Thermodynamic Equilibrium Constants for Pyridoxal and Pyridoxal 5′-Phosphate in Dioxane–Water Mixtures

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The electronic absorption spectra of pyridoxal and pyridoxal 5′-phosphate have been obtained in water–dioxane mixtures (0—70% v/v) at 25 °C, and resolved using log–normal curves. The thermodynamic values of the hydration, hemiacetal, and tautomeric equilibrium constants together with the microscopic pK values for these compounds in all the mixtures studied could then be obtained. The variation of these pK values according to solvent polarity fits Marshall's model satisfactorily.

For some years we have been studying the influence of solvent polarity on the physicochemical features of vitamin B_6 and derivatives.[1–13] We have measured the tautomerization constants and the macroscopic pK values of pyridoxine and other simple compounds in dioxane–water mixtures by u.v.–visible spectroscopy and potentiometry, respectively, and from these values we have been able to calculate the microscopic ionization constants.[5,8,9] The existence of additional hydration equilibria in the more complex molecules of the group complicates the determination of the proportion of each molecular form present in solution. However, we have recently been able to calculate the equilibrium constants involved in the case of 5-deoxypyridoxal[12] as a function of the solvent dioxane content. In this paper, the last of the series, we report the tautomeric, hydration, and microscopic ionization constants of pyridoxal and pyridoxal 5′-phosphate in water–dioxane mixtures at 25 °C. Values were obtained by using the methodology from ref. 12, the macroscopic pK values from ref. 11, and the resolved electronic spectra from ref. 8.

Experimental

Pyridoxal (Fluka puriss) and pyridoxal 5′-phosphate (Aldrich) were used without any additional purification. Dioxane (Ferosa) was purified and dried as described in earlier studies.[5,9] The concentrations of stock solutions of pyridoxal were calculated from their absorptions[14] at 318 nm (ε 8 200) after dilution in 0.1M-phosphate buffer (pH 7) and those of pyridoxal 5′-phosphate from their absorptions at 388 nm (ε 6 600) after dilution in 0.1M-NaOH. All the experimental conditions have already been described.[5,7,9]

Results and Discussion

The group in position 4 of pyridoxal can exist in aqueous solution in three different forms: as a free aldehyde, as a hydrated form, and as an internal hemiacetal created by reaction with the 5′-hydroxy group. N.m.r. studies (^{1}H and ^{13}C) have shown that the proportion of the hydrated form in aqueous solution is usually negligible in comparison with the hemiacetal forms.[15,16] Therefore, we have not considered the hydration equilibria for this compound. Other authors have followed the same approach in studies with this compound in aqueous solution.[17] The possible molecular or ionic forms for pyridoxal, except for the hydrated forms, are given in Scheme 1.

The several equilibrium constants for a given chemical species (in the same horizontal line in the Scheme) can be evaluated when the fraction of each molecular form is known. We calculated these fractions from the electronic spectra of the corresponding species in water–dioxane mixtures at 25 °C.[8] These spectra were resolved in the bands assigned to the molecular forms given in Scheme 1 by the computer methods described elsewhere.[7,17,18] The integrated intensities of the bands are given in Table 1 and are proportional to the concentrations of the corresponding molecular forms. The molar fraction, f_i, for a given form i is the ratio between the integrated intensity of its band, $a(i)$, and the corresponding molar area, $a^0(i)$, representing the integrated intensity calculated for this particular band if form i were the only form present in solution. The method of calculation of the corresponding molar areas is given in the following paragraphs.

