

## A Potentiometric Study into the Stability of the Schiff's Base Formed between Pyridoxal 5'-Phosphate and Hexylamine in Water-Dioxane Mixtures

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We have developed a fast and accurate potentiometric method to characterize the pH-dependent, Schiff's base formation equilibria in water-organic cosolvent mixtures. This method has been applied to the reaction between pyridoxal 5'-phosphate and hexylamine in water-dioxane. We have found that, at neutral and basic pH, the Schiff's base is more stable in the lower polarity media. It is suggested that this is a secondary effect, due to the fact that Schiff's base formation is accompanied by charge neutralization.

Pyridoxal 5'-phosphate (PLP), a biochemically active derivative of Vitamin B<sub>6</sub>, acts as a coenzyme in many enzymatic reactions, mainly those involved in  $\alpha$ -aminoacid metabolism.<sup>1</sup> Pyridoxal 5'-phosphate is the essential component of the enzyme active site, as shown by the fact that this substance (as well as other Vitamin B<sub>6</sub> molecules) also slowly catalyses many of these reactions in the absence of protein.<sup>1</sup> There is evidence to show that PLP-sites in, at least some, Vitamin B<sub>6</sub>-dependent enzymes have lower polarity than water<sup>2-5</sup> and several studies<sup>3,6-12</sup> have been addressed to understanding the effect of solvent polarity on the physicochemical features of Vitamin B<sub>6</sub> molecules.

Pyridoxal 5'-phosphate binds to enzymes through a Schiff's base linkage with a lysine residue;<sup>1</sup> in addition, the first step of the catalytic process of the Vitamin B<sub>6</sub>-dependent enzymes that take part in the metabolism of aminoacids, involves the breaking up of this Schiff's base and the forming of a new one with the amino group of the substrate.<sup>1</sup> In spite of this, few studies<sup>13-15</sup> have considered solvent effects on the rates and equilibria of Schiff's base formation between Vitamin B<sub>6</sub> substances and aminoacids or amines. Here we report the results of an equilibrium study of the reaction of pyridoxal 5'-phosphate with hexylamine, as a function of pH, in water-dioxane mixtures.

Previous work on Vitamin B<sub>6</sub> substances (see, for instance, refs. 12 and 16) shows that a great deal of basic information can be derived from comparative studies of many related systems; thus, in the case of Schiff's base formation, it would be desirable to study the reaction of several Vitamin B<sub>6</sub>-substances with different amines and in different types of mixed solvents. The feasibility of this long-term goal depends, however, on the availability of experimental approaches that allow us to obtain the required information about a given Vitamin B<sub>6</sub> substance-amine-solvent system in a comparatively short time. Accordingly, one of the main purposes of this work is to develop a fast and accurate method to characterize the pH-dependent, Schiff's base formation equilibrium in water-organic cosolvent mixtures.

We have found that this equilibrium information can be derived, upon the appropriate data analysis, from potentiometric titrations (with a strong base) of solutions containing the Vitamin B<sub>6</sub> substance *plus* amine *plus* the Schiff's base. This method requires comparatively low concentrations, which minimizes the possibility of ionic associations in low polarity solvents. Also, deviations from ideality are taken into account in the data analysis by using reasonable estimates of the relevant activity coefficients.

This potentiometric method may also be useful in the study of other complex equilibria in solution. Accordingly, our data

analysis is based on a very general formalism (binding partition functions), which should facilitate the application of the method to other pH-dependent reactions.

### Experimental

**Materials.**—Pyridoxal 5'-phosphate (Merck), hexylamine (Jansen) and carbonate-free potassium hydroxide (Merck) were used without further purification. Dioxane (Ferosa) was purified and dried as described in previous studies.<sup>9</sup> Distilled, deionized and degassed water was used throughout.

The concentrations of stock solutions of pyridoxal 5'-phosphate were calculated from their absorption<sup>17</sup> at 388 nm ( $\epsilon$  6600) after dilution in 0.1 mol dm<sup>-3</sup> NaOH, or from potentiometric titrations with freshly prepared KOH; both values differed by less than 1%.

Hexylamine solutions were prepared by diluting the appropriate amount of the commercial product; their concentrations were calculated from titrations with a HCl solution of known concentration. Stock solutions of chlorhydrate of hexylamine were prepared from hexylamine solutions by adding an equimolar volume of HCl solution.

KOH stock solutions were prepared by weight and their concentrations were determined by titration with potassium acid phthalate; these concentrations agreed within 0.5% with those calculated from the weights.

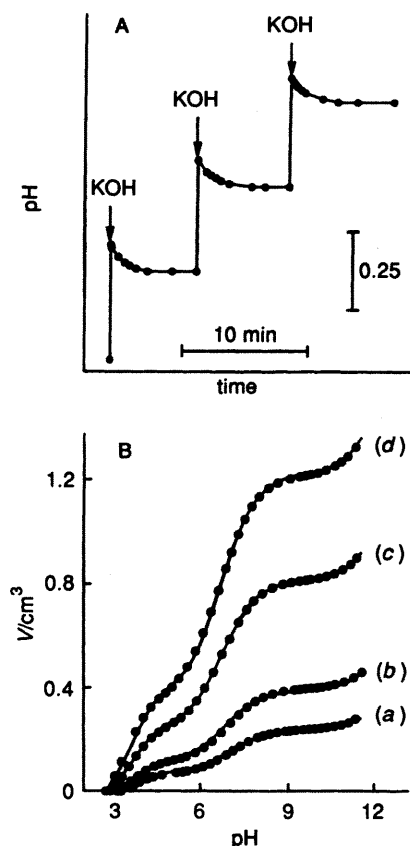
All solutions were kept under nitrogen and in the dark.

