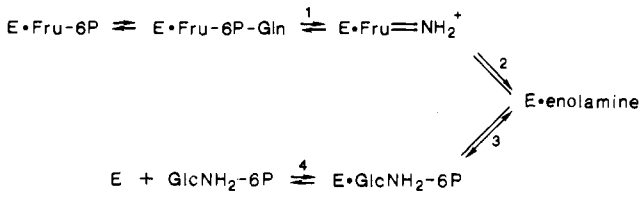
Scheme III



that of step 2. The product of the synthase-catalyzed reaction in tritiated water contains 1 mol of tritium/mol. That is, enolamine shows no discrimination between ^1H and ^3H when picking up hydrogen at C_2: this can be interpreted as a minimum for energetic barrier 3. The exchange/conversion experiment in tritiated water has shown that the intermediate collapses to tritiated product 23 times as often as it collapses to tritiated substrate. The very low tritium incorporation into fructose-6P requires the exchange to be posterior to a slow step; as step 2 is lower in energy than step 4, step 1 must be rate limiting. Therefore, the ratio 23/1 reflects the rate difference between steps 1 and 4; that is, barrier 1 is about 1.9 kcal/mol higher than barrier 4.

The precedent results raise the question about the nature of the irreversible step of the reaction. We recently demonstrated that the enzyme obeys a BiBi ordered mechanism with fructose-6P binding first and glucosamine-6P leaving last. For imine formation, the enzyme must therefore bind fructose-6P and then glutamine. Glutamate could then be released at step 1 (Scheme III); in this case the low tritium incorporation in the substrate, when the reaction is run in tritiated water, could result in imine hydrolysis into fructose-6P and ammonia, making this step virtually irreversible. Alternatively, the release of glutamate could occur at step 4 just before the release of glucosamine-6P. Whatever the position of glutamate release in this mechanism is, the energetic barrier of step 4 involves most likely an important conformational change. Although such a behavior has not been yet detected during catalysis, it is known to occur with glutamine site directed irreversible inhibitors as exemplified by half of the sites reactivity of glucosamine-6P synthase with the affinity label 6-diazo-5-oxo-L-norleucine.[9]

Acknowledgment. We thank Dr. Rousseau (CEA, Saclay, France) for his hospitality and Dr. A. M. Moustier for running the tritiation experiment in the synthesis of (1*R*)-[1-^3H]fructose-6P used in the determination of intramolecular hydrogen transfer. Financial support from Ligue Nationale Française Contre Pe Cancer to one of us (B.G.-P.) is gratefully acknowledged.

Schiff Bases and Geminal Diamines Derived from Pyridoxal 5′-Phosphate and Diamines

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Abstract: The reactions of the diamines 1,2-diaminoethane, 1,3-diaminopropane, and 1,4-diaminobutane with pyridoxal 5′-phosphate to form Schiff bases and cyclic geminal diamines have been investigated by ultraviolet-visible and ^1H NMR spectroscopy. The formation constants, pK_a values, and spectra of individual ionic species have been evaluated from the pH and concentration dependence of the spectra, and the formation constants have been evaluated independently from quantitative NMR data. The well-known system alanine:pyridoxal phosphate has been evaluated by the same techniques for purposes of comparison. Resolution of the absorption spectra into components by use of log normal distribution curves together with chemical shift values has permitted evaluation of cyclization constants for two different states of protonation of the Schiff bases of the diamines and also of tautomerization constants. Cyclization occurs readily for the diaminopropane system above pH 8 but not as readily for the diaminoethane system. For the latter, tautomerization of the ketoenamine form of the Schiff base to an enolimine form occurs to a major extent in the pH region 6-9. It is proposed that in this same pH region ~13% of the 1:1 complex may be a carbinolamine species. The data are discussed in terms of the mechanism of transimination in enzymic catalysis.

There are two important reasons for studying the interaction of the coenzyme pyridoxal 5′-phosphate (PLP) with diamines. The first is to learn what interactions might be expected with diamines and higher polyamines within cells where concentrations of the polyamines spermidine and spermine may reach 1 mM[1] and that of PLP nearly 0.1 mM.[2] Such interactions may be important both in the regulation of PLP levels and in the metabolism of these polyamines and of 1,4-diaminobutane (putrescine). The isolation of a reduced Schiff base of PLP with spermidine from sodium borohydride treated urine[3] suggests that formation of Schiff bases or geminal diamines from PLP and polyamines may occur in body fluids. It has been suggested that this interaction within the urine may be a cause of depletion of tissue PLP during pregnancy and in some pathological conditions.[4]

The second reason for studying these interaction is that geminal diamines are presumed intermediates in most PLP-dependent enzymatic reactions. Study of geminal diamines formed from 1,2-diaminoethane (ethylenediamine), 1,3-diaminopropane, and 1,4-diaminobutane may shed light on these processes. Previous investigations have dealt with the reactions of salicylaldehyde,[5,6] 5-deoxypyridoxal,[6] and PLP[7,8] with diaminoethane and diaminopropane. Kenniston[9] measured formation constants of Schiff bases of PLP at pH 7.4 for a large series of diamines. Despite this abundance of literature, there is disagreement in interpretation

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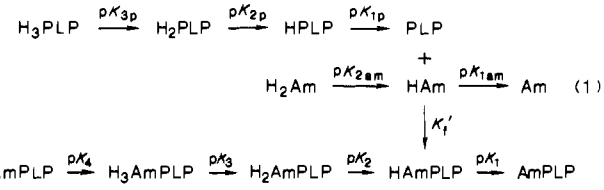
of the structures giving rise to the absorption bands around 330 nm.[6,7] These bands could all be interpreted as representing cyclic geminal diamines. However, Metzler et al. suggested that in the neutral pH range this absorption sometimes arises from a Schiff base and that protonation of the free amine group of the Schiff base of the diamine induces a tautomeric shift causing the absorption band to shift from around 420 nm to 330 nm.[6] This "electronic switch" behavior is unusual and merits further study. In the present work we have combined spectrophotometry and proton NMR spectroscopy to establish, for each state of protonation, the relative amounts of geminal diamines and of two tautomeric forms of the Schiff base for the three diamines.