The electronic spectra of the cationic species of pyridoxal do not reveal any noticeable band for the free aldehyde form under all the conditions studied. The bands observed, therefore, were assigned to the hemiacetal forms. However, the electronic spectra of the anionic species of pyridoxal do show bands for both forms (Scheme 1). Bearing in mind that only these two forms exist in solution, equation (1) can be easily obtained,[5]

$$a(^-\mathrm{h}) = a^0(^-\mathrm{h}) - [a^0(^-\mathrm{h})/a^0(^-\mathrm{a})]a(^-\mathrm{a}) \qquad (1)$$

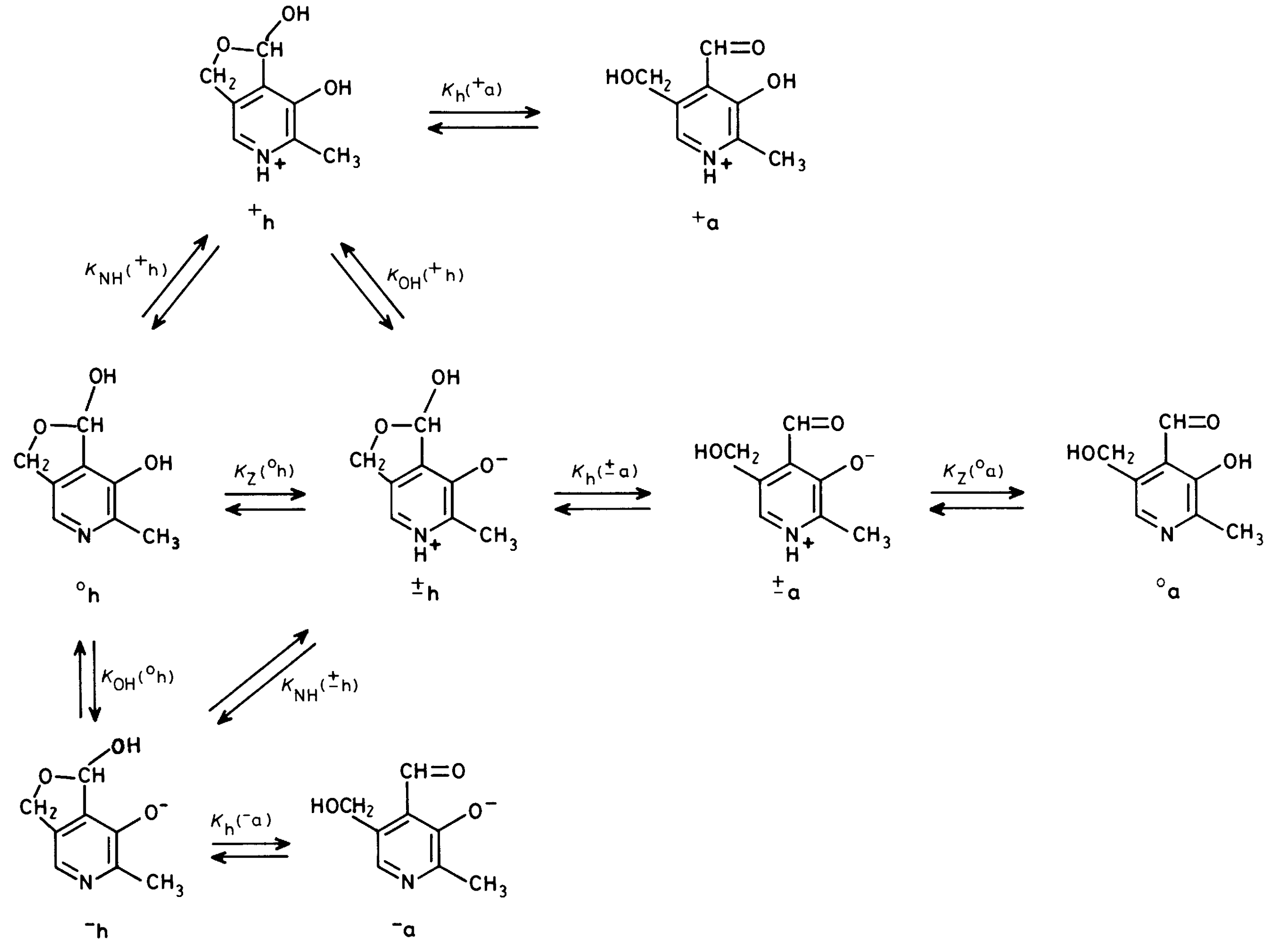
where $a(i)$ and $a^0(i)$ are the integrated intensity and the molar area of the band for the form i respectively. A plot of $a(^-\mathrm{h})$ *versus* $a(^-\mathrm{a})$ gives a straight line with a correlation coefficient of 0.9989. From the intercept and slope of this line the corresponding molar areas have been obtained (Table 1). These values agree with those obtained for the same compound by Metzler and his co-workers,[17] using a different approach (see Table 1).

