, and sodium hydroxide-sodium carbonate depending on the required pH. These buffers (0.2M) were also used as background electrolytes for solutions whose DMF contents were <50% v/v. Tetramethylammonium (0.05M) and buffers (0.15M) were used for solutions with 50% v/v DMF. Solutions with 75% v/v DMF were 0.01M in buffers and 0.19M in tetramethylammonium bromide. The high conductivity of these solutions made negligible the  $iR$  corrections. The pH values given are those measured with a

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<sup>3</sup> P. Zuman in 'The Elucidation of Organic Electrode Processes,' Academic Press, London, 1969.

<sup>4</sup> A. A. Underwood and R. N. Burnett in 'Electroanalytical Chemistry,' ed. A. J. Bard, Dekker, New York, 1972, vol. VI, pp. 1–85.

<sup>5</sup> P. J. Elving, J. E. O'Reilly, and C. O. Schmamel in 'Methods of Biochemical Analysis,' ed. D. Glick, Interscience, New York, 1973, vol. XXI, pp. 287–465.

<sup>6</sup> P. Zuman in 'Methods in Enzymology,' ed. K. Kustin, Academic Press, New York, 1969, vol. XVI, pp. 121–183.

<sup>7</sup> M. Heyrovsky and S. Vavricka, *J. Electroanalyt. Chem.*, 1972, **36**, 203.

<sup>8</sup> E. E. Snell, *Vitamins Hormones*, 1958, **16**, 77.

<sup>9</sup> E. E. Snell, P. M. Fasella, A. E. Braunsteins, and R. Fanelli in *Chemical and Biological Aspects of Pyridoxal Catalysis*, Pergamon, New York, 1963.

<sup>10</sup> E. E. Snell, A. E. Braunstein, E. E. Severin, and M. Yu. Torchinsky in 'Pyridoxal Catalysis: Enzymes and Model Systems,' Wiley, New York, 1968.

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<sup>15</sup> P. Zuman and O. Manousek, *Coll. Czech. Chem. Comm.*, 1964, **29**, 1718.

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<sup>17</sup> S. Shaltiel and M. Cortijo, *Biochem. Biophys. Res. Comm.*, 1970, **41**, 594.

<sup>18</sup> S. Shaltiel, M. Cortijo, and Y. Zaidenzaig, in 'Metabolic Interconversion of Enzymes,' eds. O. Wieland, E. Helmreich, and H. Holzer, Springer-Verlag, Berlin, 1972, pp. 72–101.

<sup>19</sup> R. C. Hughes, W. T. Jenkins, and E. H. Fischer, *Proc. Nat. Acad. Sci. U.S.A.*, 1962, **48**, 1615.

<sup>20</sup> O. L. Polyakov and B. A. Keil, *Biokhimiya*, 1963, **28**, 372.

<sup>21</sup> M. Arrio-Dupont, *Biochem. Biophys. Res. Comm.*, 1971, **49**, 653.

glass electrode after the experiments were finished. The solvent corrections to the true pH values for these solutions are not very large<sup>22</sup> except for the solution with 75% v/v DMF. Therefore, the pH values in this solution should only be taken as indicative.

the height of the wave reaches half of the total reduction current is denoted  $pK'$ . The breaks between the extrapolated linear branches going to greater steepness in the pH-polarographic half-wave potential relationship, indicating an irreversible electroreduction with preprotonation,