The major equilibria involved are conveniently represented as in eq 1. Here the four ionization states of PLP are shown with



the three macroscopic pK_a values through which they are related. We have selected, for use in eq 1, the equilibrium between the unprotonated form of PLP and the monoprotonated form HAm of a diamine to form the monoprotonated species HAmPLP of a Schiff base. This equation utilizes the predominant ionization states at a pH of ~9. The pH-independent formation constant for this reaction, as in previous publications,[6,10,11] has been given the symbol K_f', the symbol K_f being reserved for the reaction PLP + Am → AmPLP.

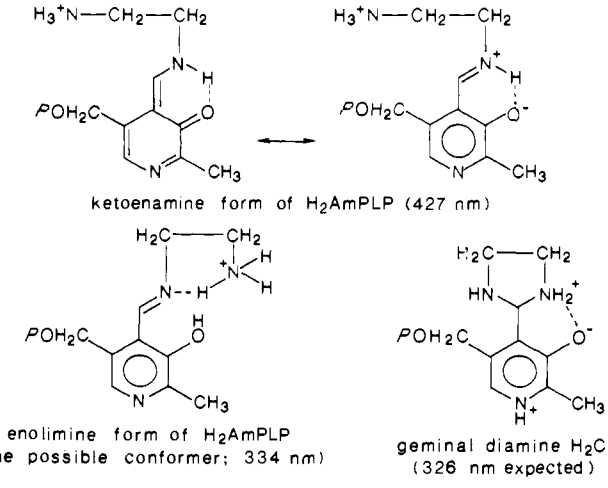
At high concentrations of PLP, Schiff bases with a 2:1 stoichiometry may be formed, for example by eq 2. This reaction will take place most extensively at lower pH values where it does not have to compete with the cyclization of the 1:1 Schiff base.

$$H_2AmPLP + PLP \xrightarrow{K_{2f}} H_2Am(PLP)_2 \quad (2)$$

It is important to recognize that each ionic form of the "Schiff base" can be made up of several different species including various tautomers, cyclic geminal diamine (C), and possibly carbinolamine. Since for a given ionic form these species exist together in a pH-independent equilibrium, they can be considered as a single form for the application of eq 1. However, from the electronic and NMR spectra we hope to be able to evaluate the equilibrium concentration ratios K_T and K_c (eq 3 and 4).

$$K_T = [\text{enolimine tautomer}]/[\text{ketoenamine tautomer}] \quad (3)$$

$$K_c = [\text{geminal diamine, C}]/[\text{ketoenamine tautomer}] \quad (4)$$



These constants are defined for each ionic species and will be designated here as K_T' for tautomerization of the monoprotonated Schiff base, K_T'' for tautomerization of the diprotonated Schiff base etc., K_c for formation of unprotonated cyclic form, K_c' for the monoprotonated cyclic form, etc.[6,12] A difficulty arises in that spectra of the enolimine tautomers, which are known to coexist with the predominant ketoenamine forms, have absorption spectra that resemble those of cyclic geminal diamines. Thus, Metzler et al.[6] concluded that ~80% of H_2AmPLP formed from 5-deoxypyridoxal and 1,2-diaminoethane is enolimine while the cyclic form contributes little if any. This conclusion was based in part on the relatively low absorption maxima and the known low molar absorptivities of enolimine forms and cannot be regarded as certain. In contrast, Tobias and Kallen[7] assigned all of the absorption near 325 nm to the cyclic form of the Schiff base of PLP with diaminoethane.

Schiff base formation with diamino acids has been studied by proton NMR spectroscopy.[13] We anticipated that use of this technique would permit a direct measurement of the amounts of the cyclic geminal diamines at various pH values. When combined with the results of UV-visible spectroscopy, the NMR data would provide a more precise description of the equilibria than would either approach alone. While the rapidity of the interconversion of Schiff base and cyclic geminal diamines has prevented a direct evaluation of the geminal diamine concentration, the values of ^{1}H chemical shifts together with the electronic spectra have allowed us to accomplish this goal.

Materials and Methods

Pyridoxal 5'-phosphate was purchased from Sigma Chemical Co. and was used without further purification. Alanine was obtained from Nutritional Biochemicals Corp., Cleveland, OH. The dihydrochloride salts of 1,2-diaminoethane, 1,3-diaminopropane, and 1,4-diaminobutane (Sigma) were prepared with concentrated HCl and were recrystallized four times from ethanol–water mixtures.

The preparation of solutions for UV-visible spectroscopy was similar to that described previously.[6] For most experiments an ionic strength of 0.2 was maintained when possible. However, in the low- and high-pH regions the ionic strength of some solutions was as high a 0.5 M. For the diaminoethane–PLP system an ionic strength of 1.0 was maintained by addition of sodium perchlorate. The electronic absorption spectra were recorded with either a Cary 1501 spectrophotometer as described previously[6] or a Cary 219 spectrophotometer interfaced to an Apple 2 computer. Most spectra were recorded with 1-cm cuvettes and total PLP concentrations of (1–2) × 10^{-4} M.

After mixing with buffered solutions of amino acid or diamine, samples were allowed to equilibrate for 10 min before the spectra were recorded. The pH of each solution was measured with a Radiometer Model PHM 84 PH meter and corrections were applied at high pH for sodium ion errors. To avoid shifts in spectral band positions caused by high chloride concentrations we used sodium perchlorate to maintain desired values of ionic strength. However, at ionic strength 1.0 this caused large deviations from expected pH values. We therefore added 0.88 to all pH readings taken at ionic strength 1.0. This returned the pH values of our reference buffers to their nominal values and gave pK_a values for PLP consistent with those in the literature (see Table I). For lower ionic strength solutions no correction was applied except for the Na^+ error at high pH.

The formation constants and spectra of individual ionic forms were determined by the method of Nagano and Metzler.[6,10] While free PLP absorbs light maximally at ~390 nm, simple Schiff bases of PLP absorb in the 415–420-nm region except at pH >11. Their presence can, therefore, be detected readily. The geminal diamines absorb in the region 290–330 nm, depending upon pH. We have recorded absorption spectra at various values of pH and for a variety of concentrations of the amine component. Using a computer-assisted iterative procedure,[6,10] we have evaluated from these data K_f' and the unknown pK_a values indicated in eq 1 as well as the molar absorptivities of the individual ionic forms AmPLP, HAmPLP, etc. When possible, experimental conditions have been chosen so that in some of the spectra at least 70% of the PLP has been converted into the unprotonated AmPLP, in other solutions at least 70% to HAmPLP, and in still others at least 70% to H_2AmPLP, etc.

