Thermodynamic and Microscopic Equilibrium Constants of Molecular Species Formed from Pyridoxal 5′-Phosphate and 2-Amino-3-phosphonopropionic Acid in Aqueous and D_2O Solution

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Abstract: Schiff base formation between pyridoxal 5′-phosphate (PLP) and 2-amino-3-phosphonopropionic acid (APP) has been investigated by measurement of the corresponding NMR and electronic absorption spectra. A value of 0.26 was found for the formation constant of the completely deprotonated Schiff base species, which is much smaller than the values reported for pyridoxal-β-chloroalanine and pyridoxal-*O*-phosphoserine. The protonation constants for the aldehyde and hydrate forms of PLP were determined in D_2O by measurement of the variation of chemical shifts with pD (pH in D_2O). The hydration constants of PLP were determined in a pD range 2–12, and species distributions were calculated. The protonation constants of the APP–PLP Schiff base determined by NMR in D_2O were found to have the log values 12.54, 8.10, 6.70, and 5.95, and the species distributions were calculated for a range of pD values. Evidence is reported for hydrogen bonding involving the phosphate and phosphonate groups of the diprotonated Schiff base. The cis and trans forms of the Schiff bases were distinguished with the aid of the nuclear Overhauser effect.

The stability constants of Schiff bases that involve vitamin B_6 and amino acids are important for the study of enzymatic and nonenzymatic reactions in which pyridoxal 5′-phosphate (PLP) or pyridoxal (PL) acts as a coenzyme or a catalyst in reactions such as transamination, racemization, β- and γ-elimination, carbon–carbon bond cleavage, and decarboxylation.[1-3] It is known that there are pyridoxal 5′-phosphate dependent enzymes in natural systems that effect the transamination of aminophosphonic acids.[4] Martell and Langohr have determined the stability constants of Schiff bases formed between pyridoxal, (aminomethyl)phosphonic acid, (2-aminoethyl)phosphonic acid (2-AEP), and 2-amino-3-phosphonopropionic acid (APP) by measurement of the corresponding electronic absorption spectra.[5] 2-Amino-3-phosphonopropionic acid (APP) is of interest since APP, 2-AEP, and its *N*-methyl derivative are the only aminophosphonic acids found in biological systems.[6-8] The work described in this paper is required to elucidate the nature of the molecular species present in APP–PLP systems over a range of pH and concentration of components. The results will be used to design the conditions necessary for catalysis of dephosphonylation and transamination, for the interpretation of the kinetics of these reactions, and for deducing possible reaction mechanisms.

Experimental Section

Materials. Pyridoxal 5′-phosphate was obtained from United States Biochemical Corp. The 2-amino-3-phosphonopropionic acid was purchased from Calbiochem-Behring Corp. KCl was obtained from MCB reagents. KOD (40%), D_2O, and DCl (20%) were obtained from Aldrich Chemical Co. KOD and DCl were diluted to the appropriate concentrations under dry nitrogen.

Potentiometric Equilibrium Determinations. Samples of about 0.15 mmol of APP and PLP were diluted with 50 and 30 mL of distilled water, respectively, in sealed, thermostatted (25 ± 0.05 °C) potentiometric titration vessels equipped with a Sargent blue glass-calomel combination electrode, N_2 inlet and bubbler outlet, and a graduated (Metrohm) microburet. The test solution, adjusted to 1.00 M in KCl, was titrated with 0.1000 M standard CO_2-free KOH while −log $[H^+]$ was measured with a Beckman Research Model 1019 pH meter calibrated with dilute standard hydrochloric acid at 1.00 M (KCl) ionic strength to read −log $[H^+]$.

NMR Measurements. The proton nuclear magnetic resonance spectra were recorded with a Varian XL200 NMR spectrometer. The chemical shifts are reported in ppm with respect to the resonance of 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) as an internal reference. Only enough DSS was used to give a small methyl peak, and the diffuse pattern of the CH_2 peaks barely showed on the base line.

The fraction of hydrate and aldehyde forms of PLP in the experimental solutions was determined by the resonances of 4′-CH and 6-CH groups when possible. The fraction of Schiff base was determined by the 4′-CH resonance. The relative errors of the NMR integrations used to compute the equilibrium constants are ~5% for the pH range 4.94–8.70 where transamination and deuteration occur at moderate rates. At pH values above 8.7 the equilibrium constants were determined by the average of five integrations for each experimental point, and consequently the experimental errors are less than 5%.

The ^{31}P NMR spectra were recorded with a Varian FT 80A NMR spectrometer. The chemical shifts are reported in ppm with respect to the resonance of 0.30 M phosphoric acid in D_2O. The reference solution of H_3PO_4 was placed in an inner tube. A spectral width of 1000 Hz was generally employed, with a pulse of 10 μs and an acquisition time of 0.5 s. The temperature of the solutions was 35.0 ± 0.1 °C.

The purity of APP and PLP and the extent of hydration of the solid material were determined by titration. The analytical concentrations of APP and PLP in D_2O were 0.100 M, prepared by direct weighing. In the low pH region less concentrated solutions were used because of limited solubility of the compounds. The appropriate amount of base necessary to achieve the desired pH was taken from the corresponding titration curve, and the ionic strength was maintained at 1.00 M by addition of KCl.

The pH values of the D_2O solutions were measured with a Corning Model 12 Research pH meter fitted with a Sargent Welch miniature combination glass electrode. The instrument was calibrated by standardization with dilute hydrochloric acid at 1.00 M (KCl) ionic strength to read −log $[H^+]$. The pD value was computed by adding 0.40 to the observed reading.[9]

The nuclear Overhauser effect (NOE) measurements were carried out with a Varian EM 390 NMR spectrometer. The lock sample, Me_4Si in chloroform, was placed in a coaxial inner tube. The APP–PLP Schiff base solutions were prepared in D_2O by direct weighing of the reagents. The analytical concentrations were 0.200 M. Purified argon was bubbled through the solutions to remove oxygen.

Spectrometric Measurements. Electronic absorption spectra were measured with a Cary Model 14 recording spectrophotometer. Matched 1.000-cm quartz cells were employed. Ionic strength was maintained at

† Abstracted in part from a dissertation to be submitted by Bruno Szpoganicz to the Faculty of Texas A&M University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Table I. Protonation Constants[a] for Pyridoxal 5'-Phosphate, 2-Amino-3-phosphonopropionic Acid, and the PLP–APP Schiff Base in D_2O (t = 35.0 °C; μ = 1.00 M (KCl)) and in Water (t = 25.0 °C; μ = 1.00 M (KCl))

compd	log K_1	log K_2	log K_3	log K_4
2-amino-3-phosphonopropionic acid	10.99	6.48[c]	2.77	
	10.406[b]	5.928[b]	2.317[b]	
pyridoxal 5'-phosphate (aldehyde form)	8.41	6.45[c]	3.56	
pyridoxal 5'-phosphate (hydrate form)	8.98	6.45[c]	4.79	
pyridoxal 5'-phosphate (macroscopic)	8.44[d]	6.45[d]	4.03[d]	
	7.915[b]	5.756[b]	3.579[b]	
APP–PLP Schiff base	12.54	8.10[c]	6.70[c]	5.95

[a] Absolute errors in log K are less than ±0.02 for NMR determinations; the absolute errors are less than 0.005 for data from the potentiometric equilibrium curves. (Constants determined by proton NMR unless otherwise stated.) [b] Protonation constants determined in water by potentiometry. [c] Protonation constants determined from ^{31}P NMR titration. [d] Macroscopic protonation constants in D_2O were determined from the microscopic protonation constants of aldehyde and hydrate forms of PLP by the equation $K_n = (K_n^a + K_{hn}K_n^h)/(1 + K_{hn})$ where K_n is the macroscopic protonation constant; K_n^a and K_n^h are the microscopic protonation constants of aldehyde and hydrate forms of PLP, respectively. K_{hn} is the hydration constant; $\beta_0^h = 0.037$ (vide supra and Scheme I).