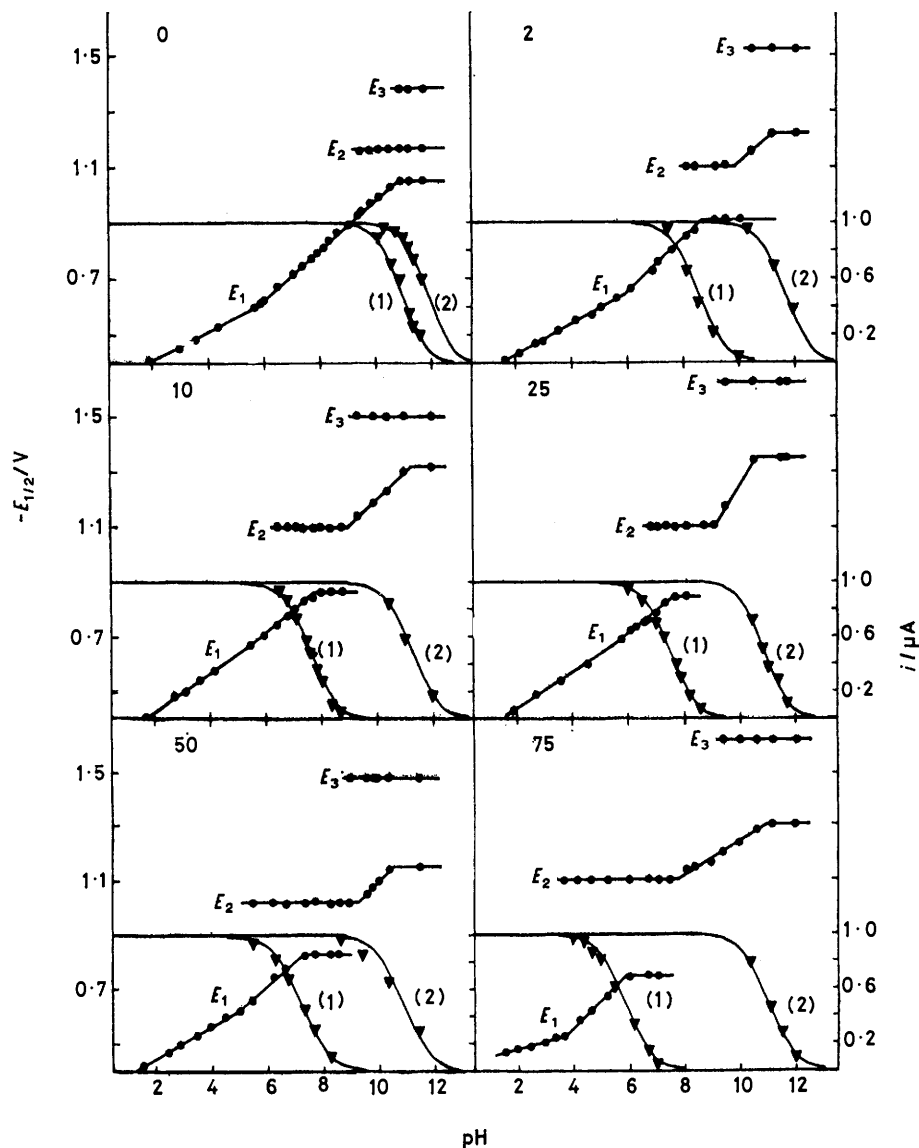


FIGURE 1 pH Dependences of the half-wave potentials (●) and wave-height of limiting currents  $i_1$ (1) and  $(i_1 + i_2)$  (2) (▼) for PLP. The DMF content of the solutions is given in the upper left corner. The experimental points were obtained from the polarograms recorded under the conditions described in the Experimental section. The dissociation curves correspond to the theoretical values for a monoprotic acid with the  $pK'$  values given in Table 2

PLP concentrations were measured by dilution of a recently prepared stock solution with 0.1M-NaOH, and measurement of the absorption at 388 nm. An absorbance index of 6 600 was used.<sup>23</sup> All the products used were from Merck. A Metromh E300B pH meter and a Radiometer polarograph were used.

