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NMR Investigation of Protonation Sites of *N,N'*-Dipyridoxylethylenediamine-*N,N'*-diacetic Acid and Coordinate Bonding in Its Metal Chelates

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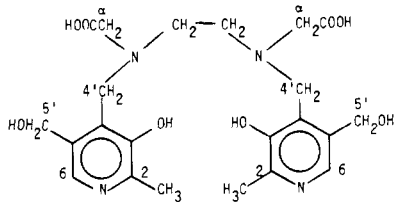
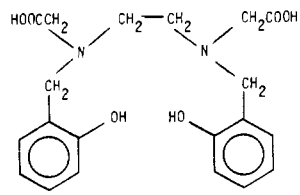
^{1}H NMR chemical shifts of *N,N'*-dipyridoxylethylenediamine-*N,N'*-diacetic acid (PLED) as a function of -log [H^+] are employed to assign the sequence of protonation sites for the eight basic donor groups of the ligand and to identify the donor groups coordinated to the diamagnetic metal ions Zn(II), Ga(III), and In(III). The pyridine nitrogens are found to be the most basic groups in the ligand, remain protonated in the metal chelates up to moderately high values of -log [H^+], and are not directly coordinated to the metal ions.

Introduction

A potentiometric study of a new chelating agent, *N,N'*-dipyridoxylethylenediamine-*N,N'*-diacetic acid (PLED, **1**), has recently been reported.[2] In this ligand, the *o*-hydroxybenzyl groups of *N,N'*-bis(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid (HBED, **2**) are replaced by pyridoxyl groups, in order



PLED, H_4L, **1**



HBED, H_4L, **2**

to increase solubility and lower the toxicity of the ligand for its potential application as a radiopharmaceutical involving Ga(III) and In(III) and as a sequestering drug for Fe(III) for the treatment of iron-overload anemias. The pyridoxyl moieties have phenolic donor groups similar to those of HBED, modified by the electron-withdrawal effect of the aromatic heterocyclic nitrogens and by the methyl and hydroxymethyl substituents in the pyridine rings. In the previous study of PLED,[2] it was shown that the metal complexes formed are somewhat less stable than those of HBED and differ from those of the latter ligand in the formation of protonated chelates that are stable to surprisingly high values of -log [H^+]. The purpose of the present investigation is to determine the relative basicities of the ligand donor groups and the nature of the protonated species formed in the presence and absence of metal ions.

The application of proton magnetic resonance to the determination of the protonation sites and conformations of amino polycarboxylic acid complexes in aqueous solutions have been investigated extensively by Kostromina,[3] Letkeman and Martell,[4] and others.[5-9] Proton magnetic resonance has also been used by Gansow and Holm[10] to study equilibria and solute structures in aqueous systems of pyridoxal-alanine-Zn(II) and -Al(III). Gansow and Holm[11] have also reported a proton magnetic resonance study of pyridoxamine-pyruvate-Zn(II) and -Al(III) systems.

Experimental Section

Materials. The ligand, PLED, was synthesized by Taylor[12] in this laboratory. The elemental analysis was determined by Galbraith Laboratories, Inc., Knoxville, TN.

Anal. Calcd for $C_{22}H_{30}N_4O_8 \cdot 3.60HCl \cdot 1.43NH_4Cl \cdot 0.61NH_4Br \cdot 4H_2O$: C, 32.30; N, 10.34; H, 6.13; O, 23.47, Cl, 21.80; Br, 5.96; Na, traces. Found: C, 32.43; N, 10.44; H, 5.78; O, 23.58; Cl, 21.88; Br, 6.14; Na, <0.03.

A stock solution of about 0.50 M reagent grade zinc(II) chloride was prepared in D_2O (99.8% D) for NMR measurements. The stock solution of 0.050 M Ga(III) for NMR studies was prepared according to the procedure previously described.[13] However, HCl was removed by the addition of DCl and repeated evaporation nearly to dryness. The final solution was prepared in D_2O.

Sample solutions for NMR measurements on the ligand were prepared by weighing out the appropriate amount of free ligand for a 0.10 M solution having a volume of 1.00 mL. The ionic strength was brought to 1.00 M by the addition of KCl. For the study by NMR of systems having a 1:1 molar ratio of metal ion to ligand, about 0.20 mL of the 0.50 M metal ion stock solution was added to about 0.80 mL of the ligand solution (calculated to be 0.10 M in the final solution of 1.00 mL) and the ionic strength was brought to 1.0 M by the addition of KCl.