**pH Measurements.**—The pH measurements were made with a combined electrode Ingold U402-M3-S7 and a pH-meter Crison-501 which can detect 0.01 unit of pH. The pH-meter was calibrated by use of standard aqueous buffers.

pH Values ( $-\log a_{\text{H}^+}$ ) were obtained from the pH-readings ( $B$ ) in water-dioxane by using the equation  $\text{pH} = B + \log U_{\text{H}^+}^0$ , and the previously published<sup>9</sup> values of the correction factor ( $\log U_{\text{H}^+}^0$ ) in water-dioxane.

**Potentiometric Titrations.**—Potentiometric titrations were carried out in water-dioxane mixtures with dioxane volume fractions of 0, 0.2, 0.4 and 0.6. All measurements were made at  $25 \pm 0.1$  °C, in the dark and under nitrogen, with recently prepared solutions obtained (except for the measurements in water) by adding a carefully measured volume of dioxane to fresh stock solutions in water.

The titration experiments started with a known volume ( $V_0 = 2$  cm<sup>3</sup>) of an 'aldehyde + amine' solution; this solution had been prepared by mixing equimolar volumes of pyridoxal 5'-phosphate and the chlorhydrate of hexylamine solutions in the desired water-dioxane mixture [note that the analytical



**Fig. 1** A: The time dependence of the pH value during the potentiometric titration of an aldehyde + amine solution with KOH solution. These data correspond to the titration (b) shown below. B: Volume of titrating solution ( $V$ ) versus pH profiles for potentiometric titrations of aldehyde + amine solutions in water–dioxane 80:20 v/v. In all cases, the initial volume ( $V_0$ ) of the solution is  $2 \text{ cm}^3$  and the concentration of KOH in the titrating solution ( $C_{\text{KOH}}$ ) is  $0.151 \text{ mol dm}^{-3}$ . The titrations differ in the value of the analytical concentration of PLP and amine ( $C_A$ ) in the initial solution. The  $C_A$  values are: (a),  $5.93 \times 10^{-3}$ ; (b)  $9.88 \times 10^{-3}$ ; (c),  $2.05 \times 10^{-2}$ ; (d),  $3.08 \times 10^{-2} \text{ mol dm}^{-3}$ . (●), experimental values; (—), theoretical  $V_{\text{calc}}/\text{pH}$  profiles calculated from the parameters given in Table 2.

concentrations of PLP and amine in these solutions ( $C_A$ ) are equal]. The reading of the pH-meter ( $B$ ) was allowed to reach a stable value and this value was taken; a known amount of KOH solution (in the same water–dioxane mixture) was added by using a calibrated syringe and a new measurement of the stable  $B$  value was taken. This procedure was continued until additions of titrating solution caused little change in the  $B$  value. For each water–dioxane mixture, four or five titrations were carried out with different  $C_A$  values within the range  $3 \times 10^{-3} - 3 \times 10^{-2} \text{ mol dm}^{-3}$ . The concentration of KOH in the titrating solution ( $C_{\text{KOH}}$ ) was ca.  $0.1 \text{ mol dm}^{-3}$ .

For any given titration, PLP, amine and the Schiff's base are present in solution and the relative amounts of these three substances change with pH (see Theory, Results and Discussion section). Schiff's base formation is accompanied by proton release<sup>18</sup> (except at very basic pH) and the rates of formation and hydrolysis of the Schiff's base are comparatively low.<sup>19</sup> As a result, the  $B$  value changes slowly with time (in an exponential-like fashion) after each addition of titrating solution (see Fig. 1A). A more detailed discussion on the time-dependence of the  $B$  values can be found in ref. 18; the important point to note here is that the values taken and analysed in this work are the equilibrium, stable ones.

**Data Analysis.**—Fitting of the potentiometric data was

carried out with a non-linear, least-squares program based on the Simplex algorithm and kindly provided by Dr. E. Freire (Johns Hopkins University). This program can fit any function to the experimental data; the specific function used, however, must be included as a subroutine. In our case, the subroutine involves an iterative procedure (see Theory, Results and Discussion section) and was written by us in C-language.

### Theory, Results and Discussion

Fig. 1B shows the volume of titrating solution ( $V$ ) versus pH profiles obtained from potentiometric titrations (with KOH solution) of aldehyde + amine solutions (see Experimental section). The data of Fig. 1 correspond to water–dioxane (80:20) v/v; similar results were obtained in the other mixtures studied in this work.

Schiff's base formation takes place in the aldehyde + amine solutions and, as a result, the  $V/\text{pH}$  profiles for these solutions are clearly different to those obtained from titrations of pyridoxal 5'-phosphate alone (results not shown), even at pH values below the  $\text{p}K$  of the hexylammonium ion. However, the equilibrium is not expected to be fully displaced towards the Schiff's base under all pH conditions within the range studied; this is suggested by the values of the apparent equilibrium constant for Schiff's base formation previously determined in aqueous solution<sup>19</sup> and by the fact that slow time-dependences of the pH value were observed (in all the water–dioxane mixtures studied) after the additions of the titrating solution (see Experimental section and ref. 18). Accordingly, an appropriate analysis of the potentiometric data should allow us to quantitatively characterize the pH-dependent, Schiff's base formation equilibrium. The theoretical basis of this analysis is described below.

**Ionization Equilibria.**—We will employ a general formalism based on the use of binding partition functions<sup>20–22</sup> (also called binding polynomials or generating functions). Our treatment closely follows that given by Poland,<sup>21</sup> the only significant difference being that we take specifically into account the deviations from ideal behaviour by introducing activity coefficients for all ionic species.

