Thermodynamic Constants for Tautomerism and Ionization of Pyridoxine and 3-Hydroxypyridine in Water–Dioxane

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Thermodynamic values of the ionization constants and tautomeric equilibrium constants of pyridoxine and 3-hydroxypyridine were determined in dioxane–water at 25 °C. The Born model is unable to explain the effect of the solvent on these constants. The logarithms of the equilibrium constants calculated change linearly according to the logarithm of the molar water content in the mixture, up to 40% dioxane in the case of pyridine ionizations, and up to 70% dioxane (maximum content investigated) for the phenol ionizations. The effect of protonation of either the phenolate or pyridinium nitrogen groups on the pK of the other group in these substances increases when the water content in the medium is decreased.

Polypeptide backbones of proteins are folded in such a way that the micropolarities of the binding sites can be very different from those of the solvent, normally water, in which the protein is dissolved. An understanding of the way in which the solvent polarity influences the physicochemical properties of any ligand is, therefore, fundamental in any study about the nature of the binding of such a ligand to any macromolecule and, in particular, the details of the protein–ligand interactions and the way that the properties of both molecules modify each other.

This consideration could be applied to any ligand, such as substrates or products of any catalytic reaction of a given enzyme, activators, inhibitors, or co-factors. It is, however, even more necessary with ligands such as vitamin B$_6$ in which there are several protonable groups and many possible tautomeric forms whose equilibrium constants can be severely affected by the change of the solvent polarity. Evidence showing that vitamin B$_6$ binding sites have less polarity than water in some vitamin B$_6$-dependent enzymes has accumulated during recent years.[1-6] Most of the physicochemical studies with these molecules, however, have been carried out mainly in water solution.[7-18] Therefore, it is advisable to undertake a study of the influence of solvent polarity on the physicochemical characteristics of the vitamin B$_6$ compounds. Furthermore, this study might help in the understanding of the solvating properties of the organic solvents and the factors that influence the cybotactic region around a given molecule, which are important fields of interest in organic chemistry.

In this paper we report some results about the ionization of a simpler compound in the group (pyridoxine) and a molecule closely related to it, 3-hydroxypyridine, in water–dioxane at 25 °C.

Results

Tautomeric Equilibrium Constants.—The uncharged species of 3-hydroxypyridine and pyridoxine are mixtures of two tautomeric forms in each case (Scheme). The neutral form (absorption maximum at 35 kK in the region transparent to dioxane) is predominant in apolar solvents, while the dipolar form (maxima at *ca.* 30.8 and 39.6 kK) is predominant in water solution. Metzler *et al.*[14] have shown that the spectrum of each tautomeric form of the compounds related to 3-hydroxypyridine can be described nearly quantitatively by summing three log-normal curves, corresponding to three $\pi \rightarrow \pi^*$ bands, called bands I—III, in order of increasing energy. The cut-off point of dioxane is just under 220 nm (see Experimental section and ref. 19). Therefore we could only disentangle the original spectra of the uncharged species of pyridoxine (Figure 1) and 3-hydroxypyridine (not shown) in four log-normal curves (see Figure 2). These four curves, in order of increasing energy, correspond to:
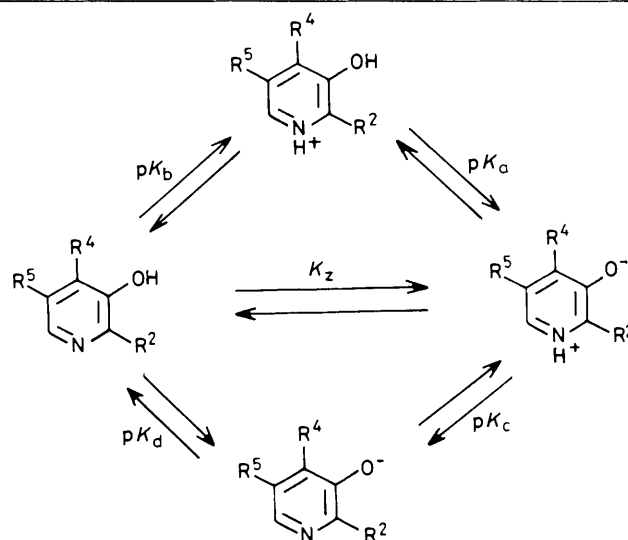
Scheme. 3-Hydroxypyridine, $R^2 = R^4 = R^6 = H$; pyridoxine, $R^2 = CH_3$, $R^4 = R^5 = CH_2OH$.