1.00 M by the addition of KCl. Adjustments of pH were made by adding small volumes of concentrated HCl or KOH from a Gilmont microburet. The reference cell contained 1.00 M KCl solution and the same concentration of amino acid as the sample cell. The temperature was maintained constant at 35.0 ± 0.05 °C by using a constant temperature refrigerated circulating VWR Scientific Inc. water bath.

Aqueous stock solutions of PLP (5.00×10^{-3} M) were stored in a refrigerator and protected from light. The stock solutions were discarded and fresh ones prepared after intervals of 2 days. Solutions for spectrophotometric study contained 1.00×10^{-4} M PLP and varying concentrations of APP.

Results and Discussion

Species of APP and PLP. The potentiometric equilibrium curves for APP and PLP were determined with the equipment described above, and their protonation constants were calculated by using the computer program PKAS.[10] The protonation constants of APP and PLP, defined by $K_H^n = [H_nL]/[H^+][H_{n-1}L]$ where L means ligand (APP or PLP), were determined by measuring -log $[H^+]$ of aqueous solutions of the ligands as a function of the moles of base added per mole of ligand. The protonation constants obtained are given in Table I and are the best available for D_2O solutions at the temperature given. They are comparable to the constants reported previously for ionic strength 0.100 M.[11]

The NMR spectra of APP appear as two sets of peaks due to the α-methine and β-methylene protons. Since the two protons of the methylene group are not magnetically equivalent, there is coupling between them and additional coupling with the methine proton and the phosphorus atom. The α-methine proton also couples with the protons of the β-methylene group and with the phosphorus atom. Figure 1 shows the NMR spectrum of APP at pD 4.85. Following Pople, the ABMX designation is used for this system.[12,13] The chemical shifts and coupling constants were calculated by spin simulation on an XL-200 NMR spectrometer based on the Fortran program, LAME, which is LAOCOON with magnetic equivalence added.[14-16] It calculates the theoretical

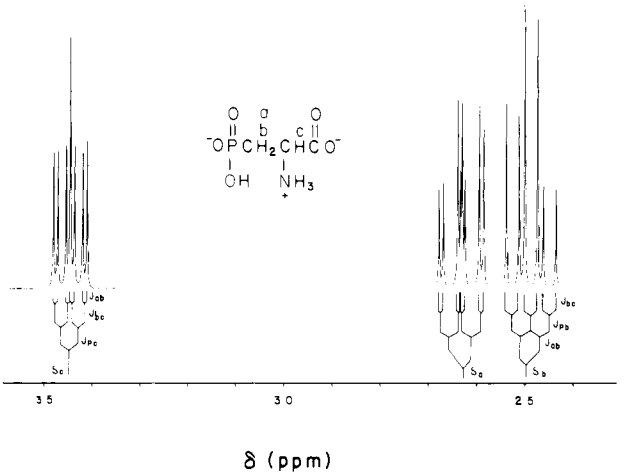
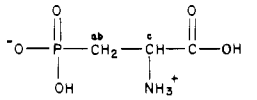
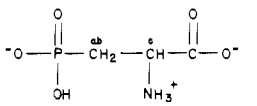
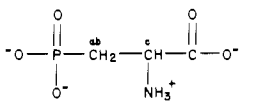
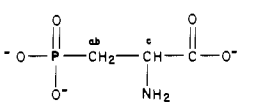


Figure 1. 200-MHz proton NMR spectra of APP at pD 4.85, t = 35.0 °C, μ = 1.00 M (KCl).

Table II. Chemical Shifts and Coupling Constants[a] for the Tri-, Di-, Mono-, and Nonprotonated Forms of APP (t = 35.0 ± 0.1 °C, μ = 1.0 M (KCl))

pD	structure	chemical shift,[b] ppm	coupling constant,[c] Hz
2.10		$\delta_a = 2.35$ $\delta_b = 2.17$ $\delta_c = 4.23$	$J_{ab} = 15.36$ $J_{ac} = 4.49$ $J_{bc} = 9.02$ $J_{Pa} = 17.54$ $J_{Pb} = 15.85$ $J_{Pc} = 16.38$
4.85		$\delta_a = 2.27$ $\delta_b = 1.99$ $\delta_c = 3.90$	$J_{ab} = 15.48$ $J_{ac} = 3.51$ $J_{bc} = 10.68$ $J_{Pa} = 17.71$ $J_{Pb} = 15.29$ $J_{Pc} = 13.88$
8.60		$\delta_a = 2.06$ $\delta_b = 1.64$ $\delta_c = 3.79$	$J_{ab} = 14.74$ $J_{ac} = 2.35$ $J_{bc} = 13.39$ $J_{Pa} = 17.14$ $J_{Pb} = 12.39$ $J_{Pc} = 9.15$
12.10		$\delta_a = 1.88$ $\delta_b = 1.47$ $\delta_c = 3.48$	$J_{ab} = 14.54$ $J_{ac} = 2.11$ $J_{bc} = 11.87$ $J_{Pa} = 17.46$ $J_{Pb} = 14.24$ $J_{Pc} = 9.94$

[a] The absolute errors in the chemical shifts and coupling constants are less than ±0.01 ppm and Hz, respectively. [b] Chemical shifts are reported in ppm; 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt (DSS), was used as an internal reference. Unless hydrogen-bonding effects are involved, a proton peak relative to Me_4Si in deuteriochloroform is within 0.01–0.03 ppm of the same peak referenced to DSS in water or deuterium oxide.[13] [c] The absolute values of the coupling constants were determined. However, the sign of the coupling constant for the hydrogens of the methylene group in amino acid has been reported to be negative.[21]

spectrum for spin $^1/_2$ nuclei, given the value of chemical shifts and coupling constants, and can adjust the values of the parameters to approach a given experimental spectrum. The chemical shifts and coupling constants were calculated for the tri-, di-, mono-, and nonprotonated species of APP (Table II). The chemical shift decreases with the increase in the negative charge on the APP

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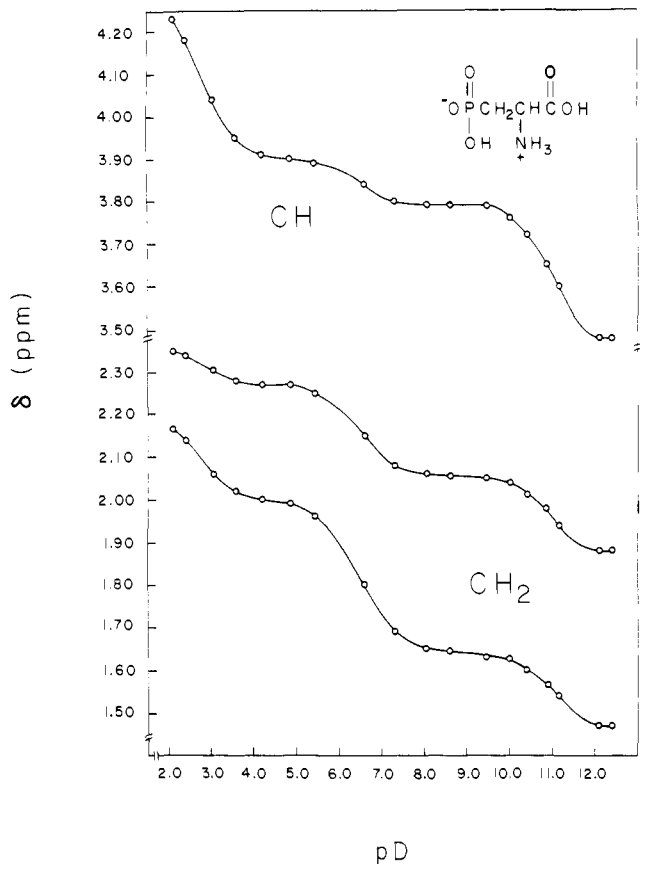


Figure 2. Variation of chemical shifts of protons of 2-amino-3-phosphonopropionic acid as a function of pD in D_2O. $t = 35.0 \pm 0.1$ °C; $\mu = 1.00$ M (KCl).

