

## EQUILIBRIUM CONSTANTS OF PYRIDOXAL 5'-PHOSPHATE SCHIFF BASES — A POLAROGRAPHIC STUDY

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The equilibrium constant for the formation of the Schiff base between pyridoxal 5'-phosphate and n-hexylamine has been evaluated from polarographic measurements. The pH dependence of the equilibrium constant has been investigated at constant ionic strength. The values thus obtained for the equilibrium constant compare well with those computed from the pK of the several ionic forms of pyridoxal 5'-phosphate, its Schiff base and the amine present in the solution.

The Schiff bases of pyridoxal 5'-phosphate are intermediate compounds formed in the reaction of vitamin B<sub>6</sub> (aldehyde form) with free aminoacids or proteins<sup>1</sup>. For this reason these imines have been extensively studied, although few stability data, especially for the phosphorylated form of the vitamin B<sub>6</sub>, are currently available.

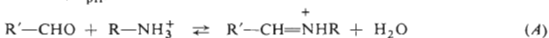
Polarography is a technique that has provided numerical results for equilibrium constants of many reactions<sup>2,3</sup>. In a recent communication<sup>4</sup> we have shown that this technique can be applied to the determination of the stability constants of these pyridoxal 5'-phosphate Schiff bases. The system fulfills<sup>4</sup> the conditions discussed by Zuman for determining equilibrium constants from polarographic measurements<sup>5</sup>.

In this paper, we have carried out a study of the equilibrium constants of pyridoxal 5'-phosphate with n-hexylamine as a function of pH at a constant ionic strength.

### EXPERIMENTAL

The imines were formed by the addition of known amounts of n-hexylamine chlorhydrate in buffered solutions of pyridoxal 5'-phosphate. The mixtures were kept in the dark for at least 12 hours. These two conditions were advisable because the reaction is slow<sup>4</sup> and because of the reported photolysis of pyridoxal 5'-phosphate<sup>6</sup>. Then, the polarograms were carried out and the pH of the mixtures were checked.

The equilibrium constants,  $K_{pH}$ , of the reaction



were calculated by the least squares adjustment of the experimental data to the equation

$$i_b/i_a = K_{pH}[\text{amine}]_f, \quad (1)$$

where  $i_b$  and  $i_a$  stand for the total limiting currents of the Schiff base and aldehyde, respectively. The amine free concentrations,  $[\text{amine}]_f$ , were calculated by subtracting the Schiff base concentrations (evaluated from their waveheights) from the total amine concentration<sup>4</sup>.

The correlation indexes of those adjustments were always greater than 0.991. KCl was added to the solutions with buffer, amine and pyridoxal 5'-phosphate in order to keep a constant value of the ionic strength. The ionic strength was 0.6, unless otherwise stated. The temperature was 20°C; instruments, chemicals and solutions were described in ref.<sup>7</sup>.

## RESULTS AND DISCUSSION

Figure 1 is an example of the polarograms obtained at a constant pH, in which four waves appear. The simpler case is that in which there are only two waves<sup>4</sup>. The more positive wave is always due to the reduction of the Schiff base formed, as checked from its half-wave potential<sup>4</sup>, and the fact that it does not appear without amine addition. The half-wave potentials of the other waves coincide with those previously reported for pyridoxal 5'-phosphate<sup>7</sup>. The total wave height of aldehyde was necessary for the calculation of  $K_{pH}$  (using equation (1)) but not the relative currents of the several waves of pyridoxal 5'-phosphate. However, a high influence of the amine concentration on the relative heights of the pyridoxal 5'-phosphate waves can be seen in Fig. 1. This influence was also observed in all the pH values studied, as is shown in the examples given in Table I. The addition of amine always increases the relative proportion of the first wave height while it decreases the relative proportion of both the second and third ones. The decrease of the third wave, however, is less than that of the second one.

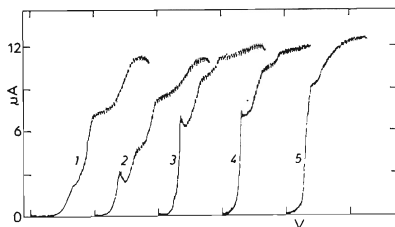


FIG. 1

Polarograms of pyridoxal 5'-phosphate ( $c = 2.8 \cdot 10^{-3}$  mol/l) with several amounts of  $C_6H_{13}.NH_3Cl$  added at pH = 11.6. 200 mV/abs. Curves start at  $-0.85$  V. N-hexylamine concentration: (mol/l.  $10^3$ ): 1 0.0; 2 0.7; 3: 3.6; 4: 5.0; 5: 8.7.