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Table I. pK_a Values and Hydration Constants for Pyridoxal 5′-Phosphate (PLP) in D_2O and H_2O[a]

ionic form	T, °C	macroscopic pK_a		hydration ratios, R_h		ionic strength
		D_2O	H_2O	D_2O	H_2O	
H_3P	25	3.61	3.62[b]	4.0	3.2[b]	0.2
	25		3.97		1.9	1.0
	50		3.95		0.85	1.0
H_2P	25		6.10[b]	0.14	0.28[b]	0.2
	25		5.90		0.24	1.0
	50		6.40		0.31	1.0
HP	25	8.59	8.33[b]	0.20	0.30[b]	0.2
	25		8.54		0.26	1.0
	50		8.36		0.20	1.0
				0.23[c]		0.2
P	25			0.087	0.09[b]	0.2
	25			0.052[c]		0.2

[a] Most values are based on spectrophotometric data. Values of pK_a in D_2O are apparent values obtained directly from pH meter readings. However, a correction of +0.88 unit was added to all pH values obtained at ionic strength 1.0. This was based on the effect of adding the same amount of sodium perchlorate to standard buffers and making the assumption that pH should remain approximately constant as the ionic strength is increased. A more extensive table giving additional data from the literature for purposes of comparison is provided in the Supplementary Material. [b] Reference 16. [c] Obtained by ^1H NMR Spectroscopy.

Comparison plots, in which each experimental spectrum is compared with that predicted by the equilibrium constants together with the spectra of individual ionic forms that have been found, have been constructed for each input spectrum.

By use of log normal distribution curves[14] the spectra of the individual species of Schiff bases have been resolved into component bands that can be assigned to individual tautomers or cyclic forms.[6,12] From the areas of the bands and the molar areas established for each species, together with ^1H NMR data, the equilibrium concentration ratios K_T and K_C have been estimated (eq. 3 and 4). Additional experimental data including the amine concentrations and pH values of experimental solutions are given in the Supplemental Material.

Proton NMR spectra were collected on a Nicolet NMC-1280 300-MHz spectrometer using 2-mL samples in 5-mm tubes at room temperature (22 °C). The external standard was a 2% solution of 3-(trimethylsilyl)-1-propanesulfonic acid (DSS) in D_2O in a 20-μL microcapillary which was held in place by a 5-mm vortex suppressor. Its chemical shift was taken as exactly 0 ppm. Spectra used to evaluate formation constants from integrated areas were collected by using a pulse length of 10 μs (corresponding to a 21° pulse), a sweep width of 2000 Hz, 16K data points, a fixed delay of 12 s, and an accumulation of 128 scans. The precision of the integration was evaluated by using a series of concentrations of PLP from 0.005 to 0.05 M at apparent pH of 11.7–12.5. The areas were found accurately proportional to concentration with a reproducibility of better than ±5%.[15] Relative areas of the 6H, 4H, and 2-CH_3 protons were in the expected 1:1:3 ratio to within ±10%. In addition, possible loss of protons from any position in PLP was investigated by observation at times up to 1000 h. Significant exchange was observed only at long times[15] and had no effect on the results reported here.

Results

Because the proton NMR studies were to be done in D_2O, spectra and pK_a values of PLP were measured in that solvent. The pK_a values found are given in Table I. The spectra of the individual ionic species were resolved as described by Harris et al.[16] to give the amounts of aldehyde and covalent hydrate. The calculated hydration ratios are also given in Table I together with values determined independently from NMR spectroscopy. The change in solvent from H_2O to D_2O has only minor effects on these equilibrium constants. The pK_a values used for the diamines were taken from the literature or were determined by a proton NMR titration and are given in Table II.

The electronic absorption spectra for the four individual ionic species of the Schiff base–geminal diamine mixture formed from PLP + 1,2-diaminoethane are shown in Figure 1, and the values of K_f' and the pK_a values found are given in Table III. The molar absorptivities for the four spectra of Figure 1 together with the equilibrium constants from Tables I–III were used to calculate the expected spectra for each experimental solution. These were plotted together with the experimental points as in Figure 2 of our previous paper.[6] Some of these curves are also shown in the Supplementary Material. The agreement was satisfactory for all solutions.

The spectra of Figure 1 have been analyzed by resolving with log normal curves.[14] Assigning appropriate molar areas (integrated intensities), we used the areas of these bands to estimate the fractions of Schiff base tautomers and geminal diamine present.[6] Figure 1 shows how the spectra of the individual ionic forms of AmPLP for the PLP–1,2-diaminoethane system were resolved.

Table II. pK_a Values of Amines, Diamines, and Polyamines Used in Computations

name	structure	pK_a		ionic strength	T, °C	ref
alanine	$H_3N^+CH(CH_3)COO^-$					
H_2O		9.81				*a*
D_2O (NMR)		9.96		0.5 (KCl)		*b*
diamino-ethane	$H_3N^+(CH_2)_2NH_3^+$					
H_2O		8.21	10.89	1.0 ($NaClO_4$)	25	*b*
H_2O		7.26	9.97	1.0 ($NaClO_4$)	50	*b*
H_2O		7.59	10.29	1.0		*c*
D_2O (NMR)		7.35	10.45	0.5		*d*
1,3-diamino-propane	$H_3N^+(CH_2)_3NH_3^+$	9.01	10.40	0.5	25	*d*
		9.03	10.94			*e*
1,4-diamino-butane	$H_3N^+(CH_2)_4NH_3^+$	9.71	11.15			

[a] Reference 20. [b] This work. See footnote *b* of Table I for information on pK_a values at ionic strength 1.0. [c] Reference 7. [d] Reference 5. [e] Reference 6.