Consider a substance ( $M$ ), which exists in solution as a mixture of ionic species which differ in the number of bound protons. A given species will be termed  $M_i$ , where the subindex stands for the number of bound protons. We take as reference the less protonated species among those significantly populated in the pH range under study:  $M_r$  ( $r$  = number of protons bound to the reference species). We define overall protonation constants ( $K_{M_i}^0$ ) as in eqn. (1) where  $[M_i]$  and  $[M_r]$  stand for the molar



concentrations,  $\gamma_{M_i}$  and  $\gamma_{M_r}$  for the activity coefficients in the molar scale, and  $a_H (= 10^{-\text{pH}})$  is the conventional activity of the hydrogen ion.

The statistical weight of a species ( $\omega_{M_i}$ ) is the ratio between its concentration and that of the reference species [eqn. (2)].

$$\omega_{M_i} = [M_i]/[M_r] = K_{M_i}^0 (\gamma_{M_r}/\gamma_{M_i}) a_H^{(i-r)} \quad (2)$$

Note that the values of  $K_{M_r}^0$  (the constant for the 'equilibrium'  $M_r \rightleftharpoons M_r$ ) and  $\omega_{M_r}$  (the statistical weight for the reference species) are unity.

The binding partition function ( $\xi_M$ ) can be defined as the sum of all statistical weights [eqn. (3)].

**Table 1** Reference species and partition function for PLP, hexylamine and Schiff's base

| Substance     | Reference species | Charge of the species | Partition function  |
|---------------|-------------------|-----------------------|---|
| PLP           | P <sub>0</sub>    | -3                    | $\xi_P = \sum_{i=0}^3 K_{Pi}^0 (\gamma_{P0}/\gamma_{Pi}) a_H^i = \sum_{i=0}^3 \frac{(\gamma_{P0}/\gamma_{Pi}) a_H^i}{\prod_{j=0}^i K_{Pj}}$             |
| Hexylamine    | A <sub>0</sub>    | 0                     | $\xi_A = 1 + K_{A1}^0 (\gamma_{A0}/\gamma_{A1}) a_H = 1 + \frac{(\gamma_{A0}/\gamma_{A1}) a_H}{K_{A1}}$   |
| Schiff's base | B <sub>1</sub>    | -2                    | $\xi_B = \sum_{i=1}^3 K_{Bi}^0 (\gamma_{B1}/\gamma_{Bi}) a_H^{(i-1)} = \sum_{i=1}^3 \frac{(\gamma_{B1}/\gamma_{Bi}) a_H^{(i-1)}}{\prod_{j=1}^i K_{Bj}}$ |

$$\xi_M = \sum_i \omega_{Mi} = \sum_i K_{Mi}^0 (\gamma_{Mr}/\gamma_{Mi}) a_H^{(i-r)} \quad (3)$$

The fraction ( $x_{Mi}$ ) of substance M present as species M<sub>i</sub>, is given by the ratio between the statistical weight of the species and the partition of function in eqn. (4) where  $[M]_T (= \sum_i [M_i])$

$$X_{Mi} = [M_i]/[M]_T = \omega_{Mi}/\xi_M = K_{Mi}^0 (\gamma_{Mr}/\gamma_{Mi}) a_H^{(i-r)} / \xi_M \quad (4)$$

stands for the total concentration of M. Note that the fraction of substance present as reference species is given by eqn. (5) as  $\omega_{Mr} = 1$ .

$$X_{Mr} = [M_r]/[M]_T = 1/\xi_M \quad (5)$$

The above formalism enables us to write compact expressions for the average quantities of interest. Thus, the average number of protons bound per molecule of substance ( $\langle i \rangle_M$ ) can be written as eqn. (6) and the average charge per molecule of

$$\langle i \rangle_M = \sum_i i x_{Mi} = (1/\xi_M) \sum_i i K_{Mi}^0 (\gamma_{Mr}/\gamma_{Mi}) a_H^{(i-r)} \quad (6)$$

substance ( $\langle q \rangle_M$ ) is given by eqn. (7) where  $q_i$  is the charge of

$$\langle q \rangle_M = \sum_i q_i x_{Mi} = (1/\xi_M) \sum_i q_i K_{Mi}^0 (\gamma_{Mr}/\gamma_{Mi}) a_H^{(i-r)} \quad (7)$$

the species M<sub>i</sub>. Given that  $q_i$  and the charge of the reference species ( $q_r$ ) are related by  $q_i = q_r - r + i$ , eqn. (7) can be written as eqn. (8).

$$\langle q \rangle_M = q_r - r + \langle i \rangle_M \quad (8)$$

Eqs. (2)–(7) are couched in terms of the overall protonation constants ( $K_{Mi}^0$ ). We shall be using, however, stepwise ionization constants ( $K_{Mi}$ ) as fitting parameters. These are defined according to eqn. (9).

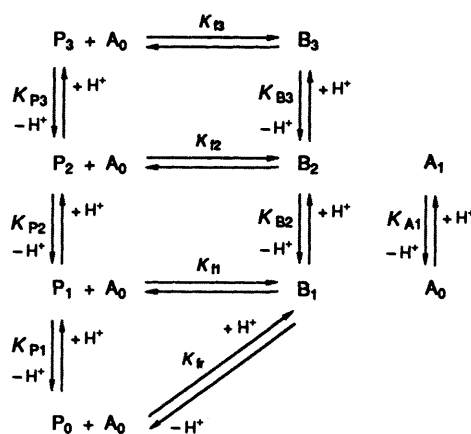


Both sets of equilibrium constants are related by eqn. (10)

$$K_{Mi}^0 = \left[ \prod_{j=r}^{j=i} K_{Mj} \right]^{-1} \quad (10)$$

where, by convention, a value of unity is assigned to the first term of the product (the 'constant'  $K_{Mr}$ ).

