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Carbon-13 nmr Spectra of Vitamin B_6 Compounds[1]

Aqueous Solution Equilibria and Determination of Microscopic Acid Dissociation Constants

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The aqueous solution equilibria and solute structure of vitamin B_6 compounds and several model compounds have been investigated using ^{13}C-nmr spectroscopy. The unsubstituted α-carbon of these compounds is a very good probe for data which permits assignment of the ionization steps to individual groups. While the ionizations of the pyridinium and phenolic groups take place simultaneously in 3-hydroxypyridine, they take place in well-separated steps in pyridoxamine (PM), pyridoxamine phosphate (PMP), and pyridoxal phosphate. It has been established that the ionization with a pK_a value of 3.7 is predominantly phenolic in origin in PM and PMP. A zwitterionic structure consistent with the earlier spectroscopic investigations is proposed for the vitamin B_6 compounds in neutral aqueous solution.

INTRODUCTION

Pyridoxal phosphate (**1**) and compounds structurally related to it play an important role in living systems. The important biological reactions that pyridoxal 5′-phosphate (PLP)[2] catalyzes include transamination, racemization, and decarboxylation of amino acids; dehydration of serine; and the desulfhydration of cysteine. A general and well-accepted mechanism of pyridoxal catalysis has been formulated by Braunstein and Shemyakin (*1*) and by Metzler and co-workers (*2*). The relationship of structure to the biological function of these vitamin B_6 compounds has also been studied (*3*). All those compounds that catalyze the reactions that are catalyzed by PLP were shown to contain an acidic hydroxyl group *ortho* to the formyl group and a strongly electronegative nitrogen so placed as to reduce the electron density about the formyl group. The ionization of the vitamin B_6 compounds has been extensively studied by electronic absorption (*4–9*) and infrared (*10*) spectroscopy. These uv and visible spectroscopic studies indicated that contrary to the popular belief, the ionization of the phenolic OH proton precedes the ionization of the heterocyclic NH proton. We have undertaken a thorough investigation of the solution equilibria of the vitamin B_6 compounds by ^{13}C-nmr spectroscopy as part of our nmr studies on metal ion–PLP interaction. Although some of our results are similar to the published ^{13}C- and ^{1}H-nmr data on these compounds (*11–19*), we do present a method for the quantitative analysis of the nmr data of these hydroxypyridines. This report includes our studies on pyridoxal

[1] Based on the Ph.D. Thesis of T.S.V. (1975).

[2] Abbreviations used: PLP: pyridoxal 5′-phosphate; PMP: pyridoxamine 5′-phosphate; PM: pyridoxamine; FT: Fourier Transform; nmr: nuclear magnetic resonance.

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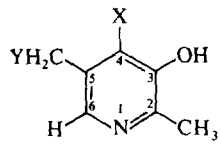
phosphate, pyridoxamine (**2**), pyridoxamine phosphate (**3**), 3-hydroxypyridine (**4**), and a number of model compounds.



1. $X = CHO$, $Y = OPO_3H_2$ pyridoxal 5′-phosphate (PLP)
2. $X = CH_2NH_2$, $Y = OH$ pyridoxamine (PM)
3. $X = CH_2NH_2$, $Y = OPO_3H_2$ pyridoxamine 5′-phosphate (PMP)
5. $X = CH(OH)_2$, $Y = OPO_3H_2$ hydrated form of pyridoxal 5′-phosphate

EXPERIMENTAL

Pyridoxamine diHCl, pyridoxamine phosphate, and pyridoxal phosphate were purchased from Sigma Chemical Company and were used without further purification. The 3-hydroxypyridine was obtained from Aldrich Chemicals and it was recrystallized from water. All other chemicals used in this work were reagent grade.

Samples for the nmr spectral determinations were obtained by dissolving weighed amounts of the pure compound in deuterium oxide and adjusting the pD of the solution with concentrated solution of NaOD or DCl in D_2O. The concentration of solute in all forms for the ^{13}C-nmr determinations are as follows: 3-hydroxypyridine, 0.25 *M*; pyridine, pyridine methiodide, and *N*-methyl-3-hydroxypyridinium iodide, 0.75 *M*; pyridoxal phosphate, 1 *M* in the pD range 4–13 and saturated solutions below pD 4; pyridoxamine, 0.5 *M* between pD 0.5 and 7.5 and above pD 12 and saturated solutions in the pD range 7.5–12; pyridoxamine phosphate, 0.5 *M*; 2-picoline, 1 *M*.