Standard base solution for use in NMR measurements was prepared from Aldrich Dilut-It potassium hydroxide concentrate under CO_2-free conditions, and the 1.0 M solution was standardized against reagent grade KHP. The KOH solution was stored in a glass bottle protected against the atmosphere with a tube of Ascarite. As an additional check, Gran's plots[14] were carried out to ensure that the base was not contaminated by CO_2. Standard acid solution was prepared from concentrated HCl, and the solution was standardized against the standard KOH solution.

Nuclear Magnetic Resonance Measurements. Nuclear magnetic resonance spectra were recorded on a Varian EM-390 instrument operated at 35.0 ± 0.5 °C.

Measurement of -log [H^+]. For the NMR experiments, the pDs of the 1-mL-sample solutions were measured with a combination microelectrode. A Corning digital pH meter was calibrated with standard aqueous HCl and KOH solutions to read -log [H^+] directly rather than activity. After calibration of the pH meter, the microelectrode was soaked in D_2O until the pH meter reading was constant and then it was immersed in the sample solution being studied by NMR.

Glasoe and Long[15] found that when a pH meter with a glass and calomel electrode combination is calibrated with standard solutions in

(1) Abstracted in part from a dissertation submitted by C. H. Taliaferro to the faculty of Texas A&M University in partial fulfillment of the requirements of the degree of Doctor of Philosophy.
(2) Taliaferro, C. H.; Motekaitis, R. J.; Martell, A. E. *Inorg. Chem.* **1984**, *23*, 1188.
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(12) Taylor, P.; Stoldt, R.; Ng, C. Y.; Samuel, S. P.; Martell, A. E.; Murase, I.; Szaboky, G., to be submitted for publication.
(13) Harris, W. R.; Martell, A. E. *Inorg. Chem.* **1976**, *15*, 713.
(14) Rossotti, F. J. C.; Rossotti, H. J. *J. Chem. Educ.* **1965**, *42*, 375.
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Table I. Protonation Constants and Metal Chelate Stability Constants of N,N'-Dipyridoxylethylenediamine-N,N'-diacetic Acid (PLED) (t = 15.00 °C; μ = 0.100 M (KCl))

equil quotient	log K PLED[a]	log K HBED[b]	equil quotient	log K PLED[a]	log K HBED[b]	equil quotient	log K PLED[a]	log K HBED[b]
[HL]/[L][H]	11.09	12.60[a]	[NiL]/[Ni][L]	18.88	19.31	[FeL]/[Fe][L]	36.88	39.68
$[H_2L]/[HL][H]$	10.68	11.00[a]	[NiHL]/[NiL][H]	9.23	8.51	[FeHL]/[Fe][H]	7.49	
$[H_3L]/[H_2L][H]$	9.52	8.44[a]	$[NiH_2L]/[NiHL][H]$	8.40	6.45	$[FeH_2L]/[FeHL][H]$	6.59	
$[H_4L]/[H_3L][H]$	7.20	4.72[a]	[CoL]/[Co][L]	16.82	19.89	[InL]/[In][L]	36.86	39.66[a]
$[H_5L]/[H_4L][H]$	3.31	2.53[a]	[CoHL]/[CoL][H]	9.29	7.77	[InHL]/[InL][H]	7.96	
$[H_6L]/[H_5L][H]$	2.57	1.74[a]	$[CoH_2L]/[CoHL][H]$	8.46	5.56	$[InH_2L]/[InHL][H]$	6.68	
[CuL]/[Cu][L]	19.87	23.69[a]	[ZnL]/[Zn][L]	16.56	18.37	[GaL]/[Ga][H]	36.31	39.57[b]
[CuHL]/[CuL][H]	8.81	8.49[a]	[ZnHL]/[ZnL][H]	8.85	8.27	[GaHL]/[GaHL][H]	7.31	
$[CuH_2L]/[CuHL][H]$	7.98	5.04[a]	$[ZnH_2L]/[ZnHL][H]$	8.22	5.99	$[GaH_2L]/[GaHL][H]$	6.38	

[a] This work. [b] Reference 13.