The above treatment is general and can be applied to pyridoxal 5'-phosphate (M≡P), hexylamine (M≡A) and the Schiff's base (M≡B). The significantly populated species of these three substances (within the pH range of the potentiometric experiments) are shown in Scheme 1; the reference species and the specific equations used for the partition functions are given in Table 1.



Scheme 1

Owing to the occurrence of pH-independent equilibria<sup>3,11,12,16</sup> (tautomerism and hydration), each ionic species is in fact a mixture of different molecular forms (the significantly populated forms for the species P<sub>1</sub> of PLP and B<sub>1</sub> of the Schiff's base are shown in Scheme 2); therefore, the equilibrium constants employed in the above treatment are macroscopic.

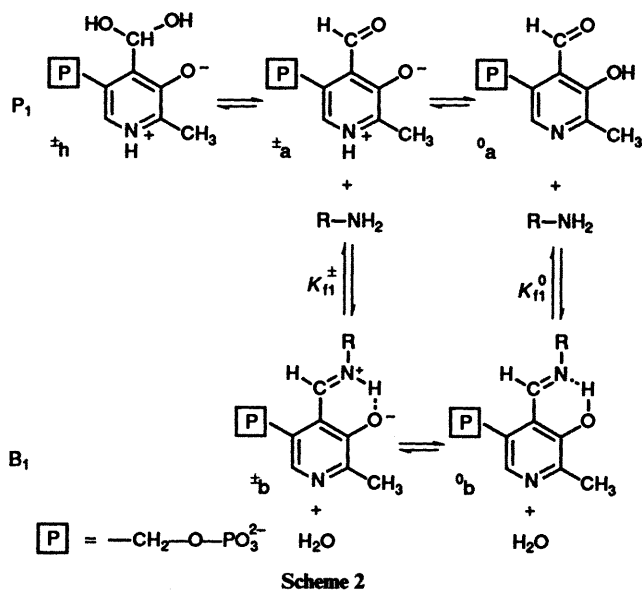
The macroscopic pK corresponding to the first protonation of the Schiff's base (pK<sub>B1</sub>) is known to be very high, reflecting<sup>16</sup> the chelate effect of the hydrogen bonding indicated in Scheme 2. In fact, pK<sub>B1</sub> is outside the pH range of the potentiometric experiments (its value, however, can be estimated<sup>16</sup> from the pH-dependence of the UV-VIS spectra of solutions containing PLP and an excess of amine: see Table 2). As a result, the non-protonated species, B<sub>0</sub>, is not significantly populated within the pH range of the potentiometric experiments and we take B<sub>1</sub> as reference species for the Schiff's base (Scheme 1 and Table 1).

*Linkage Between Ionization and Schiff's Base Formation Equilibria.*—The Schiff's base formation equilibrium can be described<sup>16</sup> by an apparent equilibrium constant, defined in terms of the total concentrations [eqn. (11)]. For a given

**Table 2** Equilibrium constants of ionization and formation of Schiff's base in water-dioxane mixtures

| Constant      | Conditions            | Reference | Dioxane volume fractions |       |       |       |
|---------------|-----------------------|-----------|--------------------------|-------|-------|-------|
|               |                       |           | 0                        | 0.2   | 0.4   | 0.6   |
| $pK_{P_1}$    | 25 °C, $\Gamma = 0$   | This work | 9.04                     | 9.23  | 10.07 | 11.23 |
|               | 25 °C, $\Gamma = 0$   | <i>a</i>  | 8.92                     | 9.19  | 9.93  | 11.29 |
| $pK_{P_2}$    | 25 °C, $\Gamma = 0$   | This work | 6.32                     | 6.93  | 7.50  | 9.00  |
|               | 25 °C, $\Gamma = 0$   | <i>a</i>  | 6.50                     | 7.15  | 7.97  | 8.91  |
| $pK_{P_3}$    | 25 °C, $\Gamma = 0$   | This work | 3.84                     | 3.97  | 3.94  | 4.36  |
|               | 25 °C, $\Gamma = 0$   | <i>a</i>  | 3.70                     | 3.87  | 3.97  | 3.85  |
| $pK_{B_1}$    | 25 °C, $\Gamma = 0$   | <i>b</i>  | 12.81                    | 14.13 | 14.60 | 15.50 |
| $pK_{B_2}$    | 25 °C, $\Gamma = 0$   | This work | 6.91                     | 7.25  | 7.88  | 9.43  |
|               | 25 °C, $\Gamma = 0.1$ | Ref. 19   | 6.60                     | —     | —     | —     |
|               | 20 °C, $\Gamma = 0.1$ | <i>c</i>  | 6.70                     | —     | —     | —     |
| $pK_{B_3}$    | 25 °C, $\Gamma = 0$   | This work | 5.99                     | 5.85  | 5.54  | 6.07  |
|               | 25 °C, $\Gamma = 0.1$ | Ref. 19   | 5.00                     | —     | —     | —     |
|               | 20 °C, $\Gamma = 0.1$ | <i>c</i>  | 5.40                     | —     | —     | —     |
| $\log K_{fr}$ | 25 °C, $\Gamma = 0$   | This work | 14.65                    | 14.85 | 15.41 | 16.81 |
|               | 25 °C, $\Gamma = 0.1$ | Ref. 19   | 13.99                    | —     | —     | —     |
|               | 20 °C, $\Gamma = 0.1$ | <i>c</i>  | 14.02                    | —     | —     | —     |
| $pK_{A_1}$    | 25 °C, $\Gamma = 0$   | <i>d</i>  | 10.58                    | 10.37 | 10.09 | 9.89  |