The dependence of the wave-height on the pH gives a dissociation curve at the pH range in which two or more waves can be observed simultaneously. The pH for which

denote the  $pK^R$  values of the oxidized form.<sup>7</sup> The break points of the branch in which the intensity current is dependent on pH,  $pK''$ , are identical with the polarographic  $pK'$  values.<sup>7</sup> The total number of protons consumed in the reduction before the rate-limiting step,  $m$ , can be obtained, as long as the polarographic wave is diffusion-controlled and no second-order reactions take place in the electrode process. In that case,  $m$  is the product of the slope of the  $E_{1/2}$ -pH plot and the slope of the dependence of  $\log [i/(i_d - i)]$  on  $E$ .<sup>7</sup>

<sup>22</sup> J. P. Shukla and S. G. Tandon, *J. Electroanalyt. Chem.*, 1972, **35**, 423.

<sup>23</sup> E. A. Peterson and H. A. Sober, *J. Amer. Chem. Soc.*, 1954, **76**, 169.

## RESULTS

Polarograms of PLP in aqueous solution with 0, 2, 10, 25, 50, and 75% v/v DMF have been recorded at different pH values from 1 to 13. A single two-electron wave ( $i_1$ ) was observed at low pH for each solvent. Second ( $i_2$ ) and third ( $i_3$ ) waves appear at high pH, while the total intensity remains constant. The total diffusion current changes with the solvent polarity as shown in Table 1. It appears that these variations could be due mainly to changes in the viscosity of the media (see Table 1). The dependence

TABLE 1  
Variation of the wave intensity of PLP with the content of DMF<sup>a</sup>

DMF (%)	$i_d/\mu\text{A}$	$\eta^b$	$i_d\eta^{1/2}$
0	1.72	1.00	1.7
2	1.66	1.05	1.7
10	1.53	1.20	1.7
25	1.40	1.64	1.8
50	1.20	2.50	1.9
75	0.96	2.30	1.5

<sup>a</sup> The PLP concentration was always  $3.9 \times 10^{-4}\text{M}$ . Acetic acid was always added to obtain a 0.1M solution. <sup>b</sup> Viscosity values from ref. 24.

of the wave intensity on concentration was always linear. A value for the diffusion current constant of 2.6 was obtained from these plots for the aqueous solution.

Wave  $i_1$  decreases upon increasing pH, at the expense of wave  $i_2$ , in the form of a dissociation curve for a monoprotic acid in all studied media (Figure 1). From these curves, the polarographic  $pK'$  values ( $pK_2'$ ) were obtained (see Table 2). At high pH values,  $i_2$  (or  $i_1 + i_2$ ) also decreases

TABLE 2  
Dependence on DMF content of  $pK$ ,  $pK'$ , and  $pK''$  values of PLP

DMF (%)	$pK_2^R$	$pK_2''$	$pK_3''$	$pK_2'$	$pK_3'$
0	5.9	10.9		10.9	11.9
2	5.9	8.6	11.1	8.6	11.7
10	5.5	7.7	11.2	7.7	11.2
25	5.4	7.6	10.8	7.6	10.8
50	5.0	7.2	10.8	7.2	10.8
75	3.7	5.8	11.1	5.8	11.2

with the same shape at the expense of the third wave  $i_3$ . From these second dissociation curves the  $pK_3'$  values given in Table 2 were obtained.

The buffer type and its concentration do not affect the results, except for the Britton-Robinson and  $\text{NH}_4\text{OH}-\text{NH}_4\text{Cl}$  buffers, which form complex species with PLP.<sup>9-16</sup>

The half-wave potentials  $E_{1/2}$  are also given as a function of pH in Figure 1. The plots for wave  $i_1$  show three linear branches. The first two have slopes corresponding to a transfer of one and two protons to PLP respectively before the rate-determining step of the electrode reaction.

The breaks between these two linear segments indicate the  $pK_2^R$  values given in Table 2. We have not observed an initial branch with slope zero at low pH, thus indicating that other  $pK^R$  values are small or negative. At pH values higher than  $pK_2''$ , the  $E_{1/2}$  of wave  $i_1$  becomes invariant with pH giving rise to another branch in the plot in Figure 1 with slope zero.