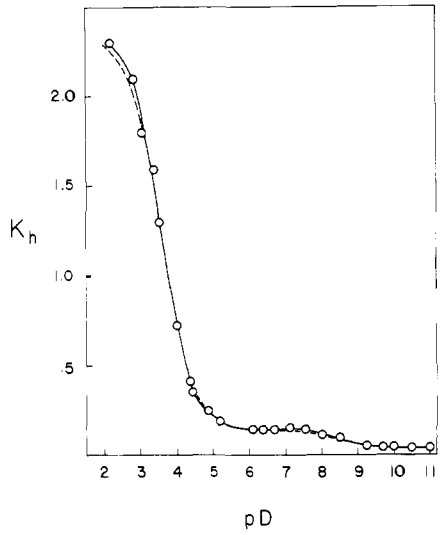


Figure 3. Variation of the conditional equilibrium constants for aldehyde and hydrate forms of pyridoxal 5′-phosphate as a function of pD: (○) calculated from the integration of 4′-CH resonance (^{1}H NMR); (---) calculated from the hydration constants β_0^h (0.037) and protonation constants from Table I.

species, as indicated in Figure 2. The plot of the chemical shift vs. pD was used to calculate the microscopic protonation constants, which are reported in Table I. Protonation constants determined in D_2O and H_2O for most acids differ by about 0.5–0.7 log unit,[17] a quantity that has been shown to increase with pK_a according to the relationship[18]

$$pK(D_2O) - pK(H_2O) = 0.41 + 0.020pK(H_2O) \quad (1)$$

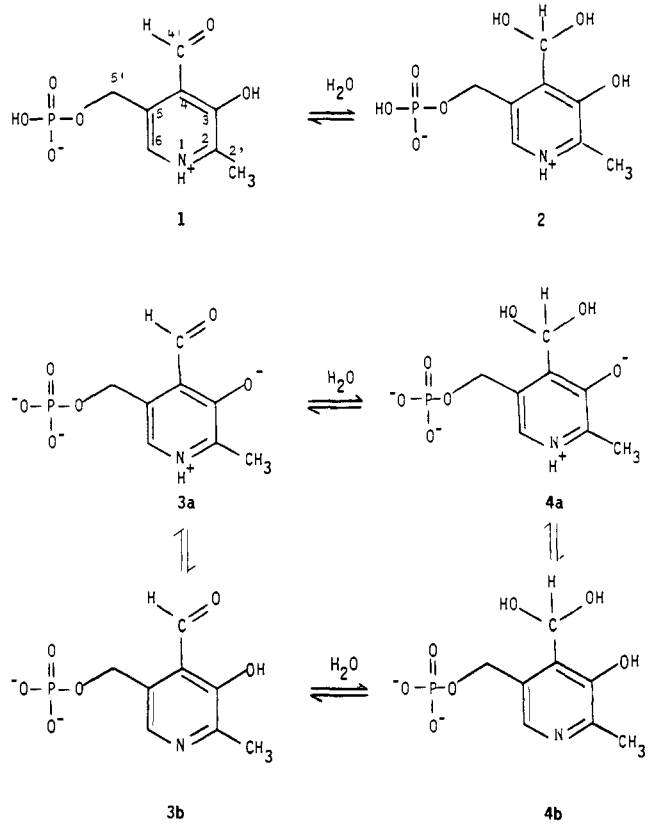
(17) Robertson, R. E.; Laughton, P. M. "Solute Solvent Interactions"; Coetzee, J. F., Ritchie, C. D., Eds.; Dekker: New York, 1969; pp 400–538.

The proton NMR spectra of PLP provide evidence for two forms, the aldehyde and the hydrate species. The ^{13}C NMR spectra of aldehyde and hydrate forms of PLP have been reported.[19,20] The proton NMR spectra of vitamin B_6 species have been reported.[22-24] From the proton NMR spectra of PLP at several pD's it was possible to calculate the conditional constant K_h for the equilibrium

$$PLP_{ald} \overset{K_h}{\rightleftharpoons} PLP_{hydr}$$

$$K_h = [PLP_{hydr}]/[PLP_{ald}] \quad (2)$$

For the equilibrium constant defined above, each bracket represents the sum of the concentrations of all possible species. Formulas **1–4** show the forms of PLP having protonated and nonprotonated phenolic oxygens. The tautomerization of the pro-



tonated pyridine nitrogen of the PLP species has been reported.[25,26] The tautomers are indicated by formulas **3a/3b** and **4a/4b**. Harris et al.[25] estimated by electronic spectra the relative concentrations of the tautomers in aqueous solution. The equilibrium constant (2) is dependent on the degree of protonation and hence on the pD of the medium, as indicated in Figure 3.

Pyridine-4-carboxaldehyde (an analogue of PLP at low pD) has a hydration constant of 1.28,[27] which is about the same in magnitude as that of PLP in this pD or pH range. A kinetic study of the hydration of PLP has been described elsewhere.[28,29] As

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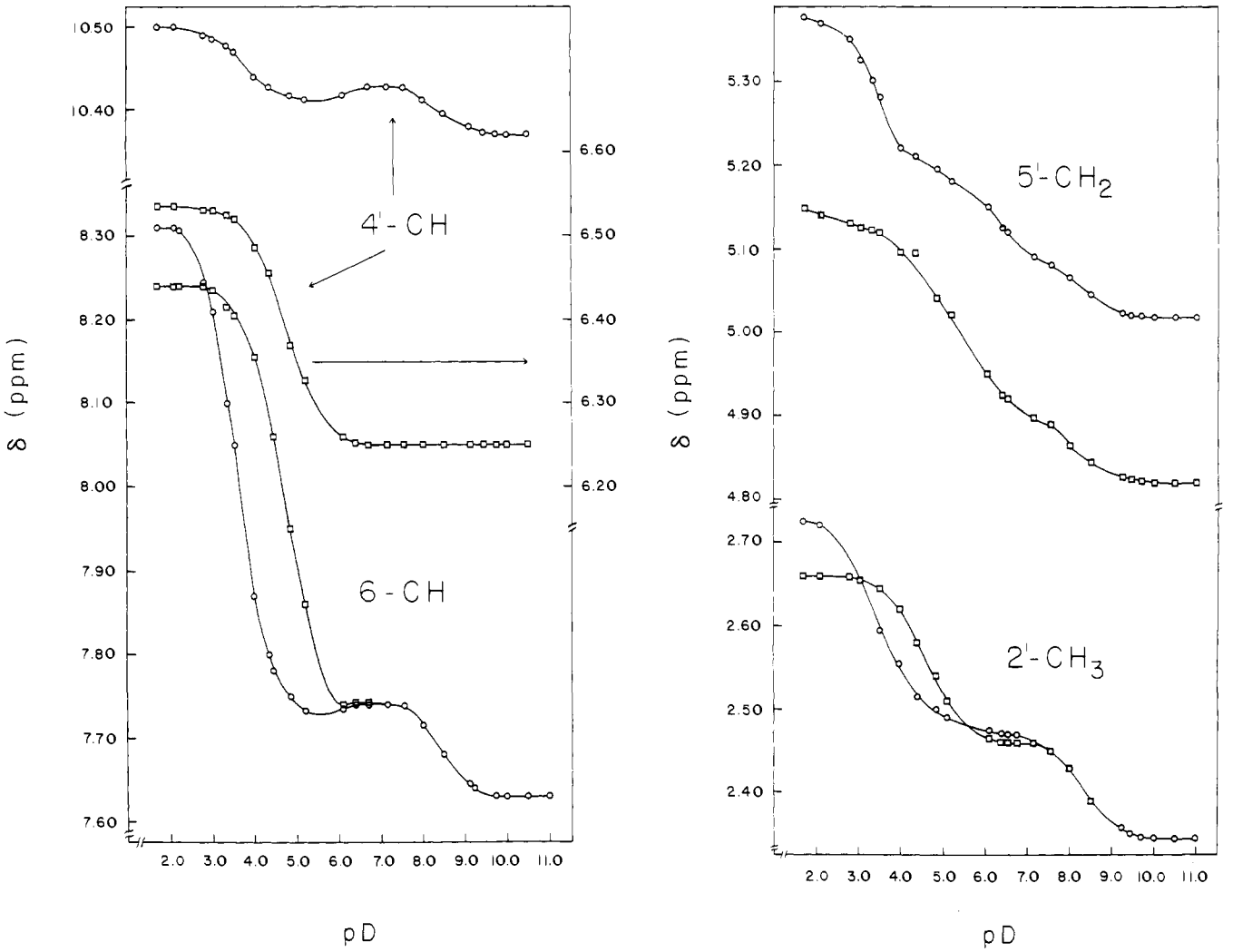
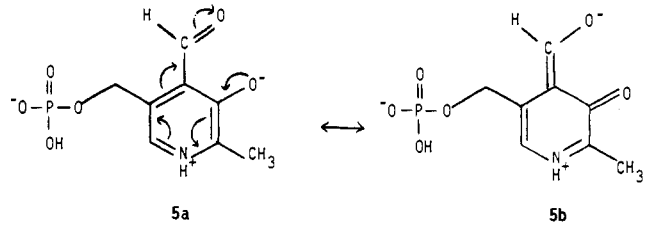