<sup>a</sup> From potentiometric titrations of PLP alone (I. M. Plaza del Pino, Doctoral Thesis, University of Granada, 1990). <sup>b</sup> From the pH-dependence of the UV-VIS spectra of solutions containing PLP and an excess of hexylamine (I. M. Plaza del Pino, Doctoral Thesis, University of Granada, 1990). <sup>c</sup> M. A. Garcia del Vado, J. Donoso, F. Muñoz, G. Echevarria and F. Garcia-Blanco, *J. Chem. Soc., Perkin Trans. 2*, 1987, 445. <sup>d</sup> J. Llor, S. Ansensio and J. M. Sanchez-Ruiz, *Int. J. Chem. Kinet.*, 1989, 21, 51.



$$K_{pH} = \frac{[B]_T}{[P]_T[A]_T} \quad (11)$$

temperature and solvent composition,  $K_{pH}$  depends on pH and ionic strength. Note that, if the  $K_{pH}/pH$  profile for a given titration is known, the  $[P]_T/pH$ ,  $[A]_T/pH$  and  $[B]_T/pH$  profiles for that titration can be easily obtained by using eqn. (11), together with the mass-balance relationships eqn. (12) where

$$C_A \cdot f = [P]_T + [B]_T = [A]_T + [B]_T \quad (12)$$

$C_A$  is the analytical concentration of, both PLP and amine in the initial solution and  $f$  is the dilution factor:  $f = V_0/(V + V_0)$ .

The ionization equilibria [described by eqns. (1)–(10)] and the Schiff's base formation equilibrium [described by eqn. (11)] can be linked by using eqn. (5), which, upon substitution into eqn. (11), leads to eqn. (13) where  $K_{fr}$  is the equilibrium constant

$$K_{pH} = K_{fr} a_H \frac{\gamma_{P_0} \gamma_{A_0}}{\gamma_{B_1}} \times \frac{\xi_B}{\xi_P \cdot \xi_A} \quad (13)$$

for the reaction between the reference species [eqn. (14)].

$$P_0 + A_0 + H^+ \rightleftharpoons B_1 \quad K_{fr} = \frac{[B_1] \gamma_{B_1}}{[P_0] \gamma_{P_0} [A_0] \gamma_{A_0}} (a_H)^{-1} \quad (14)$$

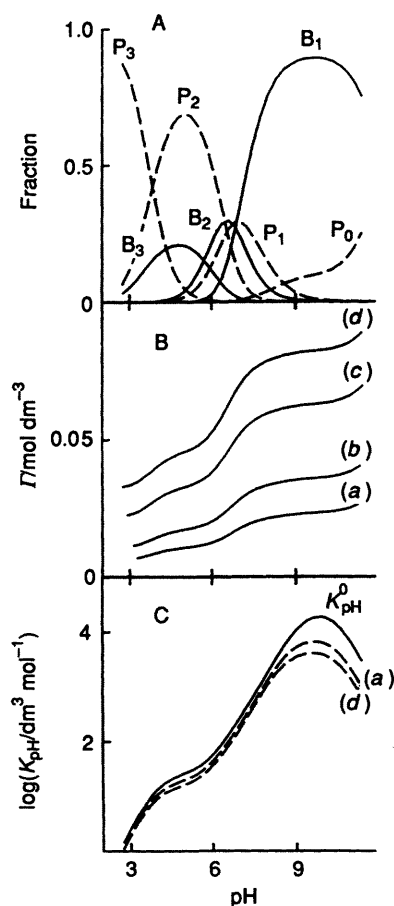
Eqn. (13) [together with eqn. (3)] gives  $K_{pH}$  as a function of pH and ionic strength (through the activity coefficients). Note, however, that  $K_{fr}$  [eqn. (14)] does not depend on pH or ionic strength.

*Calculation of the Added Volume of Titrating Solution.*— Given the values of  $V_0$  (the volume of the initial aldehyde + amine solution),  $C_A$  (the analytical concentrations of PLP and amine in the initial solution) and  $C_{KOH}$  (the concentration of KOH in the titrating solution) for a given titration experiment, and trial values of  $\log K_{fr}$  and the stepwise ionization constants of PLP ( $pK_{P_i}$ ), amine ( $pK_{A_1}$ ) and the Schiff's base ( $pK_{B_i}$ ), we wish to calculate the volume of titrating solution required to reach a given pH value. This calculated volume will be referred to as  $V_{calc}$ .

The calculation procedure can be outlined as follows: eqns. (10), (3) and (13) permit the calculation of  $\xi_P$ ,  $\xi_B$ ,  $\xi_A$  and  $K_{pH}$  at the desired pH. The value obtained for  $K_{pH}$ , together with the mass-balance relationship [eqn. (12)], leads to the total concentrations of PLP, amine and the Schiff's base. The average charge per molecule for the three substances ( $\langle q \rangle_P$ ,  $\langle q \rangle_B$  and  $\langle q \rangle_A$ ) are obtained by using eqn. (7) [or eqns. (6) and (8)]. Finally,  $V_{calc}$  is calculated from the electroneutrality condition of eqn. (15) given that the potassium ion comes from the

$$[K^+] = [Cl^-] + [OH^-] - [H^+] - \langle q \rangle_P \cdot [P]_T - \langle q \rangle_A \cdot [A]_T - \langle q \rangle_B \cdot [B]_T \quad (15)$$

titrating solution:  $[K^+] = C_{KOH} \cdot V_{calc}/(V_0 + V_{calc})$ . (Note that  $[Cl^-] = fC_A$  and that the concentrations of hydrogen and hydroxide ions can be calculated from  $[H^+] = 10^{-pH}/\gamma_H$  and  $[OH^-] = K_w \cdot 10^{pH}/\gamma_{OH}$ , where  $K_w$  is the autoprotolysis constant of water).