H_2O and is then used for the measurement of the pD of solutions of known KCl (molar) concentration in pure D_2O, a constant increment of +0.40 pH unit is observed at 25 °C.

Numerous subsequent investigations[16] have demonstrated the essential correctness of the value of 0.40 (±0.02 pH unit) and that it holds for different electrolytes at various pHs and temperatures as well as for different commercial glass electrodes from different manufacturers.

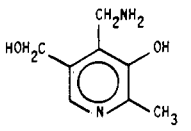
Results and Discussion

The proton, metal ion, and mixed proton–metal ion association constants of the PLED anion (L^{4-}) have been determined by potentiometric and spectrophotometric measurements and have been reported recently.[2] The equilibrium data are summarized in Table I, along with analogous data for HBED, in order that the coordination behavior of these two ligands may be compared and contrasted in the light of new microscopic information provided by NMR data.

The main difference between PLED and HBED is that PLED has two aromatic nitrogens. This result in an overall basicity of the ligand ($\sum \log K^H = 44$) that is distinctly higher than that of its analogue ($\sum \log K^H = 41$ for HBED).

If one compares the structures of PLED and HBED, one would predict that the electron-withdrawing effects of the pyridine nitrogens in PLED would lower the basicities of the aliphatic nitrogens somewhat relative to those of HBED. The same effects should have a greater influence on the basicities of the phenolate oxygens and lower considerably their hydrogen-bonding tendencies. Although the first protonation constant of PLED is considerably lower than that of HBED, the second protonation constant is just slightly lower. The third and fourth protonation constants of PLED are considerably higher than those of HBED. These observations seems to contradict what one would expect on the basis of the electron-withdrawing effects of the pyridine nitrogens in PLED.

Thus, in order to assign the calculated protonation constants to the individual donor groups of PLED, nuclear magnetic resonance studies have been carried out and the results compared to those for pyridoxamine (**3**) rather than to those for the phenolic analogue of PLED.



3 Pyridoxamine, HL

Application of proton magnetic resonance to the determination of protonation sites and conformations of amino polycarboxylic acid compounds in aqueous solutions have been investigated extensively.[5-11] If a proton combines with a ligand that has several basic groups, microequilibria are established in solution that include combinations of the protonated and nonprotonated forms. Although in principle all possible species may be considered, in most cases a relatively small number of forms make the major contributions to the system. Thus, following the addition of 1 equiv of acid, each functional group of the ligand may be protonated to a specific degree. This causes the deshielding of the covalently bound hydrogens in the immediate vicinity of the protonated functional group and a shift of the corresponding NMR signals to lower field. The magnitude of the shift depends on the nature of the electron-donating atom, i.e. on the degree of protonation of each group. In the pD range where signal shifts are observed, there is a mixture of protonated and dissociated forms. The absence of signal splitting shows that rapid proton exchange takes place between these forms.