Figure 4. Variation of chemical shifts of protons of PLP as a function of pD in D_2O. t = 35.0 ± 0.1 °C; μ = 1.00 M (KCl). (○) Aldehyde form; (□) hydrate form.

can be seen from the present work, the relative amount of hydrate form drops drastically when the pD goes from 2.0 to 5.0. The equilibrium concentration of the hydrated form of an aldehyde decreases when a substituent or other changes in molecular structure tend to release electrons to the keto form of the carbonyl group.[30] The dissociation of the phenolic hydroxyl tends to release electrons to the carbonyl carbon atoms, as indicated by the resonance forms **5a** and **5b**, thus decreasing its electrophilicity and increasing the proportion of the free aldehyde form of PLP.



5a **5b**

The effect of pH on the molecular species of pyridoxamine and pyridoxal has been studied previously by proton NMR,[31] but the relative amounts of aldehyde and hydrate forms were not determined. Figure 4 shows the variation of the ^1H chemical shifts vs. pD of the aldehyde and hydrate forms of PLP. The 6-CH proton of the aldehyde form shows a considerable decrease in chemical shift when the pD goes from 2.0 to 5.0. A corresponding decrease occurs for the hydrate form when the pD is varied from 3.0 to 6.0, indicating that the electron density in the aromatic ring is altered; i.e., it is influenced by the negative charge formed by dissociation of the phenolic hydroxyl. The protonation constants were determined from the observed chemical shifts for both forms (Table I). The hydrate has a higher pK_a than the aldehyde form because the negative charge of the dissociated phenolic group is resonance stabilized by the carbonyl group of the aldehyde. The pK_a due to the proton bonded to the pyridine nitrogen was found to be 8.41 (8.2 was found by the ^{13}C NMR technique[32]). The phenolic deprotonation affects the chemical shift of the 4'-CH proton of both the aldehyde and hydrate forms of PLP, but the deprotonation of the pyridine nitrogen only affects the aldehyde form. This result is reasonable since the carbonyl group of the aldehyde is conjugated with the pyridine nitrogen while the hydrate does not have this type of interaction. Deprotonation of the phenolic group, however, influences the 4'-CH resonance of both forms because of its proximity, whereby inductive electronic effects are sufficient to produce the observed shift independently of conjugation.

The chemical shift of the 4'-CH of the aldehyde form of PLP increases slightly when the pD goes from 6.0 to 6.8 as the result of phosphate deprotonation. The protons of the methyl and

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(30) Streitweiser, A.; Heathcock, C. H. "Introduction to Organic Chemistry"; Macmillan: New York, 1976.
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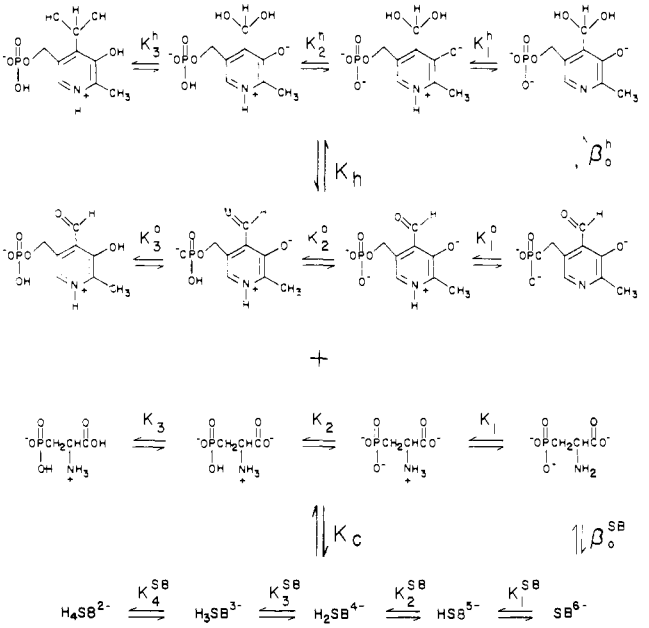
(32) Witherup, T. H.; Abbott, E. H. *J. Org. Chem.* **1975**, *40*, 2229.

Scheme I



methylene groups are also affected by this deprotonation step. The dissociation step of both forms of PLP is reflected by an upfield shift of the 5′-CH_2 protons. The protonation constants for the phosphate group were determined by phosphorus NMR. Figure 5 shows the observed variation of the chemical shift vs. pD, from which the pK_a was found to be 6.45. No difference was observed between the two forms of PLP.

The overall protonation constants of the aldehyde form of PLP are defined by eq 3. The overall hydration constants are defined

$$\beta_n^{a} = \frac{[PLP_aH_n^{n-3}]}{[PLP_a^{3-}][H^+]^n} \tag{3}$$

by eq 4. The β values were determined from the protonation

$$\beta_n^{h} = \frac{[PLP_hH_n^{n-3}]}{[PLP_a^{3-}][H^+]^n} \tag{4}$$

constants of the species involved (Table I, Scheme I).

$$\beta_n^{a} = K_1^{a}K_2^{a}....K_n^{a} \tag{5}$$

$$\beta_n^{h} = \beta_0^{h}K_1^{h}K_2^{h}....K_n^{h} \tag{6}$$

β_0^{h} is the hydration constant at high pD, K_n^{a} are the protonation constants of the aldehyde form of PLP, and K_n^{h} are the protonation constants of the hydrate form of PLP (Table I). The distributions of PLP species were calculated in the pD range 2.0–12.0 with the aid of a computer program developed by Dr. R. J. Motekaitis in this laboratory.[33] From the comparison of this distribution with the total concentrations of aldehyde and hydrate species determined from the conditional equilibrium constant for hydration, it was possible to evaluate the log protonation constant of the pyridine nitrogen of the hydrate form of PLP as 8.98.

By the use of the microscopic protonation constants of PLP (Scheme I, Table I) and the hydration constant for the completely deprotonated species of PLP (β_0^{h} = 0.037), the hydration constant (K_{hn}) for each species of PLP was determined (eq 7). K_{hn} =

$$K_{hn} = \beta_0^{h}\frac{K_1^{h}....K_n^{h}}{K_1^{a}....K_n^{a}} \tag{7}$$

$[PLP_h^{n-4}]/[PLP_a^{n-4}]$ with n = 2, 3, and 4 for this particular system (see Scheme I). The values of K_{h1}, K_{h2}, K_{h3}, and K_{h4} are 0.037, 0.138, 0.138, and 2.34, respectively. Values of 0.091, 0.24, and 3.2 have been reported by Harris et al.[25] for hydration constants of completely deprotonated, monoprotonated (pyridinic nitrogen), and diprotonated (pyridinic nitrogen and phenolic oxygen) species of PLP in water. The differences between these values and those of the present work are considered to be due more likely to differences in conditions (supporting electrolyte and temperature) than to a possible deuterium isotope effect on the equilibrium constant.