**Fig. 2** A: Effect of pH on the relative amounts of the PLP species (----) and the Schiff's base species (—) during a potentiometric titration of an aldehyde + amine solution. The fractions shown are referred to the total of PLP plus Schiff's base. These data correspond to titration (d) in Fig. 1B. B: Ionic strength versus pH profiles for the four titrations shown in Fig. 1B. C: Effect of pH on the apparent, equilibrium constant for Schiff's base formation. (----),  $\log K_{\text{pH}}/\text{pH}$  profiles for titrations (a) and (d) of Fig. 1B. (—), apparent equilibrium constant at zero ionic strength ( $K_{\text{pH}}^0$ ) versus pH profile for the water-dioxane mixture 80:20 v/v.

In applying the above procedure, however, two problems arise.

(1) Several steps in the calculation require the value of the dilution factor,  $f$  [see for instance eqn. (12)]. The correct value of  $f$ , however, is not known until  $V_{\text{calc}}$  has been obtained.

(2) Activity coefficients ( $\gamma_{\text{Pi}}$ ,  $\gamma_{\text{Bi}}$ ,  $\gamma_{\text{Ai}}$ ,  $\gamma_{\text{H}}$  and  $\gamma_{\text{OH}}$ ) appear in most of the equations involved in the calculation of  $V_{\text{calc}}$ . As previously proposed,<sup>9</sup> the activity coefficient of an ionic species can be estimated from  $\gamma = (\gamma_{\pm})^q$  where  $q$  is the charge of the species and  $\gamma_{\pm}$  is the mean activity coefficient of HCl in the same solvent and at the same ionic strength (interpolated from the data of Harned and Owen<sup>23</sup>). The ionic strength ( $\Gamma$ ) can be easily obtained if the concentrations of all ionic species in solution are known. The concentrations of the ionic species of PLP, amine and the Schiff's base can be obtained from the total concentrations ( $[\text{P}]_{\text{T}}$ ,  $[\text{A}]_{\text{T}}$  and  $[\text{B}]_{\text{T}}$ ) by using eqn. (5); note, however, that the activity coefficients appear in eqn. (5). Thus, the correct value of  $\Gamma$  (needed for calculating the activity coefficients) is not known until the activity coefficients have been obtained.

It is clear, therefore, that the procedure outlined above for the calculation of  $V_{\text{calc}}$  must be applied in an iterative way. Thus, we calculate a first, approximate value of  $V_{\text{calc}}$  using  $f = 1$ ,  $\Gamma = 0$  and  $\gamma_{\pm} = 1$  as first estimates of  $f$ ,  $\Gamma$  and  $\gamma_{\pm}$ ; once the first value

of  $V_{\text{calc}}$  has been calculated, second (and better) estimates of  $f$ ,  $\Gamma$  and  $\gamma_{\pm}$  are obtained; these, in turn, lead to a second value of  $V_{\text{calc}}$  (closer to the correct one). This iterative procedure is continued until convergence is reached [until, simultaneously, two successive values of  $V_{\text{calc}}$  (in  $\text{cm}^3$ ) and two successive values of  $\gamma_{\pm}$  differ by less 0.0001].

**Fitting the Experimental Titration Profiles.**—The iterative procedure for the calculation of  $V_{\text{calc}}$  was implemented into a general, non-linear, least-squares fitting program. This allowed us to obtain, for each solvent mixture, the optimum values of the fitting parameters, *i.e.* the values that minimize  $\Sigma(V - V_{\text{calc}})^2$ , where  $V$  is the experimental value of the added volume of titrating solution. The values of  $\text{p}K_{\text{Pi}}$ ,  $\text{p}K_{\text{Bi}}$  and  $\log K_{\text{fr}}$  were allowed to vary during the fitting process, while the  $\text{p}K$  of the hexylammonium ion was fixed in the previously published values (see Table 2). For each solvent mixture, four or five titration profiles (corresponding to different values of  $C_{\text{A}}$ ) were simultaneously fitted (*i.e.* with a single set of fitting parameters). In all cases the fits were visually excellent (see, for instance, Fig. 1B).

Note that, as a result of the fitting process, species concentrations,  $K_{\text{pH}}$  values and ionic strength values are obtained. We found that all the ionic species considered in the analysis (Scheme 1) become significant within the pH range of the potentiometric titrations (see, for instance, Fig. 2A). Fig. 2B shows the  $\Gamma/\text{pH}$  profiles corresponding to the titrations of Fig. 1B; note that the ionic strength, not only depends on the value of  $C_{\text{A}}$ , but also changes with pH for a given titration. The fact that it has been possible to fit four or five titration profiles (with clearly different values of  $\Gamma$ ) using a single set of fitting parameters supports our approach.

The values obtained for the fitting parameters are shown in Table 2. In an attempt to estimate the uncertainties associated with these parameters, we have calculated their standard errors following the method proposed by Bevington, which is described in detail in ref. 24; the important point to note here is that this method takes into account the effect of all possible correlations between the fitting parameters. The errors obtained for the parameters  $\log K_{\text{fr}}$ ,  $\text{p}K_{\text{Pi}}$  and  $\text{p}K_{\text{Bi}}$  were about 0.1 units or smaller, which indicates that these parameters are accurately determined from the fitting. Note also (see Table 2) that our values for  $\text{p}K_{\text{Pi}}$  in water-dioxane, as well as those for  $\text{p}K_{\text{Bi}}$  and  $\log K_{\text{fr}}$  in water, compare well with previous estimates (no values for  $\text{p}K_{\text{Bi}}$  and  $\log K_{\text{fr}}$  in water-dioxane have been previously reported in the literature).