TABLE I

Influence of *n*-hexylamine concentration on the relative intensities of the pyridoxal 5'-phosphate waves at two pH values

$i_1/i_T$	$i_2/i_T$	$i_3/i_T$	[amine] <sub>t</sub> , mmol
pH = 11.6			
0.222	0.458	0.320	0.00
0.307	0.393	0.300	0.15
0.376	0.356	0.268	0.57
0.456	0.362	0.218	1.03
0.620	0.270	0.110	1.99
0.692	0.246	0.062	3.29
pH = 12.0			
0.147	0.354	0.499	0.00
0.205	0.352	0.443	0.29
0.250	0.304	0.446	0.64
0.308	0.271	0.421	1.00
0.474	0.253	0.273	1.32
0.525	0.212	0.263	2.37
0.661	0.161	0.178	4.12

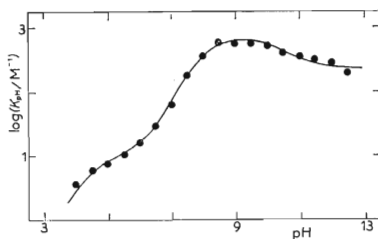


FIG. 2

Dependence of  $K_{pH}$  on the ionic strength of the medium at pH = 7.1

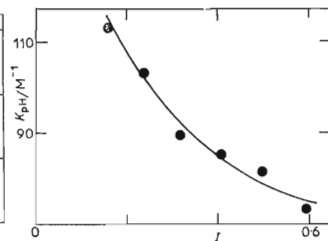
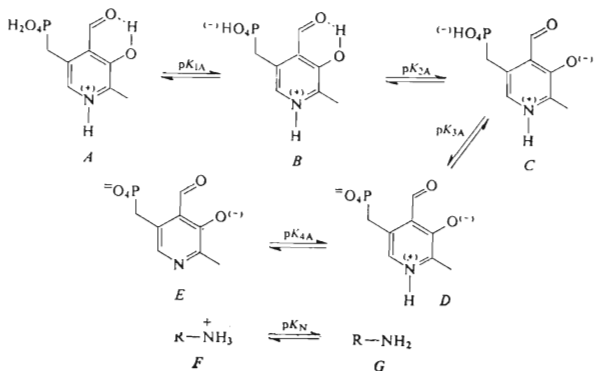
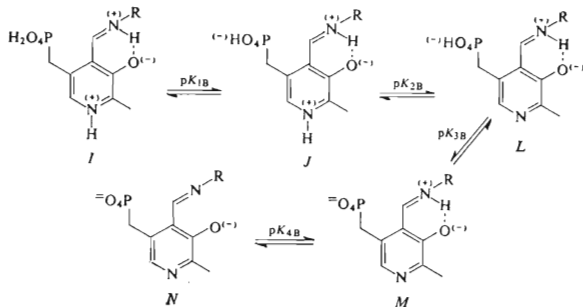


FIG. 3

pH-Dependence of  $\log K_{pH}$ . The solid curve was calculated with equation 3 using the pK values given in Table II and a  $K$  value of  $240 \text{ l mol}^{-1}$



SCHEME 1



SCHEME 2

This fact implies that the polarographic  $pK'$  values found in ref.<sup>7</sup> are functions of the amine concentration, *i.e.*, of the tensoactive agent added, confirming that the protonization reactions of pyridoxal 5'-phosphate previous to its electrode reduction do not occur in solution<sup>8</sup>.

The influence of the ionic strength of the medium on the equilibrium constant,  $K_{\text{pH}}$ , calculated from (1) is given in Fig. 2 for  $\text{pH} = 7.1$ . The minimum amount of salts needed to maintain negligible levels of migration currents do not allow to use very low ionic strengths. Therefore we could not extrapolate the values at zero ionic strength. Thus, the equilibrium constants reported in this paper must be taken as apparent ones and not true thermodynamic values.

The values of  $\log K_{\text{pH}}$  calculated as a function of  $\text{pH}$  are given in Fig. 3. The ionic species involved in this  $K_{\text{pH}}$  are depicted in the Schemes 1 and 2 with their dissociation equilibria. These schemes are simplified because there are several tautomeric forms in equilibrium for each ionic species.