The ^{13}C-nmr spectra at 25.16 MHz were recorded on a Varian XL-100 nmr spectrometer in the pulse/FT mode with proton decoupling. The temperature of the probe and the sample under these conditions was about 35°C. All carbon chemical shifts are referenced in parts per million (δ) units from internal tetramethylammonium chloride, which was our choice as a reference because of its negligible pH shift and noninterference in our metal-binding studies. These values may be converted to the TMS scale by adding 56.5 ppm to our values. Measurements of pH were made to $\pm$0.1 with Sargent–Welch Model DR pH meter. The pD of any solution was obtained from the relation (*20*) pD = pH (measured) + 0.4. Because of the high concentrations and varying ionic strengths employed in our studies, our pK_a values will differ slightly from the reported thermodynamic constants (*21*) at high pD values.

RESULTS

The assignment of the ^{13}C-nmr spectral results was a relatively difficult task for the vitamin B_6 compounds, because of the strong pH dependence of the carbon chemical shifts. The 2-methyl, 5-methylene, and C-6 carbons were easily assigned from the one bond carbon–proton couplings observed in the proton undecoupled spectrum.

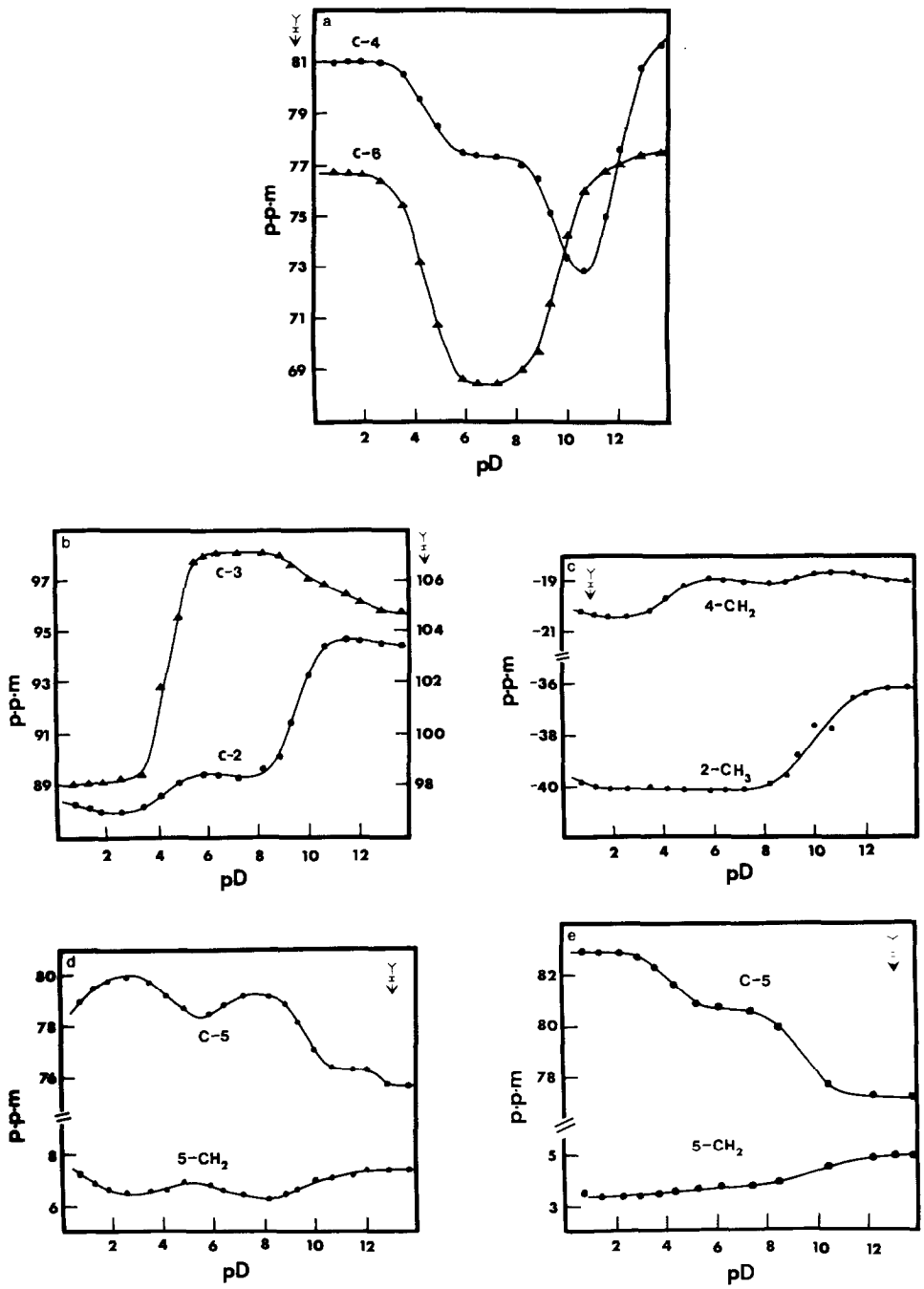


FIG. 1. The pD dependence of the ^{13}C chemical shifts of pyridoxamine phosphate at 35°C. The chemical shifts (25.16 MHz) were calculated in ppm (δ) from the tetramethylammonium chloride reference and these may be converted to the TMS scale by adding 56.5 ppm. (a) The C-6 and C-4 resonances: (b) the C-2 (left scale) and the C-3 resonances: (c) the 4-methylene and 2-methyl resonances: (d) the C-5 and 5-methylene resonances; (e) the C-5 and 5-methylene resonances in pyridoxamine.