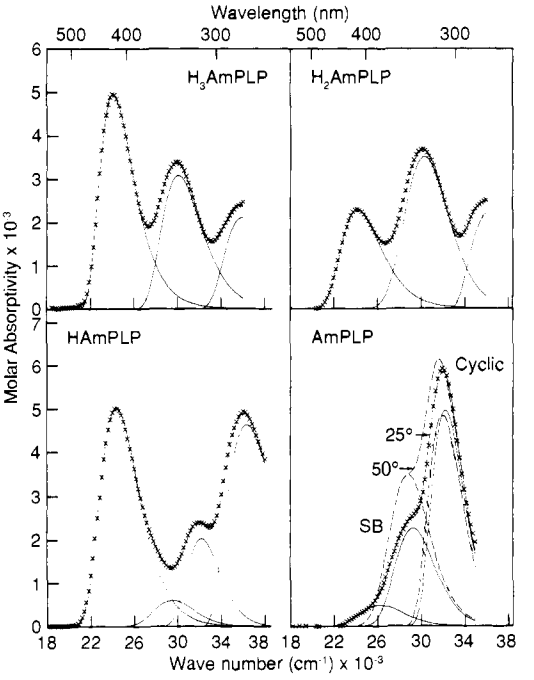

Figure 1. Electronic spectra calculated for individual ionic forms of the 1,2-diaminoethane–PLP Schiff base at 25 °C and at 50 °C, ionic strength 1.0. AmPLP is the unprotonated form, HAmPLP the monoprotonated form, etc. Each of these forms can consist of a mixture of Schiff base (SB) tautomers (AmPLP1 and AmPLP2), cyclic geminal diamine, and carbinolamine. The amounts of each have been estimated from the areas under the component log normal curves when possible and are given in Table IV.

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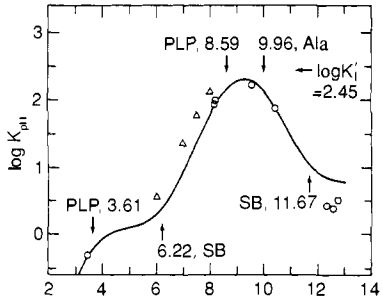


Figure 2. Plot of log K_{pH} vs pH for the PLP–alanine system in D_2O. The solid line is based on spectrophotometric evaluation of equilibrium constants at 25 °C, ionic strength 0.2. O, data from ^1H NMR at room temperature in D_2O; apparent pH values were used. Δ, data Matsuo[18] and of Lucas et al.[19] as compiled by Felty et al.[20] pK_a values are indicated by arrows.

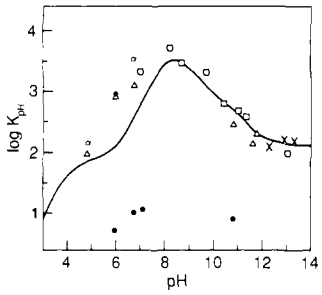


Figure 3. pH dependence of log K_{pH} for 1,2-diaminoethane. The solid line is calculated from spectrophotometric data at 25 °C, ionic strength 1.0. The points (O, 4-H; Δ, 6-H; ×, 2-CH_3) are from the NMR data, squares (□) are from Tobias and Kallen.[7] The lower points (●) represent log K_{2pH}, for formation of the 2:1 PLP–diamine Schiff base.

The corresponding curves for 1,3-diaminopropane + PLP are shown in the Supplementary Material. They resemble those for 5-deoxypyridoxal + diaminopropane described previously[6] in having high contributions of geminal diamines for the AmPLP and HAmPLP forms. The 1,4-diaminobutane–PLP system was also analyzed. The spectra resemble those of the alanine–PLP Schiff base. Little geminal diamine is formed and little interaction between the second amino group and PLP is detected at any pH. The band-shape parameters for all of the spectral bands for all of the systems studied are tabulated in the Supplementary Material. A summary of the deduced values of K_c and K_T is provided in Table IV. Plots of the pH-dependent formation constant K_{pH} (eq 5) were computed for each Schiff base including that of PLP

$$K_{pH} = [AmPLP]_{total}/[PLP]_{total}[Am]_{total} \quad (5)$$

+ alanine by using K_f' and the pK_a values for Schiff base, PLP, and amine. These are shown for the PLP–alanine system in Figure 2 and for the PLP–1,2-diaminoethane system in Figure 3.

Proton NMR spectra were run at a number of pH values using PLP and diamine in concentrations of 0.02 M. Before attempting to evaluate equilibrium constants from these data, we tested the procedure with the well-studied system PLP + alanine. The NMR spectra were easily interpretable and showed sharp resonances for the 4'-, 6-, 5'-, α-, 2'-, and β-protons (see Kallen et al.,[11] Figures 2–13). The positions of some of these peaks are plotted in Figure 6. Using the 4'- and 6-H resonances, K_{pH} was evaluated from the NMR spectra and the points obtained were plotted in Figure 2 together with the curve of K_{pH} vs pH evaluated spectrophotometrically. There is a small systematic difference of ~0.2 logarithmic unit between K_{pH} determined by the spectrophotometric and NMR methods. It may reflect the differences in the concentration range for PLP in the two sets of data.

The ^1H NMR spectra of the 1,3-diaminopropane-containing solutions (Figure 4) are relatively simple. At neutral pH and in the presence of a substantial excess of the diamine, the only major resonances are those of free PLP and of the 1:1 Schiff base. The resonance of the 4'-H of the Schiff base is at 9.05 ppm. The three methylene groups of the diaminopropane moiety display a

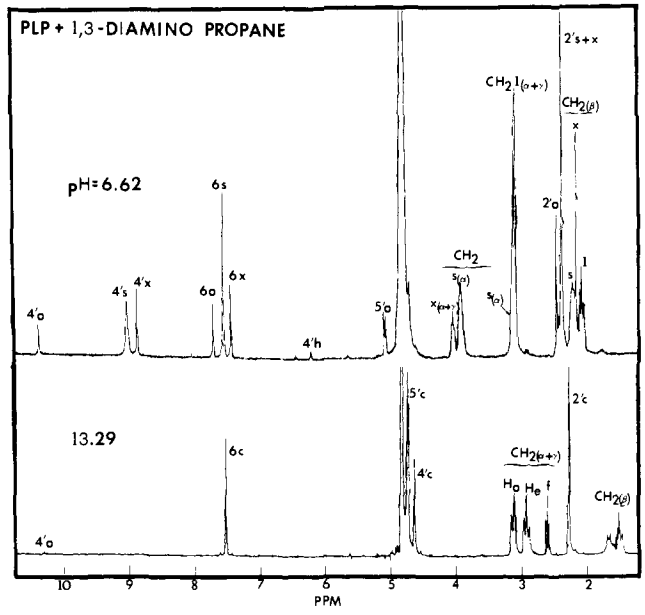


Figure 4. Proton NMR spectra of mixtures of PLP and 1,3-diaminopropane at pH 6.6 and 13.3.