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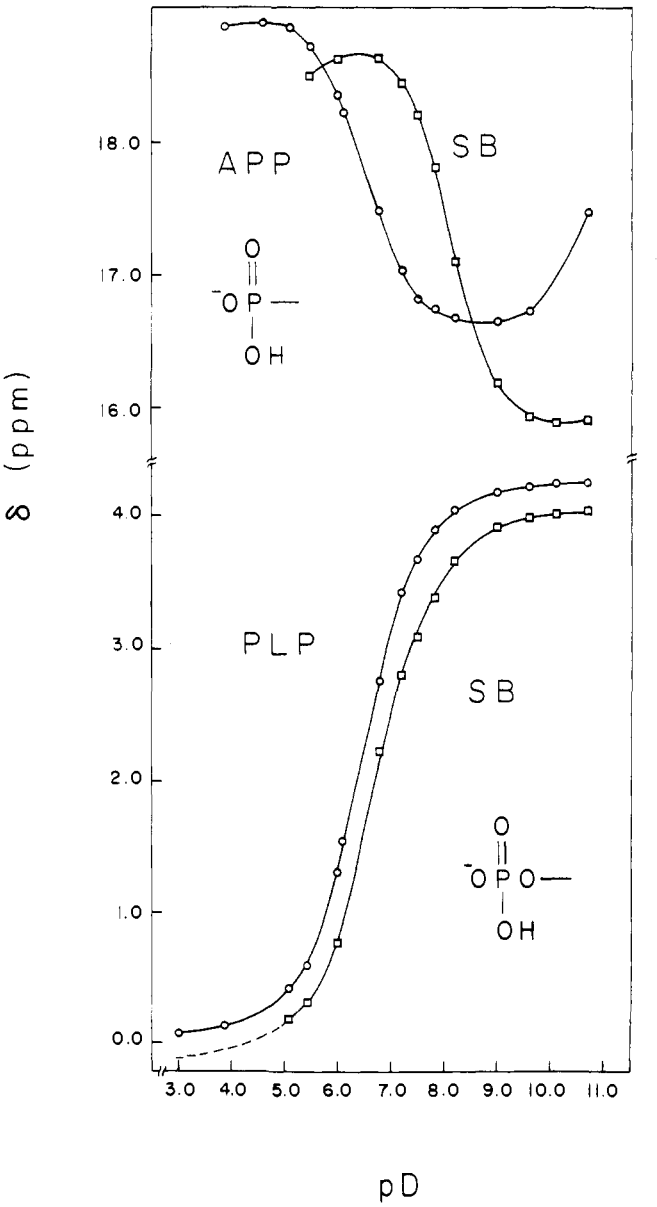


Figure 5. Variation of ^{31}P chemical shifts of phosphate group of PLP and PLP–APP Schiff base, and variation of chemical shifts of phosphonate group of APP and PLP–APP Schiff base as a function of pD in D_2O. t = 35.0 ± 0.1 °C; μ = 1.00 M (KCl).

Imine Formation. The NMR spectrum of the Schiff base of pyridoxal 5′-phosphate and 2-amino-3-phosphonopripionic acid was determined at several pD values, and appropriate assignments were made. The 200-MHz spectrum of the PLP–APP Schiff base taken 9 min after mixing at pD 7.46 is shown in Figure 6. The aldimine (4′-CH) proton appears as a singlet at 8.97 ppm and the 6-CH proton is found at 7.63 ppm, close to the 6-CH proton of free PLP. The 5′-CH_2 protons appear as a doublet at 4.94 ppm, and the 2′-CH_3 is found at 2.42 ppm, close to the 2′-CH_3 protons of free PLP. The α-methine proton resonance does not appear in this spectrum since it is at the same position as that of the HOD resonance that was decoupled. The β-methylene protons appear as a multiplet downfield with respect to the β-methylene protons of free APP.

The spectrum of the PLP–APP Schiff base 17 min after mixing at pD 9.57 shows the α-methine proton close to the HOD reso-

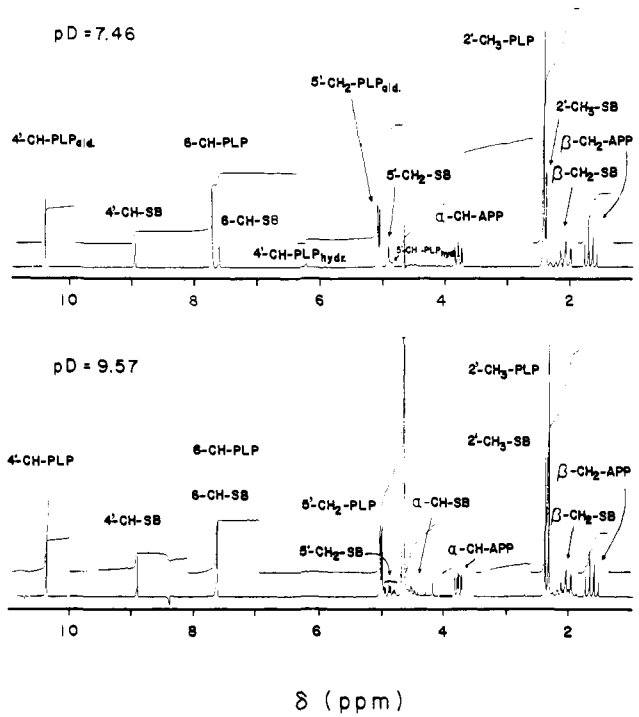


Figure 6. 200-MHz proton NMR spectra of Schiff base formed from pyridoxal 5′-phosphate and 2-amino-3-phosphonopropionic acid at pD's 7.46 and 9.57. t = 35.0 ± 0.1 °C; μ = 1.00 M (KCl).

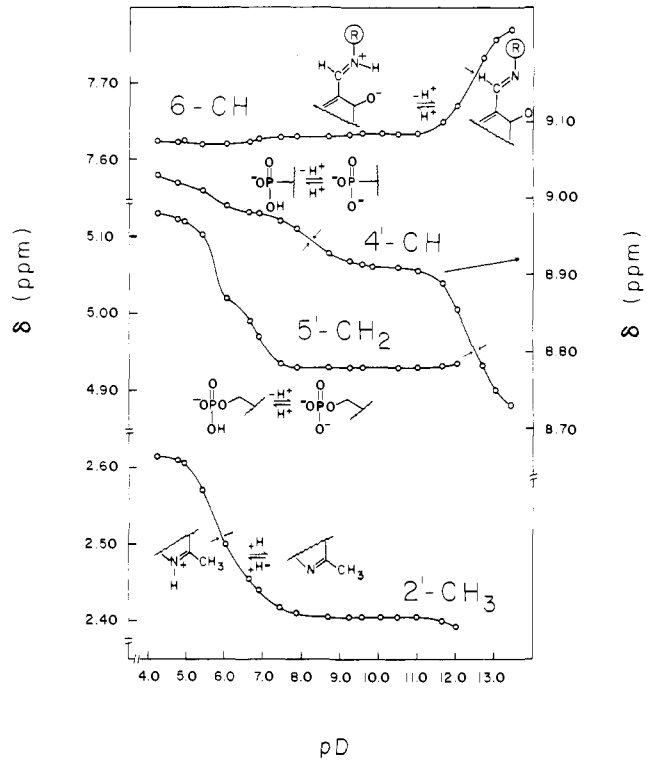
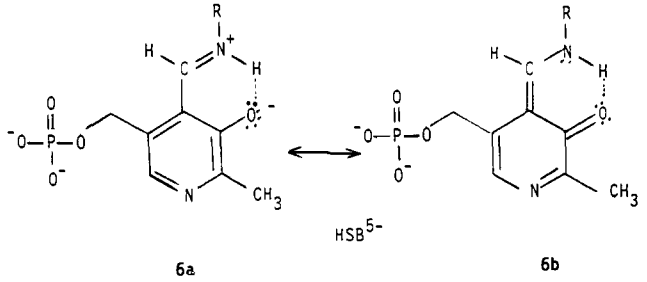