For PLP and PMP the C-5 was easily identified from its coupling ($J \approx 8$ Hz) to the phosphorus. The assignment of C-3 resonance was based on the substituent effect of the hydroxyl group, which shifts the C-3 resonance to the lowest field relative to other aromatic carbons. The C-4 resonance in PLP was assigned on the basis of its large coupling ($J \approx 22$ Hz) to the aldehyde proton. In PMP the C-4 was assigned from the large shift of this signal when the 4′-amino group ionizes. The only other carbon, C-2, was assigned arbitrarily to the remaining quaternary carbon resonance, confirmed by its coupling to 6-H and 2-methyl protons. More details about signal assignments in vitamin B_6 compounds can be found elsewhere (*22, 11–13*).

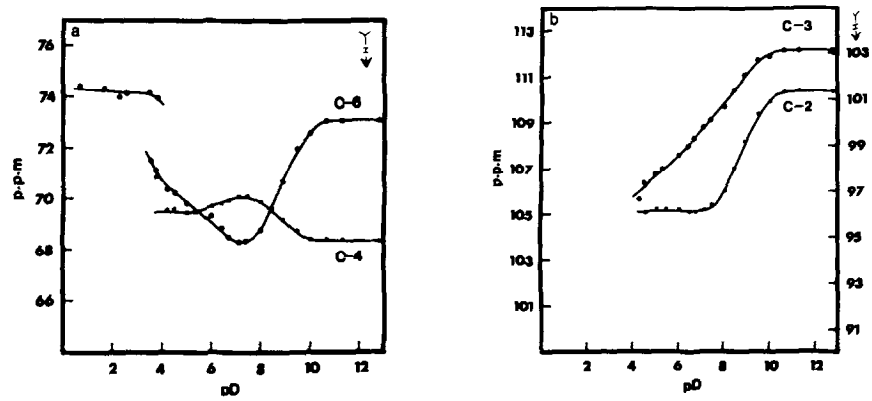


FIG. 2. The carbon chemical shifts of 1 *M* PLP as a function of the pD at 35°C. At low pD values PLP exists in the hydrated aldehyde form and above pD 5 it exists predominantly in the free aldehyde form with less than 5% of the hydrated form. (a) The C-6 and C-4 resonances; (b) the C-3 (left scale) and C-2 (right scale) resonances.

The pD dependence of the chemical shifts of all the carbon resonances of PMP are shown in Figs. 1a–d. Unlike proton chemical shifts, some carbon resonances shift upfield for one protonation and downfield for a different protonation. The magnitude of the shifts seem to depend on a lot of factors including proximity to the ionizing groups and electron density changes accompanying ionization or protonation. The curves shown in Fig. 1 for PMP are complex, since PMP has five ionizable protons. The curves for pyridoxamine, which lacks the two ionizable phosphate protons, are very similar to those for PMP (*22*), except for the C-5 and 5-methylene carbons, which are very close to the phosphate in PMP. These curves for pyridoxamine are shown in Fig. 1e. Since C-5 is apparently insensitive to the side chain 4′-amino group ionization, the curves look like a simple acid–base titration curve of a diprotic acid. The pD dependence of the C-2, C-3, C-4, and C-6 carbon resonances of PLP are shown in Fig. 2. Solubility problems and the presence of CHO $\rightleftharpoons$ $CH(OH)_2$ exchange made it difficult to observe the spectrum of the quaternary carbons below pD 4. PLP remains mainly in the free aldehyde form above pD 5 and mostly in the hydrated form below pD 3. In the intermediate pD range 3–5 both forms can be found exchanging slowly at room temperature.