For the second wave,  $i_2$ , there are also three linear branches in the  $E_{1/2}$ -pH plot, except for pure water in which there is only one branch (Figure 1). The first and third of these branches have slope zero, *i.e.* their half-wave

potentials do not change with pH. The middle branch has a slope corresponding to the transfer of one proton. The breaks between the two first linear segments appear when wave  $i_1$  has practically vanished. The  $pK_2'$  and  $pK_3'$  values virtually coincide with  $pK_2''$  and  $pK_3''$  respectively (Table 2). The half-wave potentials of the third wave do not change with pH (Figure 1). Values between  $-1.4$  and

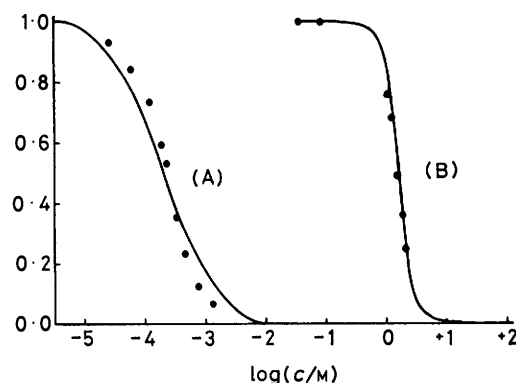


FIGURE 2 Variation of the first relative wave-height,  $i$ , with the content of DMF (A) and  $(\text{CH}_3)_4\text{N}^+$  (B). Several portions of DMF, or increasing amounts of  $(\text{CH}_3)_4\text{N}^+$ , until the final concentrations indicated were added to an aqueous solution of PLP (0.30mm). The pH, measured before and after the experiments, was 9.0. The theoretical curves for the dissociation of a monoprotic (A) and a diprotic acid (B) are also plotted

$-1.6$  V for those potentials, depending on the solvent polarity, were obtained.

At a pH value near  $pK_2'$ , an increase in DMF content produces a decrease in  $i_1$  (Figure 2). This effect is also observed with tetramethylammonium cation (Figure 2). In this case the experimental points fit a curve similar to the dissociation curve of a diprotic acid.

The influence of DMF content on  $pK_2'$  (Table 2) is also reflected in the maximal  $E_{1/2}$  values reached by the first

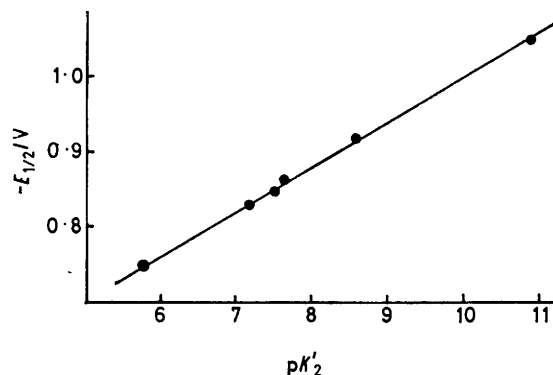


FIGURE 3 Correlation between  $pK_2'$  and the maximal values of the half-wave potentials,  $E_{1/2}$ , for the first wave. The experimental values were obtained from Figure 1 and each point represents a DMF-water mixture