(1) band I of the dipolar form, (2) band I of the neutral form, (3) band II of the dipolar form, and (4) the sum of a low-energy region of band III of the dipolar form and a low-energy region of band II of the neutral form. The optimum values obtained for the parameters of each band agree (except ε_0) with those given by Metzler *et al.*,[14] the effect of solvent on them being small and in the direction indicated by these authors.

The tautomeric equilibrium constants, K_z, can be calculated from the area under bands I, *a* (integrated intensities) (Table 1), which are directly given by the computer during the deconvolution process (see Experimental section). These areas are proportional to the concentration of the corresponding tautomeric forms. The molar areas, a° (for definition see ref. 14), of those species in which no tautomerism occurs stay constant (*ca.* 0.5%) with both changes in temperature and in solvent composition.[14]

The molar fraction of each tautomeric form, *f*, is given by equation (1) where the subscripts z and n refer to the dipolar and

$$f_z = a_z/a^\circ_z; \quad f_n = a_n/a^\circ_n \qquad (1)$$

neutral forms, respectively. From (1) the tautomeric equilibrium constant, K_z, can be expressed as in equation (2). The value of

$$K_z = (a_z/a_n)/(a^\circ_z/a^\circ_n) \qquad (2)$$
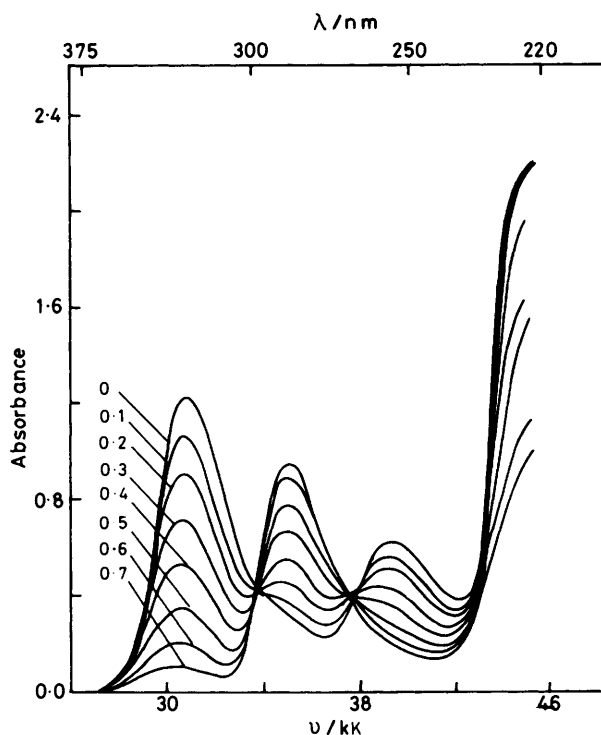
Figure 1. U.v.-visible spectra of pyridoxine at 25 °C in solution of dioxane–water mixtures at the indicated volume fractions of dioxane, φ_D. The concentration of pyridoxine was 1.00×10^{-4}M in all cases. 1 kK $= 10^3$ cm^{-1}

a°_z/a°_n can be obtained from a plot of a_z versus a_n, according to equation (3) obtained from (1), taking into account the fact that

$$a_z = a^\circ_z - (a^\circ_z/a^\circ_n)a_n \qquad (3)$$

$f_z + f_n = 1$.

These plots are clearly linear (except the point corresponding to a dioxane volume fraction of 0.7 for 3-hydroxypyridine, which has not been considered in the calculations below) showing that a°_z/a°_n does not change with the solvent composition (Figure 3). The values obtained from these calculations are summarized in Table 1.