Since the large pH shifts observed in PM, PMP, and PLP derive mainly from the heterocyclic NH and 3-OH protonations, a detailed study of a simple model compound,

3-hydroxypyridine, which contains only these two ionizable groups, would help immensely in the interpretation of the protonation shifts in vitamin B_6 compounds. The spectral assignments of the ^{13}C-nmr of 3-hydroxypyridine in our studies are similar to the published results (*23*) and differ only in the assignment of C-4 and C-5. The discrepancy in the assignment stems from solvent effects. Our assignment was based on long range carbon–proton coupling constants of C-4 and C-5 in the fully ionized 3-hydroxypyridine. It is well known that the long range coupling of ^{13}C in aromatic systems is strongest with the nuclei separated by three bonds, and C-5 has no such protons to couple with in 3-hydroxypyridine. However, it can couple strongly with the

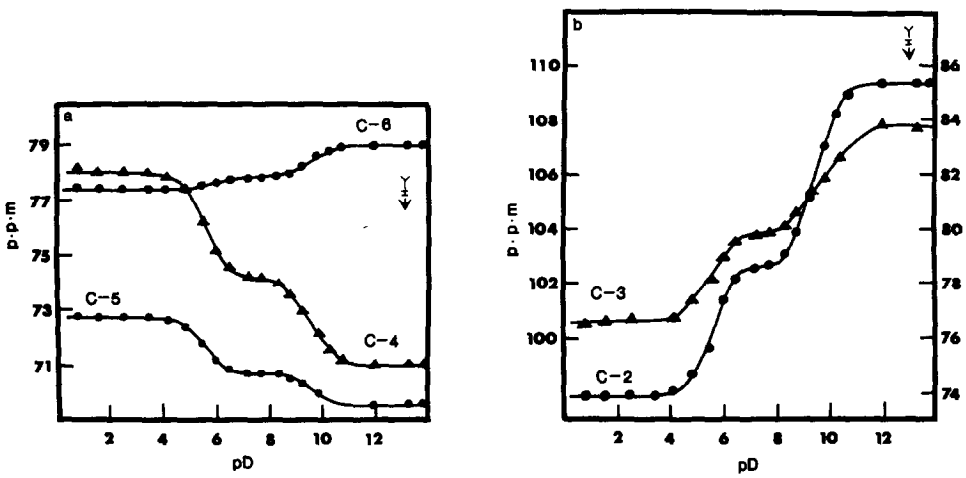


FIG. 3. The pD dependence of the carbon chemical shifts of 3-hydroxypyridine. (a) The C-6, C-4 and C-5 resonances; (b) the C-3 (left scale) and C-2 (right scale) resonances.

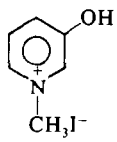
6-H proton, as observed in pyridine and its derivatives (*24*, *25*). The *X*A pattern ($J = 8.6$ Hz) obtained for the highest upfield resonance in 3-hydroxypyridine confirms its assignment as C-5. The highfield half of the proton undecoupled spectrum for C-4 shows a four line *X*AB pattern consistent with a strong ($J = 6.8$ Hz) coupling to 6-H and a moderate 4.4 Hz coupling to 2-H. The weaker coupling to 2-H occurs because of the intervening substituent. The resonances were followed closely with pD; the assignment of the high field resonance as C-5 is supported by the 20 ppm difference in the chemical shifts of the β- and γ-resonances in the fully protonated pyridine in aqueous solution, although it is reduced to 5.3 ppm in 3-hydroxypyridine by the substituent effect of the 3-OH group.

The pD dependence of the carbon chemical shifts of 3-hydroxypyridine are shown in Fig. 3. The most striking difference between this figure and Fig. 1 is in the pD dependence curves of C-6 and C-3 chemical shifts. While the C-6 resonance shows large upfield shifts ($\approx$10 pmm) for the protonation in the alkaline pD range and a downfield shift of 8.2 ppm for the protonation in the acidic pD range in PM and PMP, this resonance shows upfield shifts of only 1.2 and 0.2 ppm for the same protonations in 3-hydroxypyridine. This result indicates that the ionization of the NH and the OH protons occur simultaneously in 3-hydroxypyridine and in well-separated steps in PM and PMP. From a knowledge of the protonation shifts for the N- and O-

protonations in closely related model compounds, it is possible to calculate the relative proportion of the N-protonated and O-protonated species at a given pD. The chemical shifts and protonation shifts of pyridine, 2-picoline, *N*-methylpyridinium iodide, *N*-methyl-3-hydroxypyridinium iodide, and benzylamine are collected in Table 1. The coupling constant data for the compounds studied in this work are shown in Table 2.