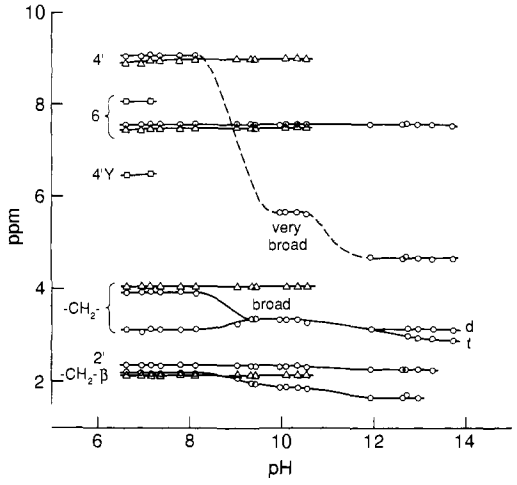


Figure 5. ^1H NMR titration curves for the mixture of PLP plus 1,3-diaminopropane at room temperature. PLP concentrations were usually 0.05 M; O, data for the Schiff base in equilibrium with cyclic forms; Δ, data for the double-headed Schiff base X; □, data for form Y.

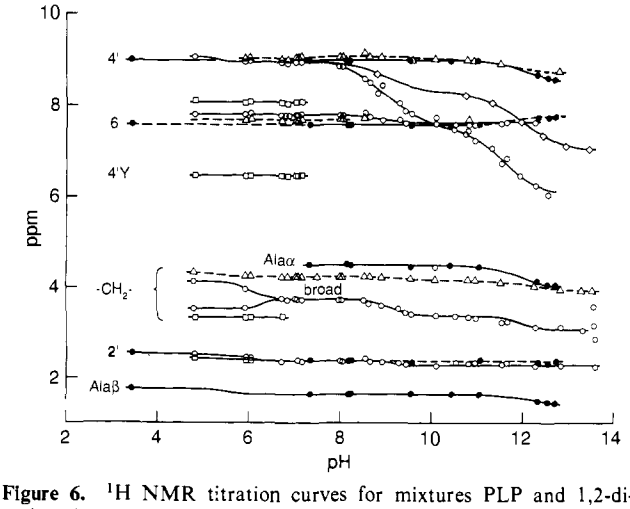


Figure 6. ^1H NMR titration curves for mixtures PLP and 1,2-diaminoethane at room temperature; O, data for the Schiff base in equilibrium with cyclic forms; Δ, data for the double-headed Schiff base X; ◇, data for 80 °C; □, data for form Y. Data for the Schiff base of PLP plus alanine are also shown (●).

Table III. Formation Constants and pK_a Values Found for Schiff Bases

amine + aldehyde (solvent)	log K_f[a]	log K_f'[a]	pK_a				ionic strength	ref
alanine								
+ PLP (H_2O)	0.53	2.50	3.02	5.44	6.57	11.78	0.5	*b*
+ PLP (H_2O)	0.46	2.62		5.38	6.89	11.97	0.2	*c*
+ PLP (H_2O)	0.56	2.59			6.01	11.86	0.2	*c*
+ PLP (D_2O)	0.74	2.45			6.22*	11.67	0.2	*c*
+ 5-deoxypyridoxal (H_2O)	0.79	2.51			6.43	11.55	0.2	*d*
1,2-diaminoethane								
+ PLP (H_2O)	2.30	3.38			8.26	11.37	1.0	*e*
+ PLP (H_2O, 25 °C)	2.10	2.89		6.14	9.61	11.68	1.0	*c*
+ PLP (H_2O, 50 °C)	1.63	2.91			9.01	11.23	1.0	*c*
+ PLP (D_2O)	2.24	3.04		5.05	8.93	11.25	0.2	*c*
+ 5-deoxypyridoxal (H_2O)	2.45	3.64		6.05	8.11	11.20	0.2	*d*
+ salicylaldehyde (H_2O)	1.34	2.37		4.87	8.83	11.04	0.2	*d*
	1.67	2.68			8.70	11.3	0.2	*f*
1,3-diaminopropane								
+ PLP (H_2O)	3.60	3.78		5.55	9.14	11.08		*c*
+ 5-deoxypyridoxal (H_2O)	3.73	3.52		6.24	9.10	10.73		*c*
+ salicylaldehyde	1.91	2.74		4.96	9.25	11.23		*f*
1,4-diaminobutane	2.70	3.27		5.80	10.41	11.71		*c*

[a] K_f = [AmPLP]/[PLP][Am]; log K_f' = log K_f + pK_1 − pK_{1am}; symbols as in eq 1. [b] Reference 23. [c] This work, spectrophotometric. Two independent determinations were made for the alanine system, one with the phosphate pK_a assumed the same as in PLP. [d] Reference 6. [e] Reference 7. [f] Reference 5.

Table IV. Estimated Cyclization and Tautomerization Constants for Schiff Bases of Pyridoxal 5′-Phosphate of 5-Deoxypyridoxal[a]

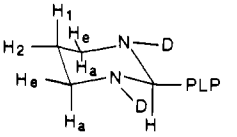
amine	aldehyde	H_3Am-PLP	H_2Am-PLP	HAm-PLP	Am-PLP
alanine[b]	PLP				
K_T		0.1	0.21	0.07	
1,4-diaminobutane[b]	PLP				
K_T		0.15	0.08	0.09	
1,3-diaminopropane[c]	DPL				
K_T		0.18	0.85[d]	1.1	
K_c				5.7	11
1,3-diaminopropane[b]	PLP				
K_T		0.12	0.22	0.33	
K_c				7.0	32
K_c (NMR)				6.1	
1,2-diaminoethane[e]	PLP				
K_T		1.04	4.0	0.22	
K_c				0.77	1.9
1,2-diaminoethane	PLP				
K_T (25 °C)		0.75	2.1[e]	0.15	
K_T (50 °C)			2.2	0.18	
K_c (25 °C)				0.52	1.9
K_c (25 °C)[f]				0.84	1.2
K_c (25 °C; NMR)			<0.01	0.57	1.6
K_c (50 °C)				0.44	1.2
K_c (80 °C; NMR)				0.19	0.67