Equation (1) can be written now as

$$K_{\text{pH}} = \frac{I + J + L + M + N}{(F + G)(A + B + C + D + E)}, \quad (2)$$

where each capital letter represents the total concentration of all tautomeric forms with the same net charge and not only those illustrated in the Schemes.

This equation can be easily converted in

$$\log K_{\text{pH}} = \log K + \log \left( \frac{[\text{H}^+]^4}{K_{1\text{B}} \cdot K_{2\text{B}} \cdot K_{3\text{B}} \cdot K_{4\text{B}}} + \frac{[\text{H}^+]^3}{K_{2\text{B}} \cdot K_{3\text{B}} \cdot K_{4\text{B}}} + \right.$$

TABLE II

$\text{p}K$  Values that fit best the experimental  $K_{\text{pH}}$  values (Eq. (3)). Literature values are also included for comparison

$\text{p}K$	This work	Spectroscopy	Potentiometry
$\text{p}K_{1\text{A}}$	1.8 <sup>a</sup>	—	1.4 <sup>b</sup> ; 2.2 <sup>c</sup> ; 2.5 <sup>d</sup>
$\text{p}K_{2\text{A}}$	4.5	4.14 <sup>e</sup> ; 4.66 <sup>f</sup> ; 3.45 <sup>g</sup> ; 3.62 <sup>h</sup> ; 3.7 <sup>i</sup>	3.4 <sup>b</sup> ; 3.9 <sup>c</sup> ; 4.1 <sup>d</sup>
$\text{p}K_{3\text{A}}$	6.1	6.10 <sup>h</sup>	6.0 <sup>b</sup> ; 6.1 <sup>c</sup> ; 6.2 <sup>d</sup>
$\text{p}K_{4\text{A}}$	8.0	8.69 <sup>e</sup> ; 8.69 <sup>f</sup> ; 8.37 <sup>g</sup> ; 8.33 <sup>h</sup> ; 8.6 <sup>i</sup>	8.4 <sup>b</sup> ; 8.3 <sup>c</sup> ; 8.69 <sup>d</sup>
$\text{p}K_{1\text{B}}$	1.8 <sup>a</sup>	—	—
$\text{p}K_{2\text{B}}$	6.0	—	—
$\text{p}K_{3\text{B}}$	6.3	6.8 <sup>i</sup>	—
$\text{p}K_{4\text{B}}$	10.9	11.2 <sup>i</sup>	—
$\text{p}K_{\text{N}}$	10.4	—	10.56 <sup>j</sup>

<sup>a</sup> Assumed; <sup>b</sup> Ref.<sup>9</sup>; <sup>c</sup> Ref.<sup>12</sup>; <sup>d</sup> Ref.<sup>13</sup>; <sup>e</sup> Ref.<sup>14</sup>; <sup>f</sup> Ref.<sup>15</sup>; <sup>g</sup> Ref.<sup>16</sup>; <sup>h</sup> Ref.<sup>17</sup>; <sup>i</sup> Ref.<sup>18</sup>; <sup>j</sup> Ref.<sup>19</sup>.

$$\begin{aligned}
& + \frac{[\text{H}^+]^2}{K_{3B} \cdot K_{4B}} + \frac{[\text{H}^+]}{K_{4B}} + 1) - \log \left( \frac{[\text{H}^+]^4}{K_{1A} \cdot K_{2A} \cdot K_{3A} \cdot K_{4A}} + \right. \\
& \left. + \frac{[\text{H}^+]^3}{K_{2A} \cdot K_{3A} \cdot K_{4A}} + \frac{[\text{H}^+]^2}{K_{3A} \cdot K_{4A}} + \frac{[\text{H}^+]}{K_{4A}} + 1) - \log \left( 1 + \frac{[\text{H}^+]}{K_N} \right), \quad (3)
\end{aligned}$$

where  $K = N/E$ .  $G$  is the apparent equilibrium constant for the reaction between the most deprotonated forms, *i.e.*, those present in solution at pH above 12 (ref.<sup>9-11</sup>).

The best fitting of the experimental values to the theoretical curve, equation (3), was obtained with the pK values given in Table II. There is a quite good agreement among the pK values given in Table II and those obtained by different techniques (except those of Schiff base and n-hexilamine that were obtained at very low ionic strength). The estimated value of  $K = 240 \text{ l mol}^{-1}$  is similar to that reported by Metzler for the reaction of pyridoxal with several amines<sup>11</sup>.

The waves of pyridoxal 5'-phosphate and its Schiff bases merge at low pH. Therefore we do not report measurements at pH values lower than 4.

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