The position of the signal is defined by

$$\delta_{av} = N_a\delta_A + N_B\delta_B \quad (1)$$

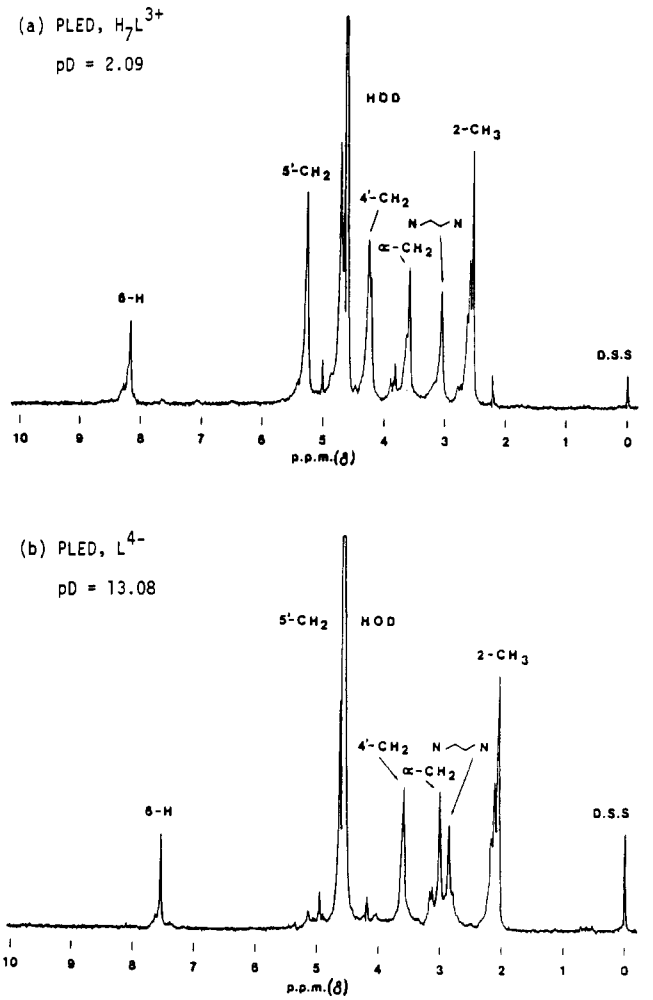


Figure 1. Proton magnetic resonance spectra (90 MHz) of 0.10 M PLED solutions in D_2O at pD 2.09 (a) and 13.08 (b) (μ = 1.00 M (completed with KCl); t = 35.0 °C).

(16) Covington, A. K.; Paabo, M.; Robinson, R. A.; Bates, R. G. *Anal. Chem.* **1968**, *40*, 700.

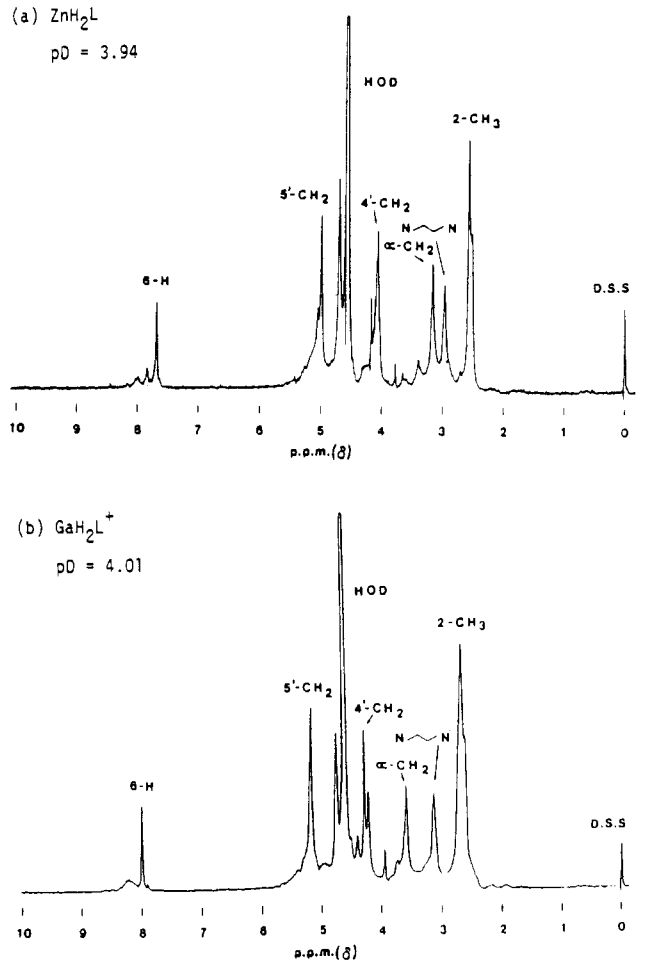


Figure 2. Proton magnetic resonance spectra (90 MHz) of 0.10 M PLED (L) solutions in D_2O, in the presence of 1:1 molar ratios of metal ion to ligand (μ = 1.00 M (completed with KCl); t = 35.0 °C): (a) ZnH_2L at pD 3.94; (b) GaH_2L at pD 4.01.

where N_A, N_B, δ_A, and δ_B are respectively the mole fractions (N) and the chemical shifts (δ) of the protonated (A) and nonprotonated (B) forms. At the point of inflection (midpoint of buffer region) where $\delta_{av} = (\delta_A + \delta_B)/2$, the concentrations of the protonated and nonprotonated forms are equal. It follows from the dissociation equation that

$$K = N_B[D^+]/N_A \quad (2)$$

When $N_A = N_B$, the dissociation constant is equal to the deuterium ion concentration.