The electronic spectra of the net zero charge species of pyridoxal have been resolved into bands assignable to the four forms given in Scheme 1 for this species.[8] The integrated intensities of these bands are given in Table 1 as a function of the dioxane content of the medium. In the case of 5-deoxypyridoxal,[12] we were able to evaluate four molar areas from a limited number of data, but this method cannot be used here as the values corresponding to both free aldehyde forms (^{0}a and $^\pm$a) are rather small and thus imply relatively large experimental errors (see Table 1). We have therefore applied a different approach here, which permits us to calculate the molar areas of the two hydrated forms. The sum of the molar fractions of the four forms of this species must be unity, giving equation (2), which can be rearranged as equation (3).

$$1 = a(^\pm\mathrm{a})/a^0(^\pm\mathrm{a}) + a(^0\mathrm{a})/a^0(^0\mathrm{a}) + a(^\pm\mathrm{h})/a^0(^\pm\mathrm{h}) + a(^0\mathrm{h})/a^0(^0\mathrm{h}) \qquad (2)$$

$$a(^\pm\mathrm{h}) + [a^0(^\pm\mathrm{h})/a^0(^\pm\mathrm{a})]a(^\pm\mathrm{a}) + [a^0(^\pm\mathrm{h})/a^0(^0\mathrm{a})]a(^0\mathrm{a}) = a^0(^\pm\mathrm{h}) - [a^0(^\pm\mathrm{h})/a^0(^0\mathrm{h})]a(^0\mathrm{h}) \qquad (3)$$

According to equation (3) a plot of the first term of this equation (henceforth denoted X) *versus* $a(^0\mathrm{h})$ should give a straight line. The X values cannot be obtained directly from our



Scheme 1.

Table 1. Integrated intensities and molar areas (in Mm mol^{-1}) under bands I for the several forms of pyridoxal and pyridoxal 5′-phosphate in dioxane–water mixtures at 25 °C; φ_D indicates the volume fraction of dioxane[a]