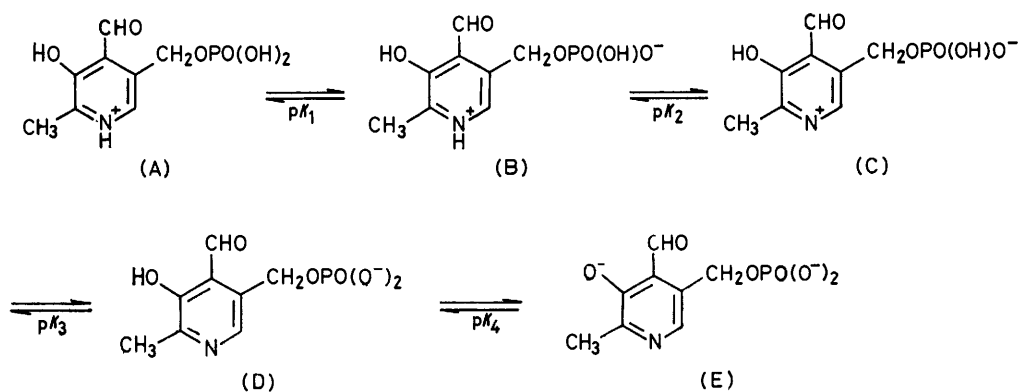
wave (*i.e.*, the  $E_{1/2}$  of the last branch at high pH). Figure 3 shows the correlation between these  $E_{1/2}$  values and those of  $pK_2'$ . The straight line obtained by the least squares method, has the equation:  $E_{1/2} = -0.40 - 0.059 pK_2'$ .

<sup>24</sup> T. Fujinaga, K. Izutsu, and K. Takaoka, *J. Electroanalyt. Chem.*, 1966, **12**, 203.

## DISCUSSION

The polarographic curves for PLP can be ascribed to the reduction of the formyl group over the whole experimental conditions used. When we compare our results with those obtained by Manousek and Zuman in aqueous solution,<sup>16</sup> the agreement is excellent, although they did not point out any linear segment for the pH dependence of the half-wave potentials.

The total intensity does not change with the pH for any given solvent. Many other aliphatic<sup>25-27</sup> and aromatic aldehydes,<sup>28-30</sup> even pyridoxal<sup>16,31</sup> show waves whose intensities increase with pH. These pH-dependences were mainly explained by dehydration reactions of the hydrated carbonyl groups. PLP is hydrated at low pH to a great extent.<sup>32,33</sup> It can be concluded that the dehydration reaction should be very fast. This conclusion is supported by direct measurements of the rate constants<sup>34,35</sup> and n.m.r. spectra.<sup>36</sup>



SCHEME 1 Ionization of PLP

The chemical species reducible at the third wave,  $i_3$ , could be a deprotonated form, say (E) (Scheme 1), due to the fact that its half-wave potential does not change with the pH. The species reducible at  $i_2$ , could be a form with one proton, say (D). Indirect evidence appears to indicate that this assumption is correct.

The transfer coefficients  $\alpha$  and the diffusion constants  $D$  of the two species that give rise to waves  $i_2$  and  $i_3$  should be similar. Therefore, the differences between their  $E_{1/2}$  values should be proportional to the activation free energy differences  $\Delta(\Delta G^\ddagger)$  of the two species that undergo reduction.<sup>37,38</sup> A very small variation in the entropy change ( $T\Delta S^\ddagger$ ) would be expected compared

with that of the enthalpy change ( $\Delta H^\ddagger$ ) in the electron transfer process. Hence,  $\Delta E_{1/2}$  should be proportional to  $\Delta(\Delta H^\ddagger)$  which is regarded as the energy difference in the lowest vacant molecular orbital of the two species.<sup>39</sup> A value of 0.46 eV is obtained from the average difference between the  $E_{1/2}$  of  $i_3$  and the first branch of  $i_2$ . This order is expected for a hydrogen bond when it is strengthened by the electrostatic and mesomeric effects.<sup>40</sup> This value also coincides with the energy differences of the forms present at neutral and basic pH (absorption maxima at 330 and 388 nm respectively).<sup>33</sup> Nevertheless, this comparison must be merely taken as indicative due to the presence of irreversible processes.