Figure 7. Variation of chemical shifts of 2′-CH_3, 4′-CH, 5′-CH_2, and 6-CH protons of Schiff base formed from pyridoxal 5′-phosphate and 2-amino-3-phosphonopropionic acid as a function of pD in D_2O. t = 35.0 ± 0.1 °C; μ = 1.00 M (KCl).

nance which was not decoupled, illustrated in Figure 6. The β-methylene protons appear as a multiplet with a chemical shift upfield relative to the β-methylene protons of free APP. The 2′-CH_3 protons are found at 2.41 ppm as a singlet. The aldimine (4′-CH) proton appears at 8.91 ppm and the 6-CH proton is found at 7.64 ppm, at the same position as that of the 6-CH proton of free PLP. The 5′-CH_2 protons appear as a doublet of quartets at 4.93 ppm, indicating that at this pD the 5′-CH_2 methylene hydrogens are not magnetically equivalent. There is coupling between them, and they couple with the phosphorus atoms of the phosphate group. This means that the dissociation of the phosphonate group (pK_a = 8.10) increases the steric hindrance between the phosphate group and the negative groups of the amino acid part of the Schiff base. The free rotation of the 5′-CH_2 methylene group is consequently slow enough to observe the coupling between the methylene protons.

Plots of chemical shift vs. pD are presented in Figure 7. As demonstrated for related systems,[31,34] plots of the type shown in Figure 7 are often useful in detecting acid–base equilibria and for determining pK_a values. It was not possible to observe pD's lower than 4.25 because of the low solubility of PLP at low pD and the small amount of Schiff base formed under such conditions. The chemical shift of the 2′-CH_3 protons is affected by the dissociation of the protonated pyridine nitrogen. Figure 7 shows this dependence clearly. The log of the protonation constant determined from these data is 5.95, which is similar to the pK_a's of the Schiff bases formed from pyridoxal and other amino acids.[35] The deprotonation of the pyridine nitrogen and phosphate groups also affects the chemical shift of the 5′-CH_2 protons. The deprotonation of the phosphate group and of the proton bound to the azomethine nitrogen is indicated by the variation of the chemical shift of the 4-CH proton. The deprotonation of the monoprotonated Schiff base (HSB^{5-}, Scheme I) affects all protons (it was not possible to observe the variations of the chemical shift of 5′-CH_2 and 2′-CH_3 protons at pD values above 12 because of the low concentrations of Schiff base present under these conditions). The curves thus obtained agree very well with each other, and the pK_a calculated from these changes is 12.54, which is in accord with the values reported in the literature for analogous systems[35] (Schiff bases of PLP with other amino acids). While the chemical shifts of the 6-CH and 5′-CH_2 protons move downfield, the chemical shifts of the aldimine 4′-CH and 2′-CH_3 protons move upfield. It has been shown that the constitution of the monoprotonated aldimine is solvent dependent, reflecting varying contributions of the resonance forms, **6a** and **6b**. This



interpretation is largely due to the spectrophotometric work of Heinert and Martell,[36-38] who showed the changes in spectrum with solvent to be mostly due to a shift in the ratio of ketoenamine to enolimine forms. More recently Ledbetter[39] found that the resonance Raman spectra of the Schiff bases of amino acids with pyridoxal-5′-phosphate and salicylaldehyde in H_2O show predominance of the ketoenamine form with the functional azomethine group –C=N^+RH··· hydrogen bonded to the phenolate oxygen. The contribution of **6a** has considerable influence on the charge distribution and bonding, thus greatly influencing the proton chemical shifts.

The deprotonation of the HSB^{5-} species to give structure **7** results in upfield shifts of the 4′-CH and 2′-CH_3 proton resonances and in downfield shifts of 5′-CH_2 and 6-CH proton resonances.

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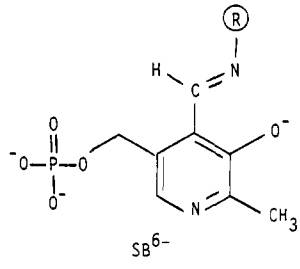
(39) Ledbetter, J. W. *J. Phys. Chem.* **1982**, *86*, 2449.

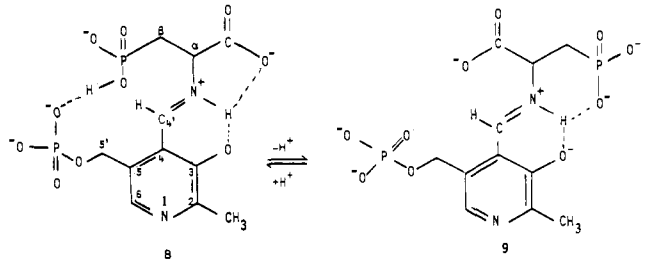


7

Formation of the quinonoid resonance form is more difficult (has higher energy), resulting in a considerable change in the charge distribution and covalent bonding of these functional groups. The negative cloud increases at the 4′-CH proton after deprotonation of HSB^{5-}, shifting the resonance upfield. The negative charge in the ring is higher for the SB^{6-} species than for HSB^{5-}, increasing the ring current. The circulating π electrons induce a magnetic field shifting the 6-CH and 5′-CH_2 proton resonances downfield. On the other hand the 2′-CH_3 resonances shift upfield. The quinonoid form, **6b**, of HSB^{5-} contributes to deshielding of the 2′-CH_3 protons due to the electronic influence of the polar C=O bond. However, after deprotonation, the quinonoid structure forms with more difficulty, resulting in the observed upfield shift of the 2′-CH_3 resonance.

The pK_a's of the phosphate and of the phosphonate group of the Schiff base were determined by the plots of chemical shift vs. pD from the proton-decoupled ^{31}P NMR spectra. Figure 5 shows the dependence of the ^{31}P chemical shift vs. pD for the phosphonate group of the Schiff base and APP and for the phosphate group of the Schiff base and PLP. The pK_a's of the phosphate group of the Schiff base and PLP are 6.70 and 6.45, respectively. While this difference is small, as expected, it is very interesting to note that the pK_a of the phosphonate group of the Schiff base (8.10) is nearly two log units higher than the deprotonation constant of the phosphate group of APP. These results provide evidence for hydrogen bonding involving the phosphonate group of the Schiff base. Since the imine nitrogen is protonated, there is no possibility for intramolecular hydrogen bonding involving the phosphonate proton. But with the azomethine proton hydrogen bonded to the carboxylate oxygen, the phosphonate proton could be bound to the phosphate oxygen as indicated by formula **8**.

In order to confirm this interpretation and to see if a structural change occurs after deprotonation of the phosphonate group, nuclear Overhauser effect (NOE) measurements were carried out on the APP–PLP Schiff base (Table III). Irradiation of the resonance frequency of the 4′-CH group gives a prominent NOE (17% on the 5′-CH_2 and 13% on the β-CH_2 of the APP–PLP Schiff base at pD 7.32; 12% on the 5′-CH_2 and 0% on the β-CH_2 of the APP–PLP Schiff base at pD 10.66), which is strongly indicative of formula **8** for APP–PLP Schiff base at pD < 8.1. At pD > 8.1 the NOE results suggest that the phosphonate group is coordinated (i.e., hydrogen bonded) to the azomethine proton, which is also hydrogen bonded to the phenolate oxygen, as indicated by formula **9**.