The values obtained for K_z in water (1.10 for 3-hydroxy-pyridine and 3.92 for pyridoxine) agree with those obtained by Metzler et al. by a slightly different method[14] at an ionic strength of 0.2 (1.05 and 3.9, respectively) but do not agree with those reported by the same authors at zero ionic strength (0.88 and 2.3, respectively) in the same publication.[14] They explain their differences as being due to changes in the activity coefficient of the dipolar form according to ionic strength. We have repeated the measurements of K_z of 3-hydroxypyridine in three phosphate buffers with different concentrations (c) and the same pH of 7. The three values of K_z: 1.10 (c 0.04M), 1.10 (c 0.13M), and 1.15 (c 0.15M) appear to indicate that the variation of the activity coefficient of the dipolar form according to the ionic strength should be small (the ionic strength of 0.15M phosphate buffer at pH 7 is near 0.3). This result could be expected given its net zero charge.

Ionization Constants.—The correction factors for the pH meter measurements in water–dioxane, log U°_H, were determined at 25 °C by a slight variation of the dilution method described by Woolley and his co-workers[20–22] (see Experi-
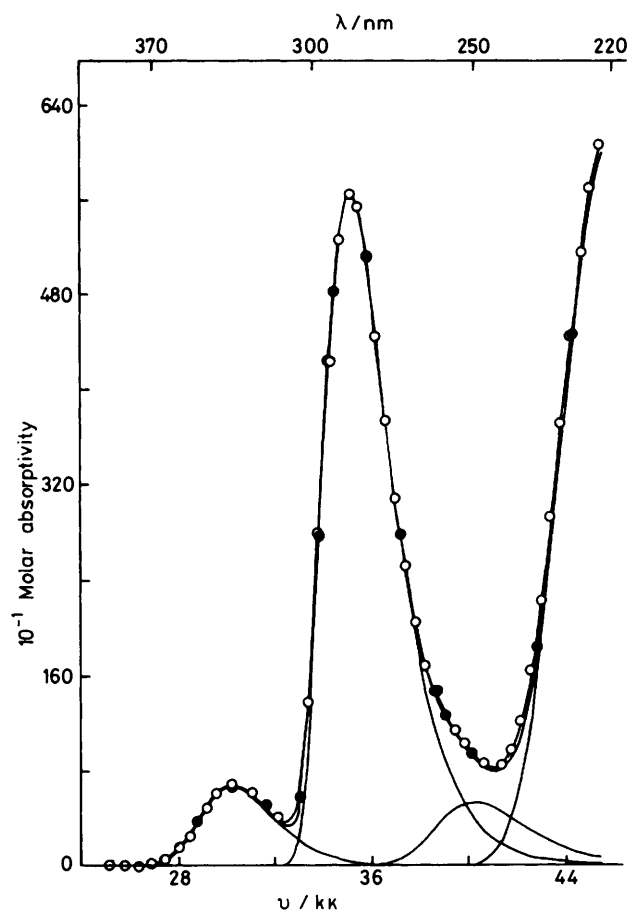

Figure 2. U.v.-visible spectrum of the uncharged species of pyridoxine in water–dioxane (30:70 v/v) at 25 °C. Deconvolution in log-normal bands (——). Original spectrum (●). Sum of log-normal curves (○). Molar absorptivity refers to one mol of the ionic species. 1 kK $= 10^3$ cm^{-1}

Table 1. Areas* under bands I of the dipolar (a_z) and neutral (a_n) forms and the tautomeric equilibrium constants (K_z) of 3-hydroxypyridine and pyridoxine in dioxane–water at 25 °C
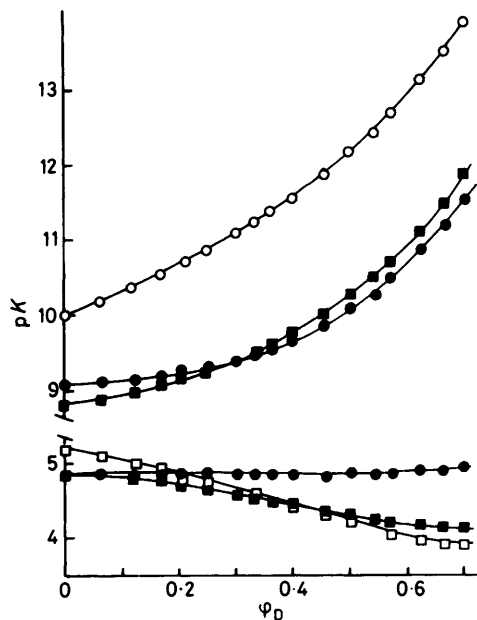
	3-Hydroxypyridine			Pyridoxine		
φ_D	a_z	a_n	K_z	a_z	a_n	K_z
0	104.3	65.8	1.10	271.8	45.6	3.92
0.1	73.2	86.0	0.59	234.4	68.8	2.24
0.2	48.4	104.7	0.32	203.1	89.5	1.49
0.3	29.5	117.3	0.17	161.0	111.8	0.95
0.4	15.3	126.9	0.084	120.4	137.9	0.57
0.5	6.7	131.8	0.035	79.7	168.8	0.31
0.6	3.9	136.6	0.020	48.7	129.6	0.25
0.7	2.1	142.3		26.5	207.2	0.084
	$a^\circ_z/a^\circ_n = 1.44$			$a^\circ_z/a^\circ_n = 1.52$		