TABLE 1

CARBON CHEMICAL SHIFTS OF 3-HYDROXYPYRIDINE AND SOME MODEL COMPOUNDS IN PARTS PER MILLION FROM TETRAMETHYLAMMONIUM CHLORIDE

Compound	pD	C-1	C-2	C-3	C-4	C-5	C-6	Other
Pyridine	2.0	—	85.67	72.05	91.83	72.05	85.67	—
	8.0	—	93.11	69.08	82.17	69.08	93.11	—
	Shift		−7.44	2.97	9.66	2.97	−7.44	—
2-Picoline	2.0	—	98.44	72.65	91.20	69.03	84.84	−36.16
	8.0	—	102.29	68.60	82.38	66.05	92.51	−32.43
	Shift	—	−3.85	4.05	8.82	2.98	−7.67	−3.73
	—	—	89.58	72.73	90.03	72.73	89.58	
	2.0	—	78.08	100.86	76.23	73.09	81.51	
	8.0	—	79.55	111.12	78.07	72.16	73.82	
	Shift	—	−1.47	−10.26	−1.84	0.93	7.69	
4A	2.0	—	73.95	100.65	78.04	72.74	77.41	
4B	—	—					85.88	
4C	—	—					69.82	
4D	10.0	—	85.35	107.75	71.00	69.58	79.02	
Benzylamine	13.8	87.20	73.23	71.92	71.46	71.92	73.23	−10.32
	1.2	77.37	73.94	73.60	73.94	73.60	73.94	−11.94
	Shift	−9.83	0.71	1.68	2.48	1.68	0.71	−1.62

DISCUSSION

The results described above show that the C-6 carbon is very sensitive to both N- and O-protonations in the 3-hydroxypyridines and in model compounds. The C-6 resonance is shifted 7.44 ppm upfield for the N-protonation in pyridine, and it is shifted 7.69 ppm downfield for the O-protonation in *N*-methyl-3-hydroxypyridinium iodide. The exact shape of the pD dependence curve of the C-6 resonance would depend on the relative pK_a values for the N- and O-protonations as well as the substituent effect on the magnitude of the protonation shifts in the 3-hydroxypyridine derivative under consideration. A simple glance at the pD dependence curve of C-6 in PMP indicates that the two protonations are well separated in this compound. In PMP the protonation in the alkaline pD region (pK_a = 8.7) results in a 8.9 ppm upfield shift of the C-6

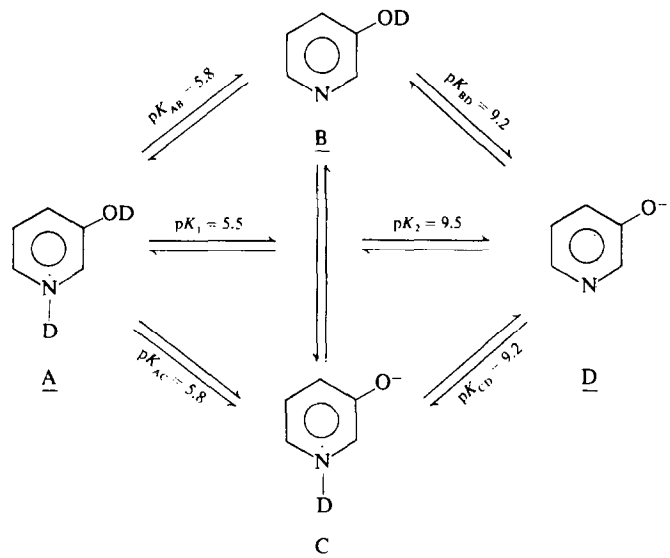
TABLE 2

GEMINAL AND LONG-RANGE CARBON–PROTON COUPLING CONSTANTS (Hz) IN 2-PICOLINE, 3-HYDROXYPYRIDINE, AND PMP

Compound	pD	C-2	C-3	C-4	C-5	C-6	C-2′	C-4′	C-5′
3-Hydroxypyridine	0.7	H2 = 189.9	H2 = 11.8 H5 = 5.0	H4 = 169.6	H5 = 174.9 H6 = 4.2 H2 = 4.2 H4 = 0.7				
	4.55	H2 = 190.4 H6 = 7.1 H4 = 1.7	H2 = 8.0 H5 = 3.5	H4 = 167.6 H6 = 6.3 H2 = 4.0	H5 = 172.9 H6 = 5.5	H6 = 190.1 H2 = 12.2 H4 = 6.1 H5 = 1.8			
	11.0	H2 = 173.0 H6 = 10.6 H4 = 3.6 H5 = 1.0	H2 = 6.4 H5 = 4.9	H4 = 164.4 H6 = 6.8 H2 = 4.4	H5 = 161.4 H6 = 8.6	H6 = 181.6 H2 = 9.8 H4 = 6.8 H5 = 2.6			
2-Picoline	6.0	H2′ < 2	H3 = 168.6 H2′ < 2	H4 = 167.6 H6 = 6.3	H5 = 169.6 H6 = 6.6 H3 = 6.2	H6 = 181.7 H4 = 6.6 H5 = 5.8	H2′ = 128.6		
Pyridoxamine phosphate	7.3	H6 = 7			P = 7.4 H6 = 3.8	H6 = 188.3	H2′ = 129.9	H4′ = 144.9	H5′ = 146.2

resonance; and the protonation in the acidic pD region ($pK_a = 3.7$) leads to a 8.2-ppm downfield shift, indicating that the former is predominantly N-protonation and the latter O-protonation. The magnitude of the protonation shifts is slightly larger in PMP than in the model compounds, a result attributable to the substituent effects.