The ^1H NMR spectra of 0.10 M PLED–D_2O solutions and those of its Zn(II) and Ga(III) chelates are shown in Figures 1 and 2. Spectrum a was measured at pD 2.09. According to the potentiometric results, at this pD value only the carboxylate groups may possibly be deprotonated. But the upfield chemical shift of 0.10 ppm of the ethylenic protons between pD 2 and 3, shown in Figure 3, suggests that one of the two carboxylate groups is still protonated at pD 2 and that its deprotonation is only complete at pD 3. If the pK_D of this carboxylate group is about 2, the corresponding pK_H would be around 1.6 and therefore could not be calculated potentiometrically. In EDTA, the deprotonation of the carboxylate groups causes a downfield chemical shift of 0.20 ppm.[8] It is well-known that the carbonyl group has both a negative and a positive long-range shielding effect depending on the orientation and distance that a hydrogen atom is separated from the carbonyl carbon atom.[17] It has been shown that the positive shielding region of a carbonyl group is close to the x axis

(17) Jackman, L. M.; Sternhell, S. "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry", 2nd ed.; Pergamon Press: New York, 1969.

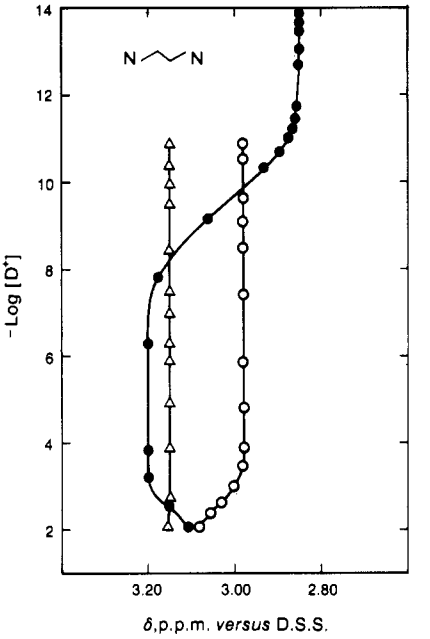


Figure 3. pD dependencies of the chemical shifts of the ethylenic protons (^1H NMR, 90 MHz) of a 1.10 M PLED–D_2O solution in the absence (●) and presence of 1:1 molar ratios of metal ion to ligand (○, Zn–PLED chelate; △, Ga–PLED chelate) (μ = 1.00 M (compted with KCl); t = 35.0 °C).

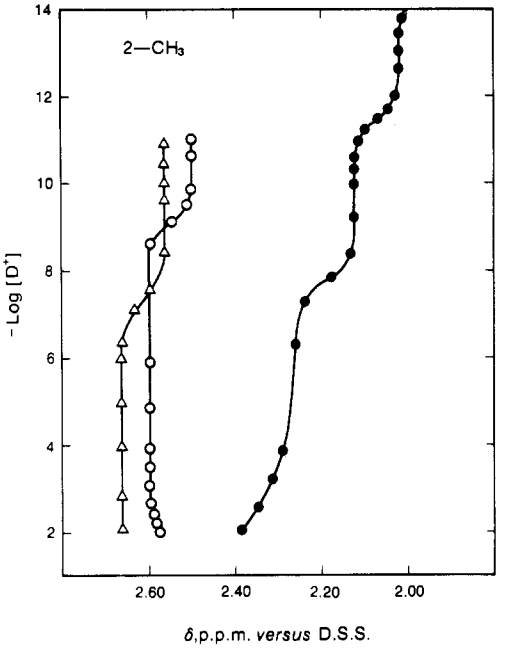


Figure 4. pD dependencies of the 2-CH_3 chemical shifts (^1H NMR, 90 MHz) of a 0.10 M PLED–D_2O solution in the absence (●) and presence of 1:1 molar ratios of metal ion to ligand (○, Zn–PLED chelate; △ Ga–PLED chelate) (μ = 1.00 M (completed with KCl); t = 35.0 °C).

at the "carbon end" of the bond and that above and below the plane of the bond, in the vicinity of the z axis, shielding is positive as well. In the case of EDTA, it has been postulated[11] that when a protonated carboxyl group swings away from the protonated nitrogen end of the molecule, it will spend most its time near the ethylene backbone of the molecule. This behavior then will introduce a measure of shielding for the ethylenic protons, which in turn exhibit a chemical shift upfield under these conditions. Thus, it is suggested that the protonation constants of the two carboxylate groups in PLED have log K_a values lower than 1.6, as is the case for EDTA.