The assignment of the wave  $i_1$  to a definite chemical species is more problematic. The pH-dependence of their half-wave potentials is similar to that described under type no. 3 by Heyrovsky and Vavricka<sup>7</sup> (see Figure 1). Then, the chemical species reduced at the

electrode, called  $AH_2$ , should be present into the solution at very low pH, and, therefore, its  $E_{1/2}$  should be invariant with changes in pH. This first branch is missing in our results. We could expect this result if the first  $pK$  were due to protonation of the carbonyl group. This previous protonation has been found in the reduction of many carbonyl compounds.<sup>27</sup> At higher pH the species present in solution,  $AH$ , has one proton less, and should be converted into  $AH_2$  (at the surface of the electrode) before electron transfer. In this case, the half-wave potentials,  $E_{1/2}$ , should change with the pH as shown by our results in Figure 1.

The  $pK_2^R$  values obtained from polarographic measurements (Table 2), should agree with those calculated by other methods. Spectrophotometric and potentiometric titrations of PLP have been carried out only in aqueous

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<sup>31</sup> O. Manousek and P. Zuman, *J. Electroanal. Chem.*, 1960, **1**, 324.

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<sup>33</sup> C. M. Harris, R. J. Johnson, and D. E. Metzler, *Biochim. Biophys. Acta*, 1976, **421**, 181.

<sup>34</sup> M. L. Ahrens, G. Maass, P. Schuster, and H. Winkler, *FEBS Letters*, 1969, **5**, 327.

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<sup>36</sup> W. Korytnyk and R. P. Singh, *J. Amer. Chem. Soc.*, 1963, **85**, 2813.

<sup>37</sup> P. Zuman, *Rev. Polarography*, 1963, **11**, 102.

<sup>38</sup> P. Zuman in 'Substituent Effects in Organic Polarography,' Plenum Press, New York, 1967, p. 25.

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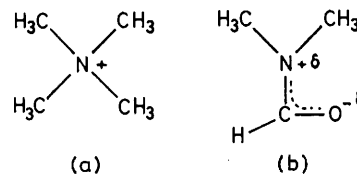
<sup>40</sup> S. N. Vinogradov and R. H. Linell in 'Hydrogen Bonding,' Van Nostrand, New York, 1971.

solution<sup>32,33,41</sup> and in dioxan-water mixtures.<sup>42</sup> In water the  $pK_2^R$  (Table 1) coincides, within experimental error, with the  $pK_3$  value obtained by Anderson and Martell,<sup>41</sup> and ascribed to the second protonation of the 5-phosphate group (Scheme 1). This protonation does not have any effect on the  $\pi$ -electron density of the aromatic ring and, consequently, is without influence on the carbonyl group electron density. Thus, this protonation is not observed in the spectrophotometric titration.<sup>33</sup> However, protonation of the other two groups, 3-hydroxy and the pyridine nitrogen, has a large influence on the spectra. The presence of a very fast three-centre intramolecular transfer, which has been found in PLP by Ahrens *et al.*,<sup>34,35</sup> could explain our results. In addition, the possible influence of the 5'-phosphate group on the acid-base reactions, before the rate-limiting step of the polarographic process, is also supported by comparison with the results obtained for pyridoxal.<sup>16</sup> Therefore we think that the more plausible form reduced at  $i_1$  is (B). Although we must bear in mind that Scheme I is a drastic simplification due to the fact that there are several tautomeric structures whose relative proportions change with solvent polarity.<sup>32,33</sup>

The effect of cations on the protonation of radicals formed in the reduction of carbonyl compounds is well

<sup>41</sup> F. J. Anderson and A. E. Martell, *J. Amer. Chem. Soc.*, **1964**, **86**, 715.

documented.<sup>27</sup> The current due to the reduction of the species formed by interaction of the radical with the cations is indistinguishable from that due to the reduction of the radical itself. Then, both waves should merge, as is the case for benzaldehyde or alkyl aryl ketones.<sup>27</sup> Tetramethylammonium cations at higher concentrations than DMF also produce a decrease of the wave  $i_2$  (Figure 2). Since both compounds have similar structures (Scheme 2) can be concluded that a charge-



SCHEME 2 Structures of tetramethylammonium cations (a) and DMF (b)

transfer complex is formed. New experiments are needed in order to elucidate the structure of this complex.

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