[a] K_T and K_c are defined by eq 3 and 4, respectively. We estimate the experimental error in most constants as no more than ±20% relative error. Unless indicated otherwise, the results are based on spectrophotometry. [b] As in previous studies,[6] we interpreted a peak band at ~340 nm to be a dipolar ionic tautomer. This was estimated to contribute 9% and 11%, respectively, to the H_2AmPLP and HAmPLP forms of the alanine Schiff base and 6% and 11%, respectively, to the corresponding forms of the diaminobutane Schiff base. An 8% contribution to the H_2AmPLP form of the diaminopropane Schiff base was assumed. [c] DPL, 5-deoxypyridoxal. Data from Metzler et al.[6] [d] The high estimate of K_T is uncertain because the intense enolimine band is not in a predictable position. It could represent a mixture of species. [e] This value could be as low as 1.6 if there is 16% carbinolamine present. [f] Reference 7.

characteristic pattern. The one closest to the Schiff base linkage is at 3.9 ppm, the one by the NH_3^+ group at 3.1 ppm, and the center one at 2.2 ppm. They are broader than the corresponding resonance of the α-proton of the PLP–alanine Schiff base at 3.76 ppm. When PLP is present in excess, another set of peaks, all of which are sharp, appears. These are designated x in Figure 4. By varying the ratios of PLP to diamine, it was established that the peaks x belong to a 2:1 $Am(PLP)_2$ compound. The positions of its 4′ and 6′ resonances are nearly identical with those of the alanine Schiff base (see Figure 6). These facts establish the 2:1 compound as a "double-headed" Schiff base with a PLP molecule at each end of the diamine.

In the pH range 12–13 the PLP–diaminopropane Schiff base exists mostly as the cyclic diamine. This is seen clearly in the electronic spectrum of the AmPLP form which has a weak Schiff base absorption band at ~341 nm and a very strong band of the geminal diamine at 310 nm. From the areas of the bands, the cyclization constant K_c (eq 4) was estimated as ~32 at 25 °C. The HAmPLP form is also mostly cyclic as judged by its spectrum, but the amount of Schiff base has increased to ~16% and K_c' is ~5.3.

The ^{1}H NMR data (Figure 4) are in agreement with this interpretation. At pH 12–13, where the AmPLP form predominates, the 4′-proton appears as a sharp singlet at 4.63 ppm and the methylene groups of the diamine as a doublet centered at 3.11 ppm, a triplet centered at 2.90 ppm, and multiplets centered at 1.47 and 1.64 ppm. The geminal diamine formed from diaminopropane contains a six-membered ring which can assume a chair conformation having the 4′-proton in the axial position



and the PLP ring in an equatorial position. The splitting pattern that is observed results from the nonequivalency of the axial and equatorial protons on C-1 and C-3 of diaminopropane with respect to the methylene protons located at C-2. The equatorial protons labeled H_e are split by H_1 and H_2, which are felt as equivalent protons, and thus a triplet is produced. The axial protons labeled H_a are split into a doublet, since H_1 and H_2 are felt by H_a as nonequivalent. No Schiff base resonances were detectable at pH 13, suggesting that the small amount of Schiff base present is in rapid equilibrium with geminal diamine.

As the pH of a solution of PLP and diaminopropane is increased from 9 to 12 the resonance of the 4′-H band shifts rapidly from 9.03 to 4.65 ppm (Figure 5). At intermediate pH values the signal cannot be detected except in the pH range 10–10.5 where it appears as a very broad band centered at ~5.65 ppm. In the same region the β-methylene carbons are represented by a single broad band centered at 3.35 ppm. If we assume that we have a mixture of cyclic and Schiff base species in rapid equilibrium, we can estimate the relative amounts as follows.

The chemical shift of the 4′-H of pyridoxamine phosphate (PMP) is observed to vary with the state of protonation of the molecule:

$$\underset{\text{PMP}}{3.70} \xrightarrow{H^+} \underset{\text{HPMP}}{4.28} \xrightarrow{H^+} \underset{H_2\text{PMP}}{4.34} \xrightarrow{H^+} \underset{H_3\text{PMP}}{4.45\ \text{ppm}}$$

The first protonation is primarily that of the primary amino group and the second that of the ring nitrogen. The same sequence of protonation steps is expected for the cyclic geminal diamine. From the observed chemical shift (4.63 ppm) of the 4'-proton at high pH, the value of $K_c = 32$, and the chemical shift of 8.53 ppm of the alanine Schiff base at high pH, we estimate that pure cyclic form C at high pH would have a 4'-H chemical shift of 4.51 ppm. Assuming that this chemical shift changes with state of protonation as in PMP, we predict

$$\underset{4.51}{C} \xrightarrow{H^+} \underset{5.09}{HC} \xrightarrow{H^+} \underset{5.15}{H_2C}\ \text{ppm}$$

(C = cyclic form with diaminopropane)

For the Schiff base of alanine and PLP we observe a chemical shift of ~8.96 ppm at a pH below ~11. Using this value for the Schiff base of PLP + diaminopropane, we estimate the fraction of cyclic form at pH 10–10.5 as (8.96 − 5.65)/(8.96 − 5.09) = 0.86. This is in agreement with the value 0.84 obtained by spectrophotometry (Table IV). In the same pH range we can estimate from the observed coalescence of the resonance of the Schiff base and cyclic adduct, which are ~3.8 apart, that the rate of interconversion must be at least 300 ($3.8\ \pi/2^{1/2}$) ≈ 2500 s^{-1}.

At pH 7.5–8 the UV-visible spectrum contains a peak at ~335 nm that was attributed by Metzler et al.[6] to the enolimine tautomer of the Schiff base. That this conclusion is correct is indicated by the fact that the chemical shift of the 4'-H is 9.05 ppm. Since at this pH the cyclic form might be expected to have a chemical shift of ~5.2 ppm, if it were present in an amount equal to 15% of the total, we would expect to see a chemical shift closer to 8.4 ppm.