8 9

Because transamination and deuteration occur at moderate rates over some of the pH range employed, the concentrations of PLP, APP, and Schiff base were determined over the pD range 4.94–8.70 by extrapolating to time zero the ratios of the integrations of the 4′-CH resonances of SB and free PLP. Since the initial concentrations of PLP and APP were the same and equal to 0.100 M, the concentrations of SB, PLP, and APP at equilibrium are determined by the expressions:

$$[SB] = \frac{4'\text{-CH(SB)}}{4'\text{-CH(SB)} + 4'\text{-CH(PLP)}}(0.100) \quad (8)$$

$$[APP] = [PLP] = \frac{4'\text{-CH(PLP)}}{4'\text{-CH(SB)} + 4'\text{-CH(PLP)}}(0.100) \quad (9)$$

The imine formation constants defined by eq 10 were calculated for the reaction

$$PLP + APP \rightleftharpoons SB$$

$$K_c = \frac{[SB]}{[PLP][APP]} \quad (10)$$

where [PLP], [APP], and [Schiff base] represent the sums of concentrations of all possible species (Scheme I). The constant K_c is dependent on pD, since the fractions of each species, PLP, APP, and Schiff base, are pH dependent. Thus K_c defined by eq 10 is a conditional equilibrium constant. The Schiff base formation constants have now been determined for the system PLP and APP over the pH range 4.94–13.02. Figure 8 shows a plot of K_c vs. pD. The calculated curve determined from the constants in Table I shows good agreement with the experimental points determined by integration of the 4′-CH resonances of the SB and PLP. The deviations from the usual bell-shaped curve reflect the existence of multiple acid–base equilibria for both starting materials and product.

The logs of the protonation constants for the pyridine nitrogen and the azomethine proton of the PLP–APP Schiff base are close to the values of 6.76 and 12.16 of the PLP–valine Schiff base and 5.85 and 11.87 for the PLP–glutamate system in water.[35] These values show that the presence of negative groups on the amino acid moiety of the Schiff base has little effect on the protonation constant of the pyridine nitrogen. On the other hand, the protonation constants for the pyridine nitrogen and the azomethine nitrogen of the PLP and APP Schiff base differ significantly from those of pyridoxal-substituted amino acid Schiff bases: 6.40 and 9.75 for pyridoxal-β-chloroalanine, 6.43 and 9.80 for pyridoxal-*O*-phosphoserine, 6.70 and 9.80 for pyridoxal-β-chloro-α-

Table III. Nuclear Overhauser Effect Measurements

nucleus irradiated	nucleus measured	APP–PLP Schiff base pD <8.1	APP–PLP Schiff base pD >8.1
4′-CH	5′-CH_2	17%	12%
4′-CH	β-CH_2	13%	0
5′-CH_2	4′-CH	25%	21%
5′-CH_2	β-CH_2	0	0

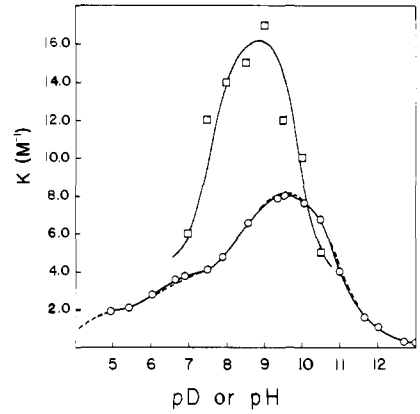


Figure 8. Variation of conditional formation constants for the Schiff bases derived from PLP and APP as a function of PD in D_2O (○) and pH in aqueous solution (□). t = 35.0 ± 0.1 °C; μ = 1.00 M (KCl). The curve (---) was calculated from the formation constant (β_0^{SB} = 0.26) and the protonation constants in Table I.

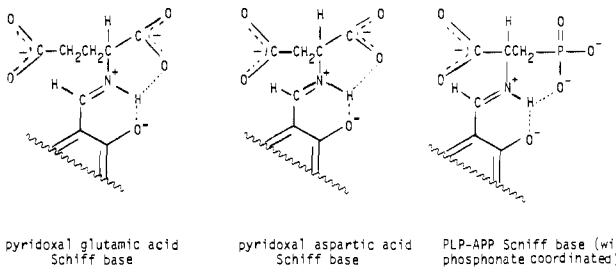
aminobutyric acid, 6.55 and 9.85 for pyridoxal-*S*-ethylcysteine,[40] and 5.88 and 10.49 for pyridoxalvaline.[35] It has been reported that the stabilizing interaction in the anion may involve hydrogen bonding between the imine nitrogen and the 5′-hydroxyl group in the pyridoxal–amino acid systems, which stabilizes the deprotonated form of the PL Schiff base relative to that of PLP.

At pD 13.02 all species are essentially completely deprotonated; therefore, the K_c calculated at this pD is taken to be pD independent, and the true imine formation constant for the reaction, β_0^{SB}, is defined (eq 11). The value of β_0^{SB} obtained is 0.26, which

$$PLP^{3-} + APP^{3-} \rightleftharpoons SB^{6-}$$

$$\beta_0^{SB} = \frac{[SB^{5-}]}{[PLP^{3-}][APP^{3-}]} \quad (11)$$

is much smaller than the values of 3.5 and 3.3 for pyridoxal-β-chloroalanine and pyridoxal-*O*-phosphoserine,[40] respectively. The amino acids of these systems differ from APP by the groups attached to the β-carbon. These results indicate steric hindrance and electron-withdrawing effects on the equilibrium constant β_0^{SB}. Substituting a methylene group at the β-carbon of β-chloroalanine results in an increase of β_0^{SB} from 3.5 to 4.2 in β-chloro-α-aminobutyric acid. A similar increase in β_0^{SB} has also been observed for the homologous pyridoxal–aspartic acid and pyridoxal–glutamic acid systems.[41]



Two effects contribute to the lower degree of formation of the Schiff base for the PLP–APP system: one factor, described above, is that the proximity of the carboxylate group to the azomethine nitrogen contributes to a lower β_0^{SB}. Another effect that may contribute to low β_0^{SB} and to low conditional equilibrium constant is the position of the amino group in relationship to the group that is coordinated to the azomethine proton (phosphonate or carboxylate group). Langohr and Martell[5] reported a β_0^{SB} of 43 for the (aminomethyl)phosphonic acid (an α-amino acid) and β_0^{SB} = 21 for the (2-aminoethyl)phosphonic acid (a β-amino acid). In the α-amino acid the azomethine proton is hydrogen bonded to the phosphonate and phenolic oxygens. Such hydrogen bonding would be expected to stabilize the protonated imine, and this stability would be reflected in the larger value of the protonation constant of the azomethine nitrogen. However, in the β-amino phosphonic acid (APP), with an additional methylene group, hydrogen bonding of the azomethine nitrogen to the phosphonate oxygen would tend to be weakened. The fact that the β-phosphonate, rather than the α-carboxylate, is hydrogen bonded to the azomethine proton is ascribed to the higher charge of the former, which increases the hydrogen bond strength, and to the greater Coulombic repulsion between the phosphonate and phosphate groups (see formula **9**), relative to the alternative structure in which the phosphate and carboxylate groups are close together.

The overall protonation constants of the aldehyde form of PLP, the hydration constants (defined previously), and the overall protonation constant of APP are defined by eq 12.

$$\beta_n = \frac{[APPH_n^{n-3}]}{[APP^{3-}][H^+]^n} \quad (12)$$

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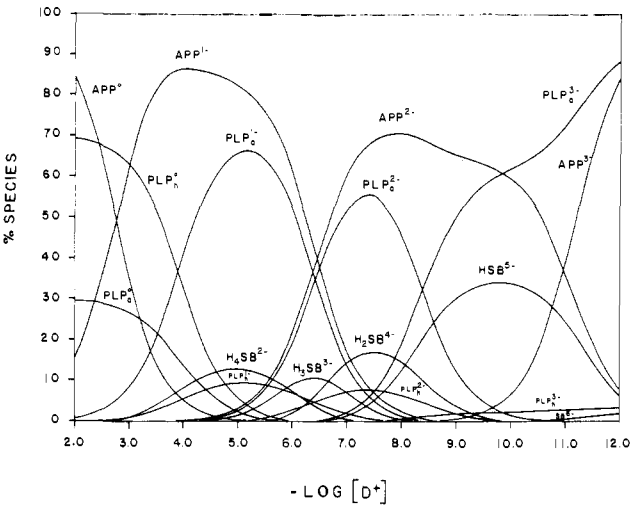

Figure 9. Species distribution of the system PLP + APP, where H_4SB^{2-}, H_3SB^{3-}, H_2SB^{4-}, HSB^{5-}, and SB^{6-} are tetraprotonated, triprotonated, diprotonated, monoprotonated, and nonprotonated, respectively, forms of the Schiff base. PLP_a^0, PLP_a^{1-}, PLP_a^{2-}, and PLP_a^{3-} represent the aldehyde species of PLP. PLP_h^0, PLP_h^{1-}, PLP_h^{2-}, and PLP_h^{3-} represent the hydrate species of PLP. APP^0, APP^{1-}, APP^{2-}, and APP^{3-} are the APP species.