* The numerical value of the area in a plot of ε (1 mol^{-1} cm^{-1}) versus 10^4 $\bar{\nu}$ (kK) gives the area in 10 Mm mol^{-1}.
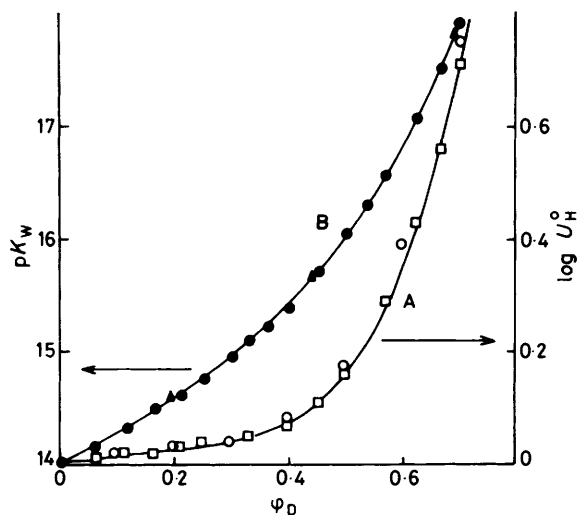
mental section) and by the method described by Van Uitert and Haas.[23] Both types of measurements practically coincide (see Figure 4) and they also agree with other values found in the literature.[24,25] The fact that our log U°_H and pK_w values coincide with those obtained by other authors using different experimental methods is a clear indication that our method is valid.
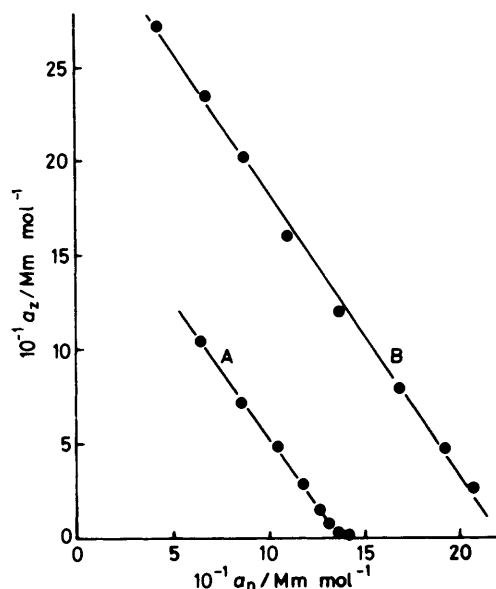
Figure 3. Plot of the area under band I of the dipolar form (a_z) *versus* the area under band I of the neutral form (a_n) of 3-hydroxypyridine (A) and pyridoxine (B). The correlation indices obtained for the straight lines are 0.9993 and 0.9986 for 3-hydroxypyridine (excluding the point at a dioxane volume fraction of 0.7) and pyridoxine, respectively. The ratios between the molar areas $(a°_z/a°_n)$ are given in Table 1
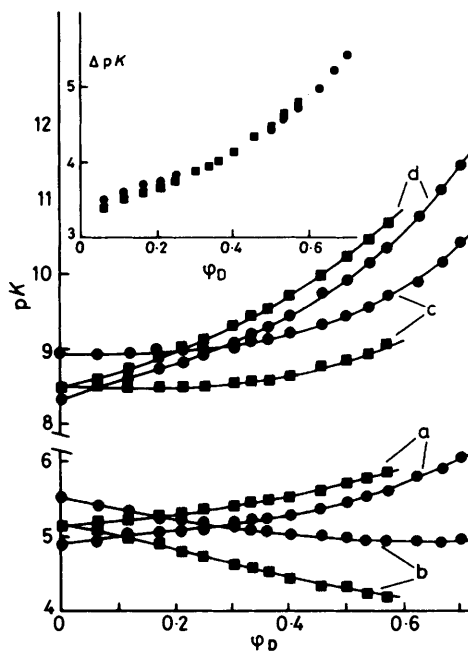