Fig. 2C shows the  $K_{\text{pH}}/\text{pH}$  profiles corresponding to the titrations of Fig. 1B. It also shows the  $K_{\text{pH}}^0/\text{pH}$  profile, where  $K_{\text{pH}}^0$  is the value of  $K_{\text{pH}}$  at zero ionic strength [ $K_{\text{pH}}^0$  values are easily calculated from the parameters of Table 2 by setting the activity coefficients equal to unity in eqns. (3) and (13)]. The  $K_{\text{pH}}^0/\text{pH}$  profiles for all the water-dioxane mixtures studied in this work are shown in Fig. 3.

**Solvent Effects on Ionization Equilibria.**—The macroscopic  $\text{p}K$  values of pyridoxal 5'-phosphate and the Schiff's base are plotted in Fig. 4A as a function of the dioxane content of the solvent. The assignments of the  $\text{p}K$ 's of PLP in water-dioxane have been previously discussed;<sup>11,12</sup> it is worth mentioning here that the second protonation of the PLP molecule ( $\text{p}K_{\text{P2}}$  in Fig. 4A) has been shown to occur on the phosphate group, irrespective of solvent composition. Thus, the fact that value of  $\text{p}K_{\text{B2}}$  is very close to that of  $\text{p}K_{\text{P2}}$  in all the water-dioxane mixtures strongly suggests that the second protonation of the Schiff's base also occurs on the phosphate group, in agreement with previous assignments.<sup>13,16</sup> The lower  $\text{p}K$  of the Schiff's base ( $\text{p}K_{\text{B3}}$ ) in water is usually attributed to protonation on the nitrogen of the pyridine ring;<sup>13,16</sup> this  $\text{p}K$  is not strongly

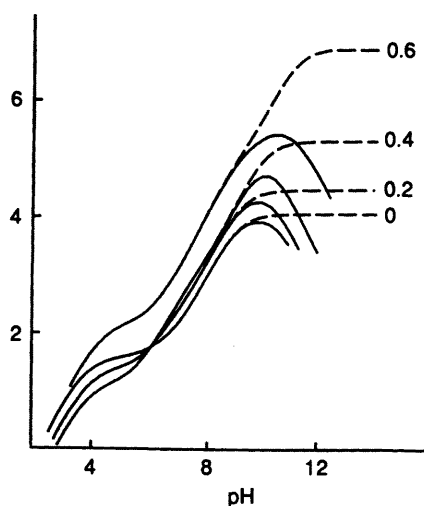


Fig. 3 Effect of pH on the logarithms of the apparent equilibrium constants  $K_{\text{pH}}^0$  (—) and  $K_{\text{pH}}^+$  (---) (see text for definitions) in several water-dioxane mixtures. The numbers alongside the curves stand for the dioxane volume fraction.

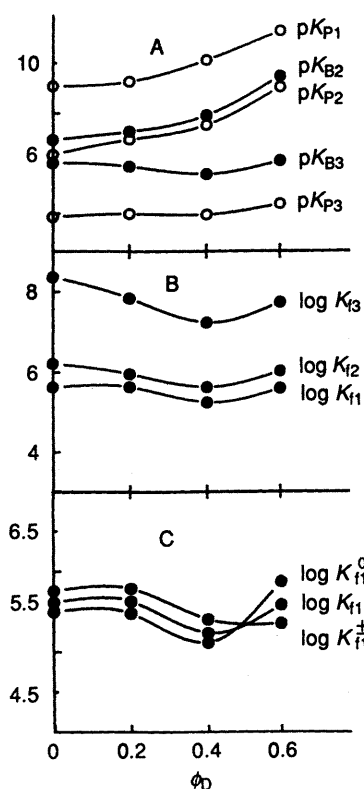
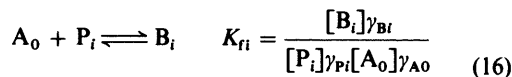


Fig. 4 Effect of the dioxane volume fraction ( $\phi_D$ ) on the following equilibrium constants. A: Macroscopic, stepwise ionization constants of PLP (○—○) and the Schiff's base (●—●). B: Macroscopic constants for Schiff's base formation. C: Macroscopic ( $K_{f1}$ ) and microscopic ( $K_{f1}^0$  and  $K_{f1}^+$ ) constants for Schiff's base formation. The lines are shown to guide the eye and have no theoretical significance.

affected by solvent polarity (Fig. 4A), as is often found for pyridine protonations.<sup>12</sup>

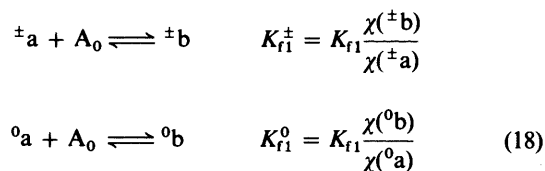
**Solvent Effects on Schiff's Base Formation Equilibria.**—From the data of Table 2, it is possible to calculate the equilibrium constant ( $K_{fi}$ ) for the reaction of the free hexylamine ( $A_0$ ) with a given ionic species of PLP ( $P_i$ ) to give the ionic species of the Schiff's base with the same number of bound protons ( $B_i$ ) [eqn. (16)] by using eqns. (17) which are readily derived from Scheme 1.



$$\begin{aligned} \log K_{f1} &= \log K_{fr} - pK_{P1} \\ \log K_{f2} &= \log K_{f1} - pK_{P2} + pK_{B2} \\ \log K_{f3} &= \log K_{f2} - pK_{P3} + pK_{B3} \end{aligned} \quad (17)$$

In all the water-dioxane mixtures studied, the  $\log K_{fi}$  values increase with the number of protons bound ( $i$ ), that is, with the electron-withdrawing character of the pyridoxal phosphate moiety. Note, however, that the values of  $\log K_{f1}$  and  $\log K_{f2}$  are similar (Fig. 4B), showing that, as was to be expected, protonation on the phosphate group does not greatly affect the Schiff's base formation equilibrium. It is also interesting that solvent polarity has a comparatively small influence on the  $\log K_{fi}$  values (Fig. 4B).