The equilibrium constants (eq 13) for Schiff base formation from APP and PLP were used to calculate the species distribution

$$PLP^{3-} + APP^{3-} + nH^+ \rightleftharpoons SBH_n^{n-6}$$

$$\beta_n^{SB} = \frac{[SB_n^{n-6}]}{[PLP^{3-}][APP^{3-}][H^+]^n} \quad (13)$$

of the Schiff base system (Figure 9). The overall protonation constants of APP were determined by NMR as was done for PLP (aldehyde form).

$$\beta_n = K_1K_2.....K_n \quad (14)$$

The Schiff base formation constant was determined by

$$\beta_n^{SB} = \beta_0^{SB}K_1^{SB}K_2^{SB}.....K_n^{SB} \quad (15)$$

(see Scheme I).

The species distribution curves show that the concentration of Schiff base is highest at pD 9.7, where the PLP is completely deprotonated and the amino group of APP is partially deprotonated. The drop in Schiff base concentration at higher pD is due to the destabilization of the Schiff base resulting from the loss of the stabilizing proton on the azomethine nitrogen. The species distributions are important for identifying the ionic species responsible for the catalytic effects of the Schiff base on the reactions of the amino acid, such as transamination, elimination, and others.

Electronic Spectral Study of Imine Formation. Evidence for imine formation is seen in the modification of the electronic spectra of PLP, in aqueous solution, when the amino phosphonic acid is present. Even at large excess of APP over PLP, the formation of SB is not complete; thus a band at 404 nm is a composite of the 391-nm band of PLP and the 413-nm band of the SB. The band at 413 nm for the PLP–APP Schiff base is comparable to the 414-nm band for Schiff bases of pyridoxal and (aminomethyl)phosphonic acid and pyridoxal and (aminoethyl)-phosphonic acid reported by Martell and Langohr.[5] At pH values above 10.0, the 404-nm imine peak undergoes a small decrease in intensity and shifts to lower wavelength. This behavior has been observed for other pyridoxylidene amino acids[5,41] and results from proton dissociation from the aldimine.

From the spectrophotometric data the Schiff base formation constants, as indicated in eq 16 (where [PLP], [APP], and [Schiff

$$PLP + APP \overset{K_c'}{\rightleftharpoons} SB$$

$$K_c' = \frac{[SB]}{[PLP][APP]} \quad (16)$$

base] represent the sum of the concentrations for all possible species), were determined. This equilibrium differs from that of eq 10 because of the fact that it occurs in water rather than in deuterium oxide medium.

The Schiff base formation constants determined for the system PLP and APP over the pH range 7-10.5 are presented in Figure 8. Increasing concentrations of APP relative to a fixed concentration of PLP results in the formation of increasing amounts of imine, as evidenced by the increase of absorption at 274 nm. The value for K_c' is obtained by measuring the changes in absorbance (ΔA at 274 nm) with increments of amino acid concentrations and by plotting $1/\Delta A$ against $1/[\mathrm{APP}]$ (eq 17). The intercept

$$\epsilon l K_c'[\mathrm{PLP}]\frac{1}{\Delta A} - K_c' = \frac{1}{[\mathrm{APP}]_T} \qquad (17)$$

of the perfectly linear plot so obtained with the abcissa[5,42,43] gave the value of K_c', where ϵ is the molar absorptivity of SB, l is the length of the cell, and K_c' is the constant for a specific pH and temperature. ΔA is the difference of the absorbance of SB and PLP. The values of K_c' are plotted in Figure 8.

The K_c' values in H_2O are in general agreement with the K_c values of the same compounds in D_2O solvent, determined by NMR. The difference observed is due to the difference in solvent. The best conditions for Schiff base formation are pH 8.8 and pD 9.7 in H_2O and D_2O, respectively. Under these conditions the difference between log K_c' and log K_c in H_2O and D_2O is 0.30. It is also seen that the protonation constants in H_2O and D_2O differ by a nearly constant amount, with the values in D_2O being roughly $0.41 + 0.020pK(H_2O)$ values higher than those obtained with H_2O as solvent.

The conditional equilibrium constants governing the degree of formation of individual Schiff base species as a function of pH or pD are not necessarily equivalent, and the small differences in the values listed are therefore reasonable.

The UV-vis measurements of formation constants in H_2O make possible comparisons of Schiff base stabilities with those of other PLP-amino phosphonic systems[5] such as aminomethylphosphonic acid and (2-aminoethyl)phosphonic acid. Of the three aminophosphonic acids studied, 2-amino-3-phosphonopropionic acid (APP) shows the lowest tendency to form Schiff bases. This lower affinity could be due principally to steric hindrance and charge repulsion between one of the negative groups of the APP residue in the Schiff base and the phosphate ester group of PLP. This interpretation seems to be supported by the use of space-filling molecular models. Coordination of the azomethine hydrogen in the APP Schiff base may occur by hydrogen bonding to either the carboxylate or phosphonate group, as indicated by formulas **8** and **9**. Because of lack of free rotation about the azomethine double bond, there will be one free negatively charged center on the Schiff base close to the monophosphonate ester at the 5′ position. Such interaction involves unfavorable charge repulsion, thus decreasing the tendency to form the Schiff base. As pointed out above, further protonation of the Schiff base at one of these positions provides an additional degree of stability (or mitigated destabilizing Coulombic repulsions) through hydrogen bonding and charge reduction.

Acknowledgment. This work was supported by a research grant, AM-11694, from the National Institute of Arthritis, Metabolic and Digestive Diseases, U.S. Public Health Service. B.S. thanks UFSC (Brazil) for fellowship support.

Registry No. 1, 54-47-7; **2**, 91239-59-7; APP, 5652-28-8; APP-PLP Schiff base, 91239-60-0.

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Organosilicon Rotanes

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Abstract: Reaction of $(CH_2)_4SiCl_2$ with 2 equiv of lithium leads to the spirocyclic organosilanes, $[(CH_2)_4Si]_n$, of which n = 5-12 have been isolated and characterized. With excess Li or K, the novel rearrangement product **3a** is formed. Reaction of $(CH_2)_5SiCl_2$ with K produces rotanes $[(CH_2)_5Si]_n$, n = 4-6. Photolysis of $[(CH_2)_4Si]_6$ or $[(CH_2)_5Si]_5$ (**5**) leads to loss of a cyclic silylene and formation of the next smaller ring. Structures of $[(CH_2)_4Si]_5$ (**1**) and of **5** were studied by single-crystal X-ray diffraction; both crystallize in space group $P2_1/c$. The silacyclohexane rings in **5** are all in the chair conformation, while the silacyclopentane rings in crystalline **1** adopt a variety of conformations.

Although the best known alkylcyclopolysilanes are the permethyl compounds, $(Me_2Si)_n$,[1-3] cyclic polysilanes containing Et, *n*-Pr, *n*-Bu, *i*-Pr, and *i*-Bu as substituents have recently been prepared.[4-6] These cyclosilanes show unusual properties which arise from electron delocalization in the Si-Si σ framework.

In this paper we describe the synthesis and properties of the novel polyspirocyclopolysilanes, $[(CH_2)_4Si]_n$, where n = 5-12, and $[(CH_2)_5Si]_n$, where n = 4-6.[7] These cyclosilanes are the first examples of rotane structures based on a ring of silicon atoms. Several of the properties of the silicon rotanes are unique among the cyclopolysilanes, probably due to the unusual steric effects of the cyclopolymethylene substituents. The X-ray crystal

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