The ^1H NMR spectrum of PLED shown in Figure 1b was measured at pD 13.08. According to the potentiometric results,

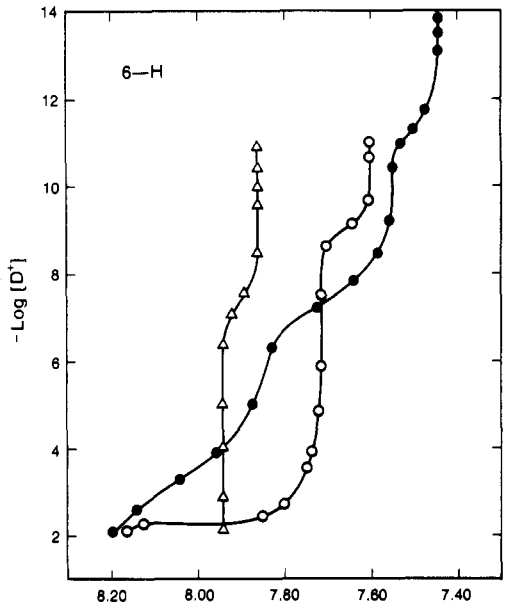


Figure 5. pD dependencies of the 6-H chemical shifts (^{1}H NMR, 90 MHz) of a 0.10 M PLED–D_2O solution in the absence (●) and presence of 1:1 molar ratios of metal ion to ligand (○, Zn–PLED chelate; △ Ga–PLED chelate) (μ = 1.00 M (completed with KCl); t = 35.0 °C).

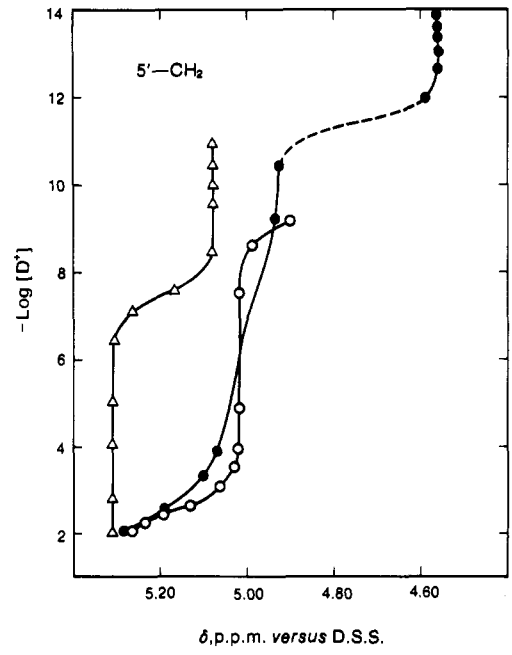


Figure 6. pD dependencies of the 5'-CH_2 chemical shifts (^{1}H NMR, 90 MHz) of a 0.10 M PLED–D_2O solution in the absence (●) and presence of 1:1 molar ratios of metal ion to ligand (○, Zn–PLED chelate; △ Ga–PLED chelate) (μ = 1.00 M (completed with KCl); t = 35.0 °C).

at this pD value the ligand is completely deprotonated. In order to elaborate a protonation scheme for this ligand, Figures 4–9 have been analyzed and the results have been compared to the protonation scheme of pyridoxamine suggested by Gansow and Holm.[18]

As seen in Figures 4–7, the third and fourth deprotonations produce a marked change in the chemical shifts of all ring substituents. The increased shielding of ring substituents indicates a net increase of negative charge on the ring in this process. The points of inflection correlate well with the logarithms of the protonation constants determined potentiometrically (2.57 and