Figure 5. Macroscopic ionization constants of phenol (O), pyridine ($\square$), 3-hydroxypyridine ($\blacksquare$), and pyridoxine ($\bullet$) in dioxane–water at 25 °C. φ_D indicates volume fraction of dioxane

Figure 4. (A) log $U°_H$ values in dioxane–water at 25 °C. Data calculated by the method of Van Uitert *et al.*,[23] O. Each point is the average of the values obtained from three different solutions of equal composition (the mean deviations were less than 0.01). The solutions contained HCl (1.85×10^{-3}M) and NaCl (1.82×10^{-2}M), $\square$. Data calculated by the dilution method (see Experimental). The concentrations of HCl and KCl in the initial water solution were 2.82×10^{-3} and 1.90×10^{-2}M, respectively. (B) Autoprotolysis constants of water in dioxane–water at 25 °C. Values obtained using the dilution method (see Experimental section) $\bullet$. Values taken from ref. 26, $\blacktriangle$. φ_D indicates volume fraction of dioxane

Figure 6. Microscopic ionization constants of 3-hydroxypyridine ($\blacksquare$) and pyridoxine ($\bullet$) in dioxane–water mixtures at 25 °C. The lower case letters refer to the microscopic pK values (see Scheme). Inset: differences between pK_d and pK_a (equal to pK_c − pK_b), ΔpK, as a function of the volume fraction of dioxane, φ_D

The macroscopic pK values obtained for phenol, pyridine, 3-hydroxypyridine, and pyridoxine are given in Figure 5. These values are the average of at least two independent measurements, using different initial concentrations of each compound (5—15 mM). The mean deviations were always equal to, or less than, 0.03. The pK values in water for phenol and

pyridine (9.97 and 5.18, respectively) agree with those given in the literature.[27] The values obtained in water for 3-hydroxy-pyridine (4.86 and 8.78) and pyridoxine (4.84 and 9.04) are close to those reported by Meztler *et al.*[14] at an ionic strength of 0.2 (4.91 and 8.62 for hydroxypyridine, and 4.94 and 8.89 for pyridoxine) and the differences can be easily explained considering the activity coefficients at this ionic strength.

Table 2. k Values obtained by fitting the experimental pK values in several dioxane–water mixtures to the Marshall model: log K = log K° + k log c_w. All the data given in Figure 6 were used in the fitting for phenols, and the data with φ_D equal to or lower than 0.4 in the fitting for pyridinium ions. r Correlation coefficient. The figure alongside k indicates the 90% confidence limit

Substrate	pK in water	k	r
Phenol	9.97	7.56 ± 0.03	1.000
3-Hydroxypyridine (pK_d)	8.45	6.23 ± 0.12	0.999
Pyridoxine (pK_d)	8.35	6.01 ± 0.21	0.997
3-Hydroxypyridine (pK_a)	5.13	1.98 ± 0.06	0.999
Pyridoxine (pK_a)	4.94	2.13 ± 0.12	0.993
Pyridoxine (pK_c)	8.98	1.23 ± 0.23	0.960
3-Hydroxypyridine (pK_c)	8.51	0.60 ± 0.22	0.874
Pyridoxine (pK_b)	5.53	−2.19 ± 0.24	0.986
3-Hydroxypyridine (pK_b)	5.19	−3.45 ± 0.16	0.998
Pyridine	5.18	−3.63 ± 0.08	1.000

The assumptions and approximations implied in the method which we are using to calculate the pK values are the same as those pointed out by Panichajakul and Woolley[22] as regards the dilution method.[20–22] Therefore we believe that the total limit of error (0.08 in pK) given by these authors[22] might also be applied to our values.