Since 3-hydroxypyridine is expected to be free from substituent effects on protonation shifts, it is possible to determine quantitatively its microscopic ionizations constants. The ionization of 3-hydroxypyridine is shown below:



The observed chemical shift v_{Oi} of the ith carbon is the weighted average of the chemical shifts of that carbon in all the four species, A, B, C, and D in equilibrium:

$$P_a v_{ai} + P_b v_{bi} + P_c v_{ci} + P_d v_{di} = v_{Oi}, \quad [1]$$

$$P_a + P_b + P_c + P_d = 1. \quad [2]$$

The mole fraction P_j of the four species at a given pD is determined by the macroscopic acid dissociation constants K_1 and K_2 and the microscopic dissociation constants K_{AB}, K_{AC}, K_{BD}, and K_{CD}:

$$P_a 10^{(pD-pK_1)} - P_b - P_c = 0, \quad [3]$$

$$P_b + P_c - P_d 10^{(pK_2-pD)} = 0, \quad [4]$$

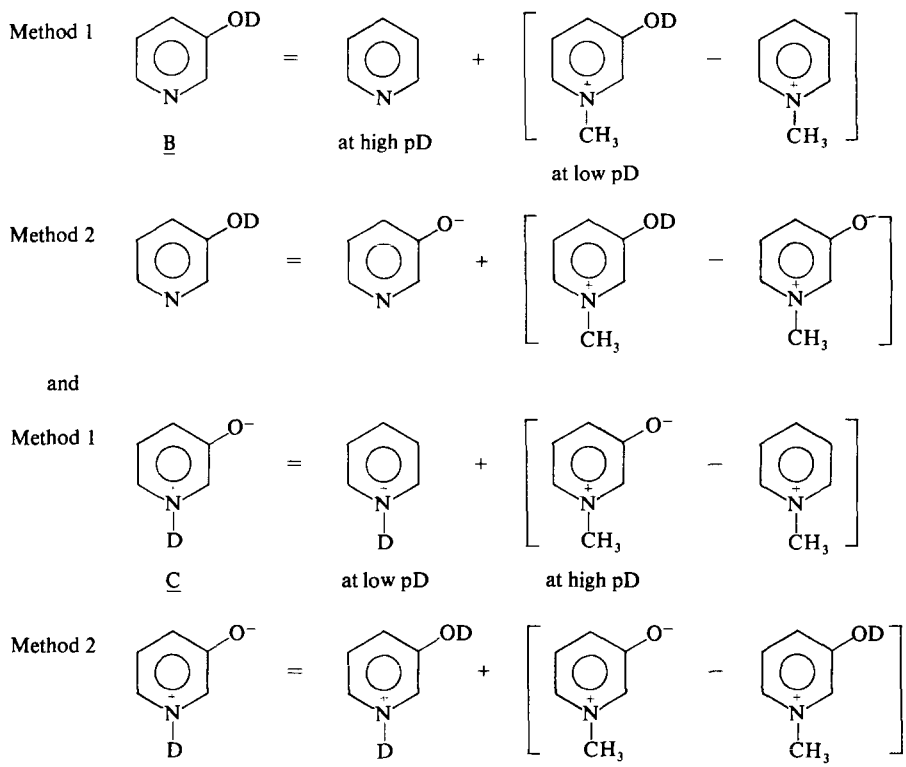
$$K_1 = K_{AB} + K_{AC}, \quad [5]$$

$$1/K_2 = 1/K_{BD} + 1/K_{CD}. \quad [6]$$

The ratio, K_z, of the concentration of the neutral form B to the zwitterionic (also called dipolar) form C of 3-hydroxypyridine is:

$$K_z = K_{AB}/K_{AC} = K_{CD}/K_{BD}. \quad [7]$$

The chemical shifts of the carbon resonances of species A and D correspond to the experimental values at very low and at very high pD respectively. However, there is no simple way of obtaining the ^{13}C-nmr spectrum of species B and C. We employed the principle of the additivity of ^{13}C chemical shifts to obtain the spectrum of B and C. Although this rule breaks down for complex molecules, it should be valid for 3-hydroxypyridine since the substituent effects are calculated from closely related compounds. The two methods used for calculating the spectrum of species B and C are shown below:



The chemical shifts of the C-6 resonance in species C as computed by the method 1 and method 2 are 69.91 and 69.74 ppm respectively. Corresponding values for the species B are 85.04 and 86.71 ppm. The agreement is not as good with B as for C but, it is certainly within reasonable limits. Since both methods are basically equivalent, the average of the values computed by the two methods was taken to obtain the spectrum of species B and C. The macroscopic ionization constants K_1 and K_2 were obtained from the titration curves shown in Fig. 3.