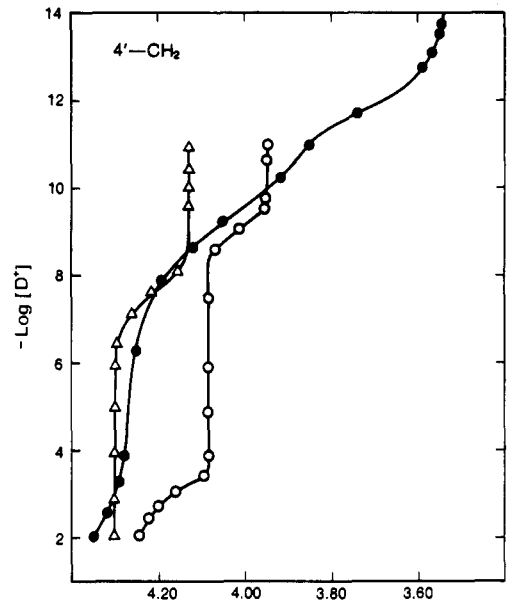
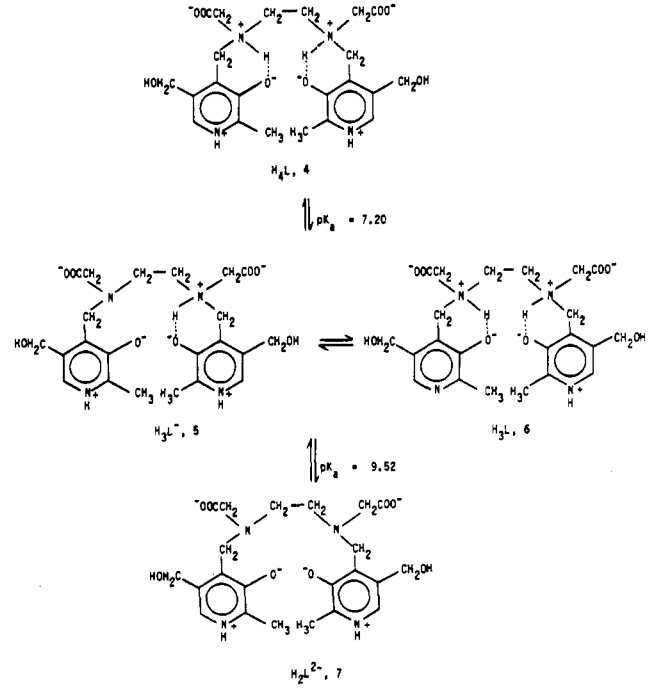


Figure 7. pD dependencies of the 4'-CH_2 chemical shifts (^{1}H NMR, 90 MHz) of a 0.10 M PLED–D_2O solution in the absence (●) and presence of 1:1 molar ratios of metal ion to ligand (○, Zn–PLED chelate; △ Ga–PLED chelate) (μ = 1.00 M (completed with KCl); t = 35.0 °C).

3.31),[2] if one takes into account the conversion of pK_H to pK_D, the change in temperature from 25 °C (potentiometric experiment) to 35 °C (NMR measurements), and the change in ionic strength from 0.10 to 1.0 M KCl. These observations are consistent with the loss of phenolic or pyridinium[19] protons in these two steps. When taken in conjunction with ultraviolet spectral observations of pyridoxamine,[20] the chemical shift data can be interpreted satisfactorily in terms of the loss of the two phenolic protons in these two steps. This product is represented by formula **4**. In



this structure, the negative charge on the oxygen atom is stabilized by hydrogen bonding with the protonated amino group and by the positive group on the pyridine nitrogen. The third and fourth pK's of PLED (2.57 and 3.31) correlate well with the first pK

(18) Gansow, O. A.; Holm, R. H. *Tetrahedron* **1968**, *24*, 4477.

(19) Gil, V. M. S.; Murrell, J. N. *Trans. Faraday Soc.* **1964**, *60*, 248.
(20) Matsushima, Y.; Martell, A. E. *J. Am. Chem. Soc.* **1967**, *89*, 1322.