It must be noted, nevertheless, that the  $K_{fi}$  constants are macroscopic; that is, they are defined [see eqn. (16)] in terms of the total concentrations of the ionic species. Owing to the possibility of tautomerism and (in the case of PLP) hydration, each ionic species is, in fact, a mixture of different molecular forms. Microscopic constants for Schiff's base formation (defined in terms of specific molecular forms) can be obtained from the macroscopic ones if the molar fractions ( $\chi$ ) of molecular forms are known for each ionic species. This information in water-dioxane mixtures is available for all ionic species of PLP,<sup>11,12</sup> but only for the species  $B_1$  of the Schiff's base.<sup>3</sup> The significantly populated forms for the species  $P_1$  ( $^{\pm}a$ ,  $^0a$ ,  $^{\pm}h$ ) and  $B_1$  ( $^{\pm}b$ ,  $^0b$ ) are shown in Scheme 2, and the following microscopic equilibrium constants\* [eqn. (18)] could be obtained



from the  $K_{f1}$  values and the published molar fractions.<sup>3,11,12</sup> The values of these microscopic constants are close to that of the macroscopic one ( $K_{f1}$ ) (Fig. 4C). It appears, therefore, that, whether one considers microscopic or macroscopic constants, solvent polarity (at least, in water-dioxane mixtures) has only a moderate effect on the Schiff's base formation equilibrium.

**Solvent Effect on the Apparent, pH-dependent Equilibrium Constant for Schiff's Base Formation.**—It is interesting that, in spite of the above results,  $K_{\text{pH}}^0$  does increase (at constant pH) with the dioxane content, at least at pH values close to that corresponding to the maximum of the  $K_{\text{pH}}^0/\text{pH}$  profiles (Fig. 3). The origin of this effect deserves some attention, given that low-polarity environments in the active sites of Vitamin B<sub>6</sub>-dependent enzymes might contribute to the tight binding of pyridoxal 5'-phosphate.<sup>13</sup>

The macroscopic and microscopic equilibrium constants discussed above ( $K_{fi}$ ,  $K_{fi}^{\pm}$ ,  $K_{fi}^0$ ) correspond to processes in which the charge and the number of bound protons are conserved. However, in discussing the solvent effect on  $K_{\text{pH}}^0$  at a given pH,

\* The equilibrium processes depicted in eqn. (8) and Scheme 2 have been chosen in such a way that the separation of charges of the PLP form and the Schiff's base form are, at least, similar. Thus, any variation of  $K_{f1}^{\pm}$  and  $K_{f1}^0$  with the dioxane content is more likely to be attributable to a solvent effect on the Schiff's base formation process itself (rather than to a concomitant charge redistribution). It may also be of interest to note that tautomers  $^{\pm}b$  and  $^0b$  are among those found in the active sites of Vitamin B<sub>6</sub>-dependent enzymes.<sup>16</sup>

the significantly populated ionic species at that pH must be taken into account. Thus, hexylamine is protonated in most of the pH range studied and it is illustrative to define an apparent, pH-dependent equilibrium constant for Schiff's base formation from PLP and the hexylammonium ion [eqn. (19)] where  $x_{A_1}$

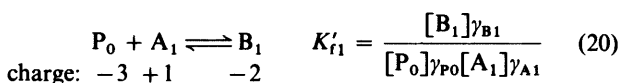


is the fraction of hexylamine present as hexylammonium ion.  $\log K_{\text{pH}}^+/p\text{H}$  and  $\log K_{\text{pH}}^0/p\text{H}$  profiles are compared in Fig. 3. Several features are immediately apparent.

(1) The  $\log K_{\text{pH}}^+/p\text{H}$  profiles do not show a maximum (as do the  $\log K_{\text{pH}}^0/p\text{H}$  profiles) but, instead, they reach a plateau at basic pH; thus, the maximum in the  $\log K_{\text{pH}}^0/p\text{H}$  profile is due to hexylamine ionization (the effect of which is corrected for in the  $\log K_{\text{pH}}^+/p\text{H}$  profiles).

(2) Although amine ionization prevents the  $\log K_{\text{pH}}^0$  values from reaching the plateau, the  $K_{\text{pH}}^0$  values at the maximum are close to those corresponding to the plateau, except for the 40:60 water-dioxane mixture.

(3) The plateau values of  $K_{\text{pH}}^+$  are obviously equal (see Fig. 2A) to the equilibrium constant ( $K'_{t1}$ ) for the reaction between non-protonated PLP and hexylammonium ion to give the monoprotonated Schiff's base [eqn. (20)].



Note that the above reaction involves charge neutralization; therefore, the  $K'_{t1}$  values (and, hence, the  $K_{\text{pH}}^0$  values at the maximum) are expected to be higher in low-polarity media, as is in fact observed in Fig. 3.\* It appears, therefore, that the stabilization of the Schiff's base as the dioxane content increases (Fig. 3) is caused by the fact that the pK values of the substances involved in the reaction are such that Schiff's base formation is accompanied by charge neutralization.

\* This reasoning is intended to explain (qualitatively) the general trend observed. The situation for the 40:60 v/v mixture is somewhat different. In this mixture (see Table 2), the pK value for the hexylammonium ion ( $\text{p}K_{A_1}$ ) is clearly lower than the first pK of the PLP molecule ( $\text{p}K_{P_1}$ ); as a result, the value of  $K_{\text{pH}}^0$  at the maximum is close to that of the equilibrium constant ( $K'_{t1}$ ) for the reaction between monoprotonated PLP ( $P_1$ ) with the non-protonated amine ( $A_0$ ) to give the monoprotonated Schiff's base ( $B_1$ ).

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