Equations [1] to [4] are four simultaneous equations in four unknowns, P_a, P_b, P_c, and P_d, and they can be solved exactly from the data for each carbon. The values of pK_{AB} etc. calculated from the C-6 chemical shift data are shown in Table 3. The pK values are constant for the data in the pD region 3–10, and the values of 5.5 for pK_1 and 5.8 for both pK_{AB} and pK_{AC} calculated from the nmr data are about 0.4 unit higher than the values of 5.1, 5.44, and 5.37 reported by Metzler and Snell (*5*) for these

quantities. The difference of 0.4 unit is expected since the electronic absorption measurements were done in H_2O and the nmr studies were done in D_2O (*20*). Our pK_2 value and the values of pK_{CD} and pK_{BD} calculated from pK_2 are, surprisingly, 0.5 unit higher than the reported (*5*) values. We believe that our pH measurements are less accurate in highly alkaline solutions due to the high concentration of solute, the increased ionic strength resulting from the titration with NaOD, and the relative insensitivity of the pH electrode used by us in alkaline solutions. The slight inaccuracy in the magnitude of pK_2 should not, however, affect the determination of the ratio of the neutral form to the zwitterionic form of 3-hydroxypyridine. We obtained a value of 1.0 for K_z, which is in excellent agreement with a value of 0.95 obtained recently by Metzler *et al.* (*6*) for 3-hydroxypyridine. This result shows that the neutral and the zwitterionic forms are present in nearly equal amounts in an aqueous solution of 3-hydroxypyridine

TABLE 3

THE MICROSCOPIC EQUILIBRIUM CONSTANTS OF 3-HYDROXYPYRIDINE

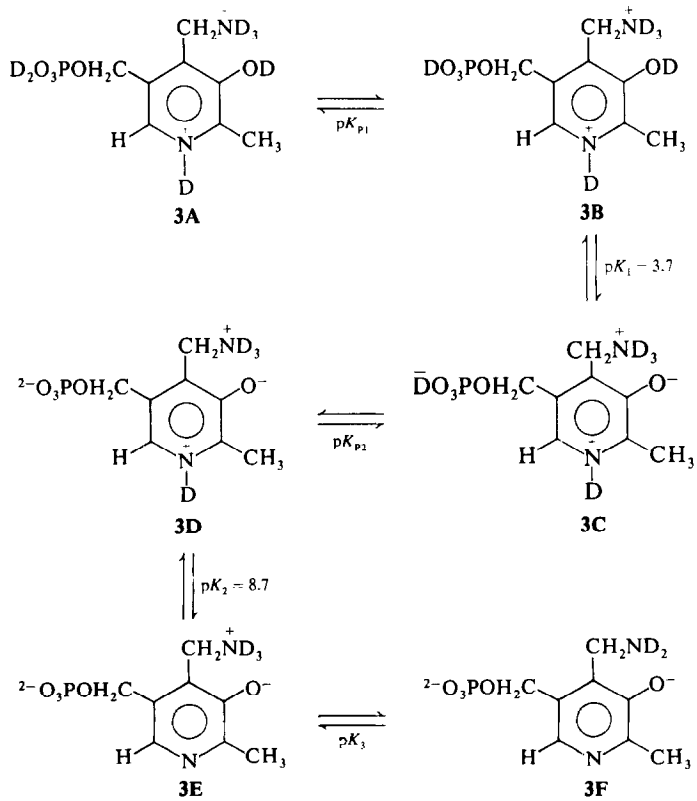
pD	pK_{AB}	pK_{AC}	pK_{BD}	pK_{CD}	$K_z = P_B/P_C$
3.43	5.84	5.84	9.21	9.21	0.99
5.47	5.84	5.83	9.20	9.22	0.97
5.96	5.84	5.84	9.20	9.21	0.98
6.43	5.84	5.84	9.20	9.21	0.98
7.15	5.84	5.84	9.21	9.21	0.99
7.68	5.84	5.84	9.21	9.21	0.99
8.28	5.84	5.84	9.21	9.21	1.00
8.72	5.84	5.84	9.21	9.21	1.00
9.20	5.84	5.84	9.21	9.20	1.02
9.8	5.8	5.8	9.2	9.2	1.0
10.2	5.8	5.8	9.2	9.2	1.0
Average	5.8	5.8	9.2	9.2	1.0 ± 0.1