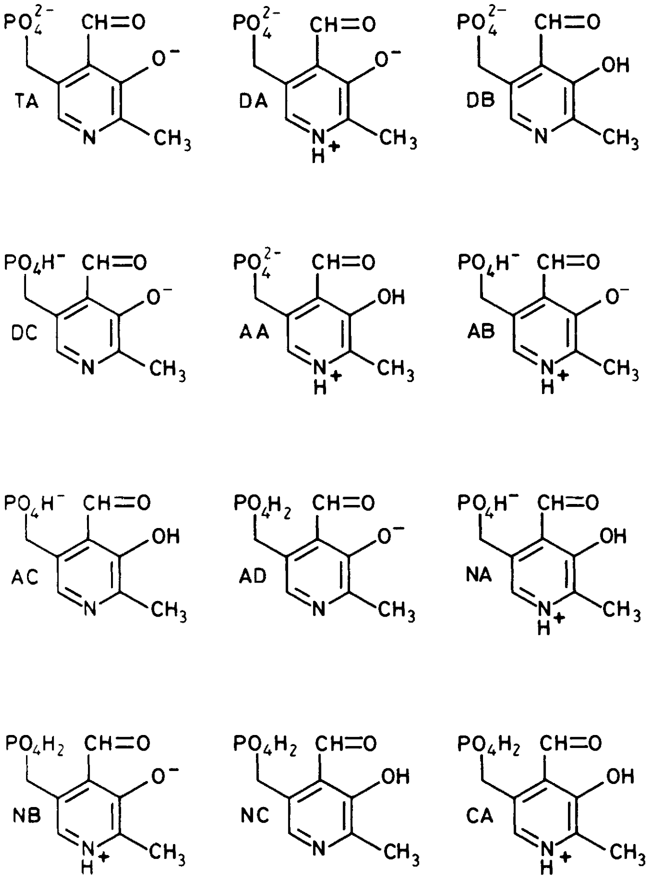
		φ_D									
Compound	Form	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	a^0	$a^{0\,b}$
Pyridoxal	$^{-}$a	92.5	115.1	142.1	165.5	196.3	225.3	246.2	268.5	375	345
	$^{-}$h	196.7	177.8	157.0	143.9	126.2	103.2	87.2	73.8	260	284
	^{0}a	4.2	4.3	7.1	7.5	9.2	11.9	16.4	18.8	215^{c}	
	$^{\pm}$a	4.7	4.8	6.9	8.1	8.8	8.3	9.9	9.4	260^{c}	407
	^{0}h	16.0	24.8	42.7	64.9	95.1	129.4	146.0	160.1	210	224
	$^{\pm}$h	314.0	288.1	261.3	212.3	158.3	105.1	62.7	36.6	350	373
	$^{+}$a	0		0		0		0	0		297
	$^{+}$h	304.5		312.9		308.7		306.6	299.9		325
Pyridoxal 5′-phosphate	$^{-}$a	315.3		321.0		327.2		348.2		375^{c}	326
	$^{-}$h	32.2		27.0		20.1		21.7		260^{c}	272
	^{0}a′	58.9		82.0		111.8		75.9		216^{c}	173
	$^{\pm}$a′	208.1		178.3		145.8		263.1		261^{c}	385
	$^{\pm}$h′	57.5		41.0		25.7		13.5		350^{c}	348
	^{0}a	47.2		79.3		117.5		160.7		216^{c}	166
	$^{\pm}$a	193.1		166.1		112.5		53.5		261^{c}	385
	$^{\pm}$h	56.8		42.8		32.2		30.1		350^{c}	348
	$^{+}$a	72.6		66.8		67.9		93.5		292^{c}	284
	$^{+}$h	249.2		252.6		228.5		227.8		340^{c}	308
	NB	0		3.7		11.1		12.2			

[a] See Schemes for symbol meanings. [b] Values taken from ref. 17. [c] Assumed values.

Table 2. Thermodynamic hydration (K_h), hemiacetal (K_h, pyridoxal case), and tautomeric (K_z) equilibrium constants for pyridoxal and pyridoxal 5′-phosphate in dioxane–water mixtures at 25 °C; φ_D indicates the volume fraction of dioxane[a]

		φ_D								
Compound	Const.	0	0[b]	0.1	0.2	0.3	0.4	0.5	0.6	0.7
Pyridoxal	$K_h(^-a)$	3.1	2.49	2.2	1.6	1.2	0.93	0.66	0.51	0.40
	$K_h(^\pm a)$	50	64	44	28	19	13	9.4	4.7	2.9
	$K_z(^0a)$	(0.9)		(0.9)	(0.8)	(0.9)	(0.8)	(0.6)	(0.5)	(0.4)
	$K_z(^0h)$	12	11	7.0	3.7	2.0	1.0	0.5	0.3	0.1
Pyridoxal 5′-phosphate	$K_h(^-a)$	0.15	0.09		0.12		0.09		0.09	
	$K_h(^\pm a')$	0.20	0.30		0.17		0.13		(0.04)	
	$K_h(^\pm a)$	0.22	0.28		0.19		(0.21)		(0.42)	
	$K_h(^+a)$	2.9	3.2		3.2		2.9		2.1	
	$K_z(^0a')$	2.9	1.9		1.8		1.1		(2.9)	
	$K_z(^0a)$	3.4	2.1		1.7		0.8		0.3	