The 1,2-diaminoethane–PLP system is the most difficult to interpret. At a high ratio of diamine to PLP, the UV-visible spectrum of the high-pH form AmPLP (Figure 1) shows that cyclization occurs, but to a smaller degree ($K_c = 1.9$) than with diaminopropane. However, the mono-, di-, and triprotonated forms show much *larger* absorption bands in the 305–335-nm region than with diaminopropane. These results are in agreement with those reported previously.[5-7] About the same relative amounts of the two Schiff base tautomers and of geminal diamine C appear to be formed from PLP (Figure 1) and from 5-deoxypyridoxal,[6] judging by the intensities of the absorption bands.

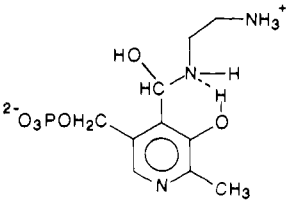
The NMR spectra of PLP–diaminoethane mixtures at low pH (Figure 6) are similar to those with diaminopropane. The 4'-H chemical shift falls to ~6.05 ppm at high pH. From the ultraviolet–visible spectrum (and ignoring the minor 381-nm band) there is about 66% cyclic form and 34% Schiff base ($K_c = 1.9$). This predicts a chemical shift of 4.77 ppm for the pure cyclic form, assuming a rapid equilibrium. In fact, at a very high pH of 13.9 separate 4'-H resonances for Schiff base and cyclic form are seen at 8.6 and 4.5 ppm, respectively. The latter is the same as the value we estimated for the geminal diamine of diaminopropane. Assuming that the 4'-H chemical shifts for the HAmPLP and H_2AmPLP forms are also the same for both geminal diamines, we can use the values estimated in the preceding discussion (4.51, 5.09, and 5.15 ppm for the three cyclic species) to estimate cyclization constants. From the observed chemical shifts of 6.05 and 7.55 ppm at pH ~13 and 10.5, respectively, we estimate K_c and K_c' as 1.6 and 0.57, respectively. The latter is in agreement with the estimate from the electronic spectrum of the HAmPLP form (Figure 2) of 31% cyclic form (from the area of its band centered at 310 nm) and $K_c' = 0.52$. It was estimated that 9% was enolimine tautomer absorbing at 339 nm. We are unable to judge the amount of cyclization in the H_2AmPLP form by spectrophotometry, but from the NMR data we conclude that it is very small. The large 329-nm band must be the enolimine tautomer. The relative amount of cyclic form decreases as the temperature is increased (Table IV). The effect on these ratios of changing the solvent from H_2O to D_2O is minor. All band positions are shifted, at most about 100–400 cm^{-1} to a lower energy in D_2O.

When possible (in the lower and higher pH regions), the peaks in the NMR spectra were integrated and the resultant data were used to evaluate formation constants according to eq 5. The latter values for the diaminoethane–PLP system are plotted in Figure 3 together with the spectrophotometric data and with some data from the literature. The 6-H resonances, which are well separated, were especially favorable for this purpose. Some 4'-H resonances were used in the lower pH range. When significant amounts of the double-headed Schiff base $Am(PLP)_2$ were present, values of the pH-dependent constant K_{2pH} (eq 6), which is related to K_{2f} of eq 2, were also calculated from the NMR data and are plotted in Figure 3. A similar plot for the diaminopropane–PLP system is included in the Supplementary Material.

$$K_{2pH} = [Am(PLP)_2]_{total}/[PLP]_{total}[AmPLP]_{total} \quad (6)$$

The rate of exchange between the Schiff base of diaminoethane and cyclic forms was calculated from the NMR spectra by utilizing the fact that the line width of the 4'-proton is broadened by exchange. A two-site exchange program, based on the original work of Gutowsky and Holm[17] and run on a Nicolet 1280 computer, was used to calculate the rate from observed line widths and inferred positions for Schiff base and cyclic forms in the absence of exchange. These positions were estimated for the cyclic form in the preceding section and for the Schiff base by comparison with that of alanine (Figure 6). A line width of 0.2 Hz was observed for non-exchange-broadened peaks. The calculated exchange rate varies with pH and temperature. Interconversion at room temperature reaches a maximum rate of 100 000 s^{-1} near pH 10 and decreases to ~20 000 s^{-1} at pH 7 and 12. Above pH 12 the peak broadens greatly as the rate decreases further. At pH 13.9 there is essentiallly no exchange and separate resonances are seen for Schiff base and cyclic forms. At 80 °C the maximum exchange rate is 220 000 s^{-1} near pH 10.5. Plotting ln (exchange rate) vs temperature yields a straight line from 275 to 315 K with an activation energy of 6.8 kcal/mol. Above this temperature the rate does not increase. The line width exhibits a strong field dependence, going from 3.8 Hz at 2.4 tesla to 32 Hz at 7.2 tesla.

At high ratios of PLP to diaminoethane a set of sharp NMR resonances representing the $Am(PLP)_2$ species X appears. In addition, at pH <7.3, there is a new set Y which contains sharp peaks at 8.05 and 6.45 ppm. As the pH is decreased the relative amount of Y increases while that of X decreases. The presence of the peak at 6.45 ppm near that of the hydrate of PLP suggests that Y is an adduct to the C=N double bond. There is no corresponding peak in the NMR spectra of the system (PLP + alanine) and only a small amount in the system (PLP + 1,3-diaminopropane, Figure 5). Thus, there may be some special stabilization of compound Y with diaminoethane. We established that the ratio of Y to Schiff base is nearly constant in the pH region 6–7: [Y]/[H_2AmPLP1] ≈ 0.19. We suggest that compound Y may be the following carbinolamine:



carbinolamine structure proposed for compound Y

This would be expected to absorb at ~295 nm and to account for ~7% of the 329-nm absorption band of species HAmPLP shown in Figure 1. It is significant that this absorption band is unusually wide (4.76×10^3 cm^{-1}), suggesting that it may represent two components.