at any pD. The information available from the pD dependence of the other carbon resonances of 3-hydroxypyridine is less precise but leads to the same conclusion. The C-2 resonance shifts 7.44 ppm upfield for N-protonation and 1.47 ppm upfield for O-protonation, for a total of 8.9 ppm in model compounds. The total protonation shift of 11.4 ppm observed for the C-2 resonance in 3-hydroxypyridine in steps of 4.7 ppm for K_1 and 6.8 ppm for K_2 exceeds the calculated value by 2.5 ppm. Consequently, the C-2 shifts may not be used to calculate the microscopic ionization constants accurately. The C-3 resonance shifts 2.97 ppm downfield for the N-protonation and 10.26 ppm upfield for the O-protonation in the model compounds to a net upfield shift of 7.3 ppm. In 3-hydroxypyridine the C-3 resonance shows a net upfield shift of 7.2 ppm with 3.2 ppm for K_1 protonation and 4.0 ppm for K_2 protonation. A simple calculation yields a value of 0.9 for K_z in 3-hydroxypyridine from this data. The C-4 and C-5 shift data also support this result qualitatively.

The conclusion that PMP and PM exist mainly in the dipolar form in neutral aqueous solution, arrived at from the C-6 chemical shift data, is reinforced by the pD dependence of the other carbons in these compounds. The C-3 and C-2 resonances of PMP are shifted 9.0 and 1.4 ppm upfield respectively for the protonation with $pK_a = 3.7$,

while the corresponding upfield shifts for O protonation in the model compounds are 10.3 and 1.5 ppm respectively. However, the downfield protonation shift of the C-4 resonance in PMP and PM (3.6 and 3.8 ppm respectively) for this protonation deviates substantially from the upfield shift of 1.84 ppm for the O-protonation in model compounds. Similarly, the protonation with $pK_a = 8.7$ shifts the C-2 resonance 5.3 ppm upfield in PMP compared to the upfield shifts of 3.85 and 7.44 ppm in 2-picoline and pyridine respectively for the N-protonation. These observations show that the magnitude of the ^{13}C protonation shifts are very sensitive to substituents and that the shifts in model compounds cannot be used to calculate microscopic ionization constants in more complicated systems such as PMP and PLP.

There has been some uncertainty (*5*) regarding the order of ionization of the phosphoric acid groups and the phenolic OH group in PMP. Comparison of the pD dependence of the C-5 resonance in PMP (Fig. 1d) and PM (Fig. 1e) shows that the upfield shifts of C-5 observed in PMP on lowering the pD below 3 and on lowering the pD from 8 to 6 should be due to the protonations of the phosphate moiety. The proximity of the C-4 carbon to the 4′-amino group makes the C-4 carbon sensitive to the protonation of this functional group at pD $\approx$ 11.7. The upfield shift of (>8.8 ppm) the C-4 resonance of PMP and PM when the pD is lowered from 14 to about 10.5 is similar to the upfield shift of 9.8 ppm observed for this carbon in benzylamine. Thus, the ^{13}C-nmr studies of PMP and PM reveal the exact sequence of ionization of all the ionizable protons in PMP. The ionization scheme of PMP is shown below:



The pD dependence of all the carbon chemical shifts of PLP except C-4 and C-3 are similar to those observed for PMP. Solubility problems and the conversion of the free aldehyde form to the hydrated aldehyde form in acidic solutions made it difficult to study thoroughly the ionization of any one species of PLP. The 'V' shaped pD dependence observed for the pD dependence of C-6 in the free aldehyde form of PLP as compared to a 'U' shape in PMP probably indicates the presence of appreciable amounts of O-protonated forms in neutral aqueous solutions of PLP, confirming the conclusions of Harris *et al.* (*7*) and Bazhulina *et al.* (*8*). The predominant species, however, is the N-protonated form.

COUPLING CONSTANTS

Phosphorus–carbon couplings in PLP and PMP were studied as a function of pD. The coupling constant for the phosphorus–C5 coupling was found to be higher than for the phosphorus–C5′ coupling. Both coupling constants were nearly pD independent, and a value of 7.7 ± 0.4 and 3.3 ± 0.5 Hz may be assigned to $J_{C5\text{-}P}$ and $J_{C5'\text{-}P}$ respectively. The large vicinal coupling (*26*) observed for $J_{C5\text{-}P}$ strongly suggests that conformations in which the phosphorus atom is *trans* to the ring carbon C-5 are preferred in aqueous solutions of PLP and PMP.

In conclusion, evidence has been presented to show that the vitamin B_6 compounds, PM, PMP, and PLP, exist mainly in the N-protonated zwitterionic form in neutral aqueous solution. This conclusion and the K_z value derived for 3-hydroxypyridine are in excellent agreement with recent spectroscopic studies of these compounds (*6*). It seems that the 2-methyl and 5-methylene functional groups play an important role in altering the acid–base properties of 3-hydroxypyridine to provide maximum stability for the Schiff base intermediates derived from pyridoxal phosphate at physiological pH.

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