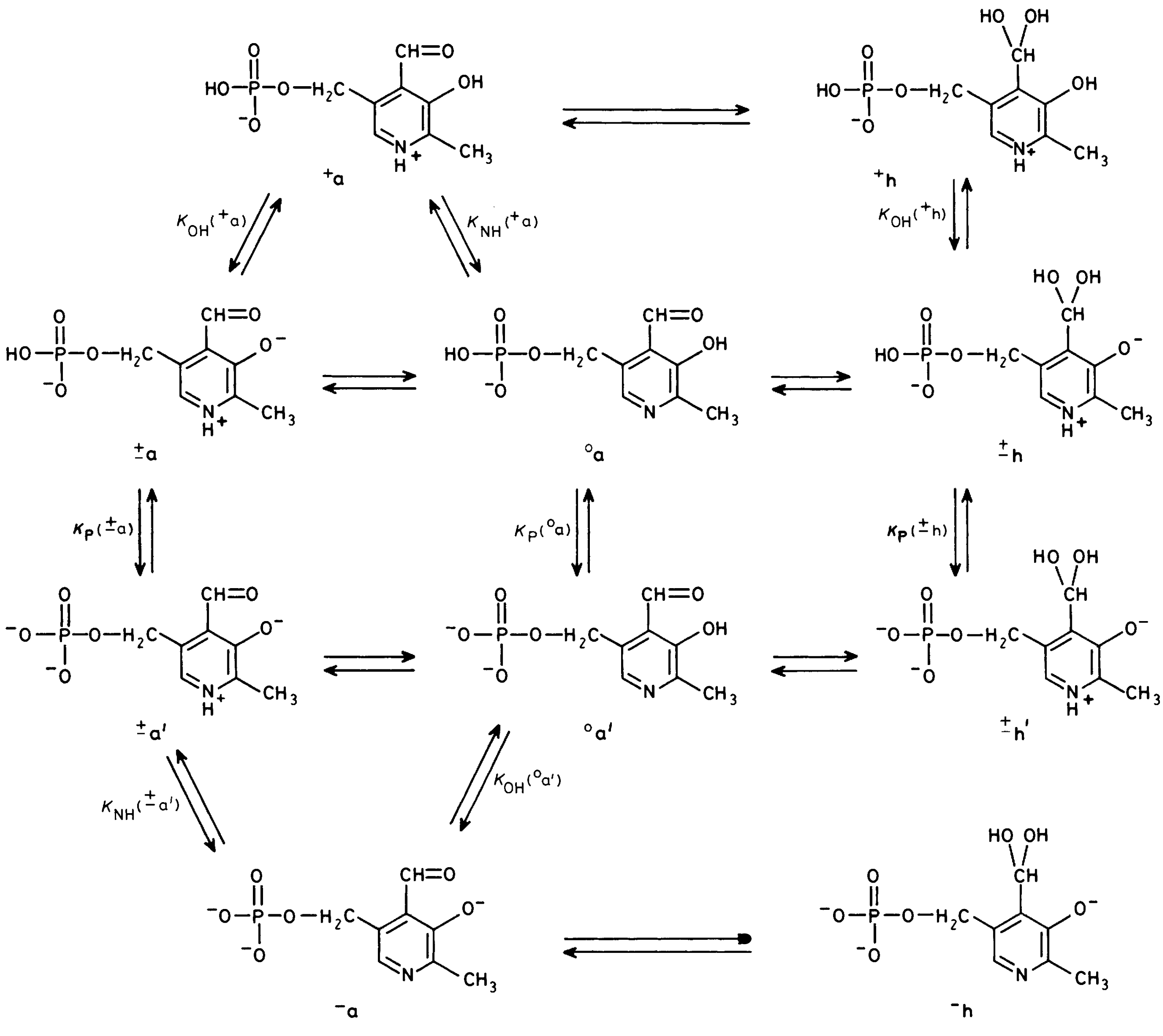
[a] The values in parentheses are regarded as less reliable. [b] Literature values[17] in aqueous solution at $\mu = 0.1$.