It is hard to predict exactly where the 4'-H resonance of this carbinolamine should come. However, Kobayashi and Makino[21]

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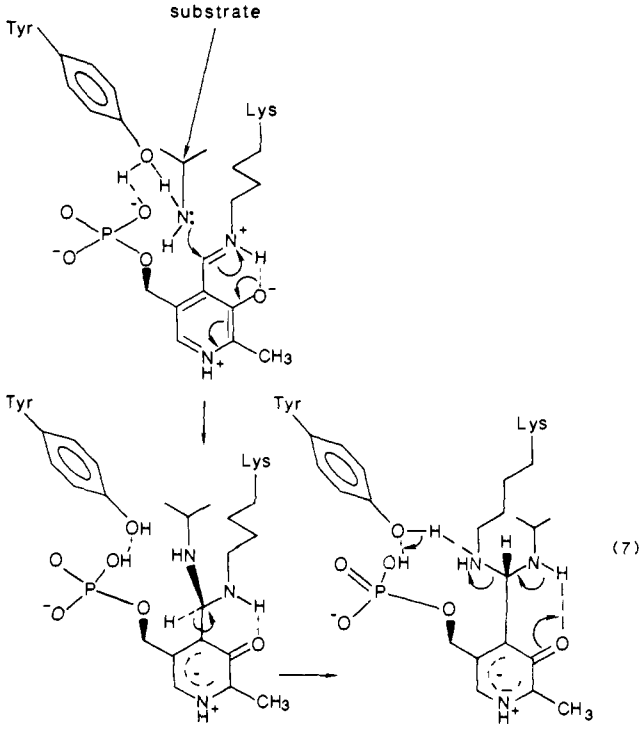
reported (and it has been confirmed)[22] that the Schiff base of tris(hydroxymethyl)aminomethane and PLP at high pH shows a strong 4′ resonance at 6.22 ppm. This is presumably a carbinolamine. The position at 6.45 ppm for Y in a lower pH range seems reasonable. The position of the 6-H resonance at 8.05 ppm suggests that the phenolic oxygen is protonated as shown in the drawing.

Discussion

The cyclization constants for the unprotonated and monoprotonated Schiff bases of diaminopropane are estimated as 32 and 5.3, respectively, and those for the corresponding Schiff bases of diaminoethane as 1.6–1.9 and 0.45–0.6, respectively. The NMR data suggest little or no cyclization of the more highly protonated species. The greater stability of six-membered rings as compared with those containing five atoms explains the difference between cyclization constants of the two diamines. Diaminobutane, which could form a seven-membered cyclic geminal diamine, yields Schiff base species that undergo little or no cyclization. The diprotonated species of Schiff base also appear not to cyclize to a measurable extent. This may reflect the fact that the corresponding cyclic form would be protonated either on both of the closely spaced geminal diamine nitrogen atoms or on one of these atoms and on the pyridine ring nitrogen. Either arrangement may be unstable. For example, protonation on the ring nitrogen would withdraw electrons from the phenolate oxygen into the ring, weakening the hydrogen bond to the protonated geminal diamine nitrogen.

The low stability of the protonated cyclic geminal diamine relative to its Schiff base may be of biochemical significance. Most PLP-dependent enzymes act via a transimination reaction in which the amine substrate adds to a Schiff base of the PLP with a lysine side chain of the protein. The initial step, illustrated here for aspartate aminotransferase, is undoubtedly the addition of an unprotonated NH_2 group to the N-protonated imine group (eq 7). The formation of the geminal diamine would be facilitated



(7)

by the indicated synchronous removal of a proton from the entering amino group to give the unprotonated form of the geminal diamine shown in eq 7. The resulting unprotonated geminal diamine group may be able to undergo easier rotation to bring the amino group of the substrate into a position coplanar with the pyridine ring than if one nitrogen remained protonated.

It has been proposed[23,24] that the phosphate group of PLP participates in a proton shuttle mechanism during transimination. Because of its position in aspartate aminotransferases, the phenolic group of tyrosine 70 may also participate as is indicated in eq 7. The fact that a mutant with tyrosine 70 replaced by phenylalanine retains some catalytic activity[25] does not invalidate the idea because a water molecule might be able to fit in and substitute for the phenolic group. Reversal of the postulated shuttle would allow protonation of the lysine nitrogen. This would facilitate its elimination to form the substrate Schiff base. This shuttle mechanism would account for the fact that analogues of PLP with a single negative charge on the side chain, e.g., the phosphate monomethyl ester of PLP, are catalytically inactive with some enzymes and do not undergo transimination.[26-29]

The present results support the proposal that the forms of the Schiff base of 1,2-diaminoethane and PLP with an N-protonated free amino group tend to tautomerize to the enolimine. The indicated hydrogen bonding (see preceding structural formula suggested for the H_2AmPLP form) presumably competes with hydrogen bonding with the phenolic OH and tautomerization of the latter to the ketoenamine form. If the chemical shifts of the 4′-H of the two tautomers are slightly different, a relatively slow rate of exchange between them could account for some of the observed broadening of the 4′-H resonance in both the diaminoethane and diaminopropane Schiff bases in the low-pH range. Thus, the peak marked 4′s in Figure 4 is distinctly broader than 4′x, the corresponding peak for the double-headed Schiff base, or than the peak for the alanine Schiff base. However, broadening may also result from a small amount of cyclization, as proposed for the high-pH range. Ketoenamine–enolimine tautomerization may conceivably have a significance in enzymology. However, in the case of aspartate aminotransferase the enzyme effectively locks the coenzyme ring into a dipolar ionic state preventing O-protonation of the form of the coenzyme that reacts with N-protonated amino acid substrates.

The rates of interconversion of Schiff base and cyclic species at pH 10 are remarkably rapid. This is in agreement with the previous results of Tobias and Kallen,[7] who pointed out that the nonenzymic transimination reaction occurs at rates greater than those of overall enzymic reactions.

Acknowledgment. We thank William A. Le Clair for computer programming.

Registry No. Pyridoxal 5′-phosphate, 54-47-7; 1,2-diaminoethane, 107-15-3; 1,3-diaminopropane, 109-76-2; 1,4-diaminobutane, 110-60-1; alanine, 56-41-7.

Supplementary Material Available: Additional experimental information including compositions of solutions studied, an extended version of Table I, a table of band-shape parameters for spectra resolved with log normal curves and estimated fractions of various species together with explanations of calculations, and figures showing typical comparison plots, spectra of individual ionic forms of diaminopropane–PLP Schiff base, NMR spectra, and a plot of log K_{pH} for the diaminopropane system (18 pages). Ordering information is given on any current masthead page.

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