The calculation of the microscopic pK values [pK_a, pK_b, pK_c, and pK_d (see Scheme)] from the macroscopic ones and from the tautomeric equilibria K_z is straightforward and the K_z values were interpolated from a plot of log K_z versus φ_D.[14] The results are given in Figure 6.

Discussion

Effect of the Solvent on the Microscopic Ionization Constants and on the K_z Values.—The Born model is unable to explain the effect of the solvent on the microscopic pK values calculated in this work, as can be shown by the fact that the plots of pK versus the inverse of the dielectric constants of the media are markedly curved (not shown). The plots of pK versus the logarithm of water molar concentration in the media are, however, practically straight (Table 2). The parameter log c_w has been used by Marshall to describe the variation of many equilibrium constants according to the proportion of water in several water–organic solvent mixtures.[28,29] According to the Marshall model the slopes ($-k$) of the plots of pK versus log c_w could mean net changes in the number of water molecules solvating the acid, conjugated base, and proton, *i.e.*, changes in the 'true' solvation numbers.[29] On the other hand, the k values can be taken as indices that reduce the number of parameters necessary for the description of the dependence of a given equilibrium[29] with the solvent composition. We prefer to use the k values in this second way, due to the fact that they change with the type of organic solvent used in the mixture.[20,28]

The k values obtained in this work (Table 2) appear to be correlated with the value of the corresponding pK in water for each type of ionization. The pK values in water are a function of the corresponding substituent constants in a Hammett equation, and thus the k values might also be a function of these substituent constants. Work is in progress in order to check the overall validity of this finding.

The logarithms of the tautomeric equilibrium constants, K_z, can be expressed as differences between two microscopic pK value (see Scheme) and therefore they can also be adjusted to the Marshall model.

Interactions through the Pyridine Ring.—The strong interaction of the pyridinium nitrogen and the phenolic groups in the

3-position is well established.[12] From the values of pK_a, pK_b, pK_c, and pK_d given in Table 2 it is clear that on protonation of any of these two groups in water solution the acidity of the other group is altered by over three orders of magnitude. Such an effect can be important in the biological action of vitamin B$_6$.[12,30]

It is worth noting here that this effect is clearly enhanced when the polarity of the medium is decreased (see inset of Figure 6). A possible immediate consequence of the existence of a hydrophobic binding site for this vitamin is the increased efficiency of the transmission of the effects of protonation of each group through the ring, that is, an increase in the significance of the 'switching mechanism', postulated by Ivanov and Karpeisky.[31]

Experimental

Materials.—Concentrated HCl was added to dioxane (Ferosa) (*ca.* 10% w/w) and refluxed during 3 h. The aqueous layer was separated and the dioxane layer was shaken up with solid KOH. After separation of the solid material dioxane was refluxed with sodium for 12 h under a gentle flow of nitrogen. It was distilled and kept frozen at 4 °C under nitrogen. Its m.p. coincided with that given in the literature.[19] Dioxane purified by this mode has no significant acid or base impurities and is totally transparent above 300 nm. Its cut-off point is 220 nm, lower than that normally obtained from commercial sources (u.v. spectroscopic grade). Reichhardt reports a similar cut-off point for spectroscopic measurements. 3-Hydroxypyridine (Merck; synthesis grade) was transformed into its chlorohydrate and recrystallized from acetone–ethanol–ether. KOH, free of carbonates, was prepared by the method described in refs. 32 and 33. All the other chemicals used were of the best quality available. The water used was degassed under vacuum and deionized. All measurements were made under nitrogen.

The concentrations of the stock solutions of pyridoxine were calculated from their absorption at 308 nm after dilution in 0.1M-NaOH (ε 7 000).[34] Three independent measurements were made each time. Stock solutions of pyridine, phenol, and 3-hydroxypyridine were prepared by weighing the appropriate amount of the substance and using 50 ml volumetric flasks.

pH Measurements.—The pH measurements were made with a combined electrode Ingold EA120 and a pH-meter Crison which can detect 0.01 unit of pH. The scale of the pH-meter was adjusted by use of standard aqueous buffers (Radiometer).