Scheme 2.

experimental data and therefore we have assumed that the functions $a^0(^\pm h)/a^0(^\pm a)$ and $a^0(^\pm h)/a^0(^0a)$ are equal to those obtained for 5-deoxypyridoxal,[12] *viz.* 1.35 and 1.63, respectively. These two presumed values must be near the true values, but any differences will have little influence on the value of X because of the low values for the areas of the two free aldehyde forms in this particular case (see Table 1). The values obtained from the intercept and the slope of this plot are 350 and 210 Mm mol^{-1} for the molar areas of the $^\pm$h and ^{0}h forms, respectively. These values are close to those estimated by Harris *et al.*[17] (Table 1) for this compound using a different method, and the $a^0(^\pm h)$ value coincides with that calculated by us for 5-deoxypyridoxal.[12] The value of $a^0(^0h)$ could not be calculated for this compound.

An internal hemiacetal clearly cannot be formed in the pyridoxal 5′-phosphate molecule. The number of possible molecular or ionic forms for this compound is, however, larger than for pyridoxal because of the presence of two additional protonatable groups in position 5′. Only half of the possible forms for this compound appear in Scheme 2; an equal number of hydrated forms should be added to give the whole picture. Previous ^{1}H, ^{13}C, and ^{31}P n.m.r. studies[15,16,19–21] appear to demonstrate the absence of the forms NC, NB, AA, AD, DC and also the corresponding hydrated forms (see Scheme 2) in aqueous solution. Metzler *et al.*[17] made this assumption implicitly when they studied this system in aqueous solution. The simplified situation is described by Scheme 3, although the presence of other forms can be detected in solvents with a different polarity from water (see later). The electronic spectra of the ionic species of pyridoxal 5′-phosphate have been resolved[8] on the assumption that the unprotonated and the most protonated species (bottom and top of Scheme 3, respectively) have an identical ring structure (and therefore analogous electronic spectra) to the anionic and cationic species of 5-deoxypyridoxal, respectively. The two species in the centre of this Scheme are then similar to the net zero charge species of 5-deoxypyridoxal. One form additional to those depicted in Scheme 3 had to be considered in order to resolve the spectra of the cationic species studied (top of the Scheme) when dioxane was present in the solvent. The spectrum of this additional form is only compatible with that expected for the aldehyde NB form (Scheme 2). The possible hydrate of this form would show electronic bands indistinguishable from those of the aldehyde form with the cationic ring (NA of this Scheme). This possible overlap has not been taken into account in the evaluation of the integrated intensities given in Table 1. These values should be regarded as estimates, especially in cases with high dioxane contents, as the presence of other molecular forms with spectra indistinguishable from those for the forms proposed (Table 1) cannot be ruled out. Thus, for instance, the high value found for the intensity of the band due to the form $^\pm$a′ at $\varphi_D = 0.6$ might be explained if we admit the existence of a significant proportion of the form DC (Scheme 2). This form might be expected to be favoured at high dioxane levels, owing to its smaller charge separation. For the calculation of the molar areas here we could not follow the same methods as those used for 5′-deoxypyridoxal and pyridoxal. We have therefore followed the approach used by Metzler and his co-workers,[17,18] assuming values equal to those obtained for similar compounds.



Scheme 3.

The values given in Table 1 have been used to calculate the tautomeric and hemiacetal (with pyridoxal) or hydration (with pyridoxal 5′-phosphate) equilibrium constants given in Table 2. There are no reported values for these constants in dioxane–water mixtures, but a comparison with those published for aqueous solution (see Table 2) indicates that the approximations used were probably correct. The hydration and hemiacetal constants decrease as the water content of the medium decreases, reflecting the greater proportion of the free aldehyde forms. There is only one exception to this general rule, corresponding to the more cationic form of pyridoxal 5′-phosphate, in which the amount of free aldehyde increases only at very high dioxane content. If we had taken into account the presence of some proportion of the hydrated form, corresponding to the form NB, then this effect would be even more pronounced. A similar behaviour can be seen with the cationic species of 5-deoxypyridoxal in both dioxane–water[12] and *N*,*N*-dimethylformamide–water[22] mixtures, and it may be explained by taking into account the values for the substituent constants on the pyridine ring.[13]

The microscopic p*K* values for these compounds can be calculated from the values given in Table 2 and their macroscopic p*K* values (see ref. 11) (Figure). For the reasons already discussed, the p*K* values for pyridoxal 5′-phosphate must be considered as estimates, especially at high dioxane contents. As occurs with the other compounds of the vitamin B_6 group the variations of the microscopic p*K* values and of log K_z with solvent polarity do not follow Born's model,[23] but they do fit that of Marshall[24] (Figure).

Marshall's model takes into account the number of water molecules (*k*) involved in a given ionization process [equation (4)]. The model assumes that the 'complete' equilibrium constant defined as in equation (5), where c_w is the molar water concentration, does not change with solvent composition. Accord-

$$AH + kH_2O \rightleftharpoons A^- + H^+ \qquad (4)$$

$$K^\circ = \frac{[A^-][H^+]}{[AH]c_w^{\,k}} = \frac{K}{c_w^{\,k}} \qquad (5)$$

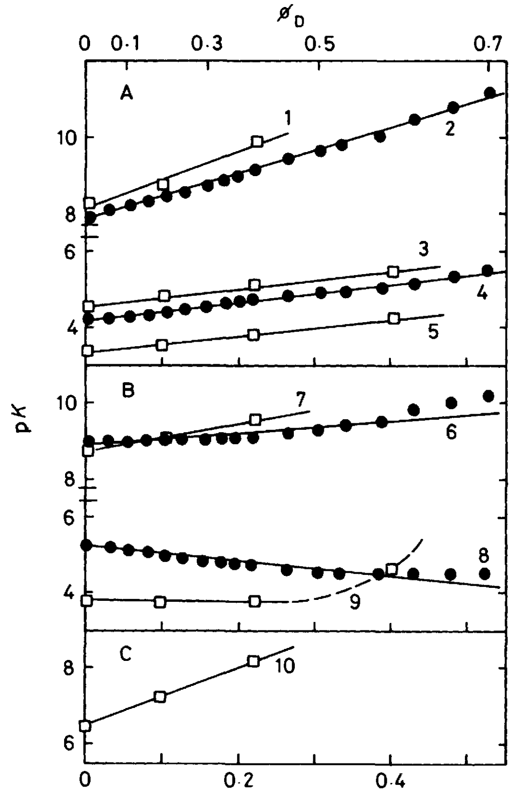


Figure. Microscopic pK values for ionizations of the phenol group (A), the pyridinium ion (B), and the 5′-phosphate group (C) in pyridoxal (●; 2, 4, 6, 8) and pyridoxal 5′-phosphate (☐: 1, 3, 5, 7, 9, 10). Numbers alongside the curves stand for: 1, pK_{OH} (^{0}a′); 2, pK_{OH} (^{0}h); 3, pK_{OH} ($^+$h); 4, pK_{OH} ($^+$h); 5, pK_{OH} ($^+$a); 6, pK_{NH} ($^\pm$h); 7, pK_{NH} ($^\pm$a′); 8, pK_{NH} ($^+$h); 9, pK_{NH} ($^+$a); 10, pK_P. The sets of microscopic pK values for pyridoxal 5′-phosphate were calculated by using spectrophotometric and potentiometric macroscopic pK values. Given that the values obtained usually differed less than 0.2 units, the average value is plotted. Three 5′-phosphate group pK values were calculated: pK_P ($^\pm$a), pK_P (^{0}a), and pK_P ($^\pm$h). The values were identical within experimental error, and again the average was plotted (C); c_w is the molar concentration of water in the mixture; φ_D indicates the volume fraction of dioxane; 55.34 is the molar concentration of water in pure water

ingly, the pK value for a given microscopic ionization in water–dioxane mixtures should change linearly with log c_w. We have previously pointed out,[12] however, that the k values for a given ionization reaction in several mixed solvents are clearly different. We have therefore proposed[12] that k has no real structural significance, and that log c_w must be considered as an empirical solvent parameter.

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