The pH-meter readings in dioxane–water (B) were converted into pH ($-\log a_H$) using equation (4)[23] where the correction

$$\text{pH} = B + \log U^{\circ}_H \qquad (4)$$

factors log U°_H were calculated as described (Figure 4). Our symbols are the same as those used by Van Uitert and Haas.[23] Other authors[35] prefer to use pH*, pH, and $-\delta$ instead of pH, B, and log U°_H, respectively.

U.v.–Visible Spectra.—The electronic spectra of 3-hydroxypyridine and pyridoxine in water–dioxane mixtures at pH within the two macroscopic pK values of these compounds were recorded with a Cary 210 spectrophotometer at 25 °C. Their concentrations were *ca.* 10⁻⁴M and the solutions contained no other substance except the necessary amount of KOH or HCl to obtain a given pH. The solutions were prepared by dilution of a freshly prepared stock solution in water (usually *ca.* 10⁻²M).

Resolution of overlapping spectral bands was accomplished with the weighted least-squares minimization computer program developed by Metzler *et al.*[14,16,36] This program was

adapted to an Eclipse (General Data) computer. 'Excellent' fits, as defined by Metzler et al.,[14] were obtained in most cases.

The program gives the optimum parameters of each log-normal curve (band position, v_o, peak height, ε_o, width, W, and skewness, ρ) and the area (integrated intensities) under each curve (a plot of ε versus $\bar{v}$).

Potentiometric Measurements.—The potentiometric data were obtained by a method similar to that described by Woolley and his co-workers[20-22] based on the dilution of an aqueous solution with the organic co-solvent.

The experiments started with a given volume (usually 3 ml) of an aqueous solution of known concentration (ca. 10^{-2}M), arrived at as described above for the stock solutions. The reading of the pH-meter, B, was taken; a known amount of dioxane was added and a new measurement of B was taken. The procedure was repeated until the composition of the mixture reached 70% dioxane (v/v). The concentration in each mixture was calculated by taking the dilution into account. The densities of the water–dioxane mixtures were interpolated from the data given by Harned and Owen.[37]

This method was applied to the calculation of correction factors for the measurement of the pH. Equation (4) can be written in the form (5).[23] The values of log U°_H can be

$$-\log U^\circ_H = B + \log[H^+] + \log \gamma_\pm \qquad (5)$$

calculated from B obtained by successive dilutions of an initial solutions (in water) of HCl and KCl, the concentration of both components being precisely determined beforehand. The mean activity coefficients, $\gamma_\pm$, were obtained by interpolation of the values given by Harned and Owen.[37]

Water autoprotolysis constants, pK_w. Taking into account equation (4), the pK_w in a given solvent can be defined as (6). In

$$pK_w = B + \log U^\circ_H - \log[OH^-] - \log \gamma_\pm \qquad (6)$$

this case the starting solution in water was a solution of KOH (carbonate free) at a precisely determined concentration. The $\gamma_\pm$ values for each solution were assumed to be equal to those of HCl at the same ionic strength and in the same media.

Ionization constants. These were calculated from equation (7).

$$pK = B + \log U^\circ_H + \log [A]/[B] + \log \gamma_A/\gamma_B \qquad (7)$$

The initial[A]/[B] ratio was kept near 1 in order to minimize errors in the determination of the pK values. Then, at the initial concentration of a given substance (calculated as indicated above for the stock solutions), a known quantity of HCl or KOH was added, depending upon the pK under investigation and the available ionic species of the substance. For example, to study the higher pK of 3-hydroxypyridine KOH was added to a solution of its chlorohydrate until their analytical concentrations were c and ca. $1.5 c$, respectively. The molar concentrations of the acid (A) and conjugated base (B) species were calculated from charge and material balances. The activity coefficients of the uncharged species were always taken as 1 and those of the chemical species with a net charge of $+1$ or -1 were assumed to be equal to those of HCl at the same ionic strength and in the same media (interpolated from the data of Harned and Owen[37]).

This method can be applied if the consecutive pK values of a given substance are widely enough separated, as in our case (see Figure 5).

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