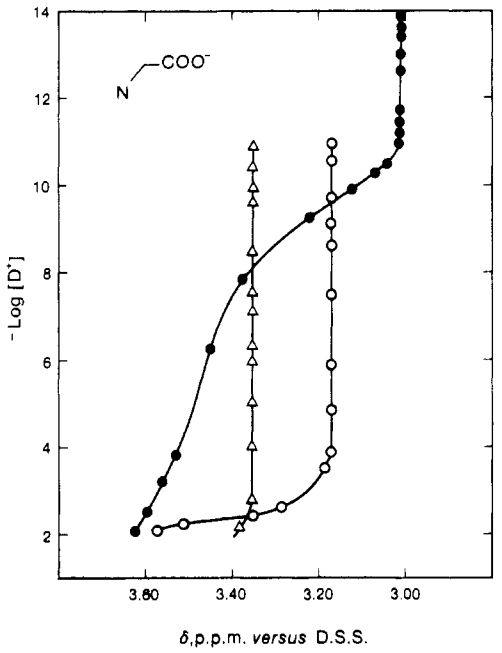


Figure 8. pD dependencies of the α-CH_2 chemical shifts (^1H NMR, 90 MHz) of a 0.10 M PLED–D_2O solution in the absence (●) and presence of 1:1 molar ratios of metal ion to ligand (○, Zn–PLED chelate; △ Ga–PLED chelate) (μ = 1.00 M (completed with KCl); t = 35.0 °C).

of pyridoxamine and are therefore assigned to the phenolic groups.

As seen in Figures 3–8, the fifth deprotonation produces a small chemical shift of the 5′-CH_2 protons, but it produces marked chemical shifts in all other ring substituents, in the α-CH_2 protons, and in the ethylenic protons. However, the deprotonation of an amino nitrogen should not affect the ring substituents to such a large extent, and the deprotonation of a pyridine nitrogen should not affect the α-CH_2 protons and the ethylenic protons. Therefore, analogous to the equilibria suggested by Matsushima and Martell[20] and Gansow and Holm[18] for pyridoxamine, it is here suggested that the fifth deprotonation yields two species in equilibrium, represented by formulas **5** and **6**. The points of inflection (midpoints of buffer regions) correlate well with the logarithm of the protonation constant determined potentiometrically (7.20),[2] if one takes into account the conversion of pK_H to pK_D and the changes in temperature and ionic strength.

As seen in Figures 3–8, the sixth deprotonation produces changes in the chemical shifts of 4′-CH_2, α-CH_2, and the ethylenic protons. This observation makes it clear that an amino nitrogen is being deprotonated, yielding the species represented by formula **7**. Again, the points of inflection correlate well with the pK determined potentiometrically (9.52).[2] The second deprotonation constant of pyridoxamine is reported to be 8.01, which falls exactly between the two corresponding pK's of PLED (7.20 and 9.52).[2]

Finally, the seventh and eighth deprotonations of PLED cause marked chemical shifts in all ring substituents. The fact that these deprotonations do not produce chemical shifts of the α-CH_2 and ethylenic protons makes it clear that the pyridine nitrogens are being deprotonated in these last two steps, with pK's of 10.68 and 11.09.[2] The corresponding pK of pyridoxamine is 10.13.

Metal Chelate Formation Constants and Species Distribution of PLED Complexes in Solution. The results in Table I show that the chelate formation constants of PLED with the divalent metal ions are much lower than those of HBED. This is reasonable if one takes into account the fact that the two most basic groups of the ligand (pyridine nitrogens with pK's of 11.10 and 10.68) are not involved directly in the coordination of the metal ion. If one considers the sum of the pK's of the amino groups (9.54 and 7.21) and the phenolic groups (3.31 and 2.57), the resulting basicity of the ligand is actually much closer to that of EDTA than to that of HBED. The high formation constant of the Cu(II) chelate of PLED relative to those of Ni(II), Co(II), and Zn(II) is probably due to lower steric repulsions arising from substituents, primarily the 2-CH_3 groups of the pyridine rings. The coordination sphere of Cu(II) contains only four strong coordinate bonds, thus allowing the basic donor groups of the ligand to come more fully into play in determining the stability constant of the metal chelate.

The chelate formation constants of PLED with the trivalent metal ions are also much lower than those of HBED, because of the much lower basicity of the phenolic groups in PLED. An important difference between the chelates of PLED and those of HBED is the existence of protonated chelates at neutral and low pH. Coordination sites for two additional protons are provided by the pyridine nitrogens, thus allowing protonation without the breaking of coordinate bonds of the metal ion. The thermody-

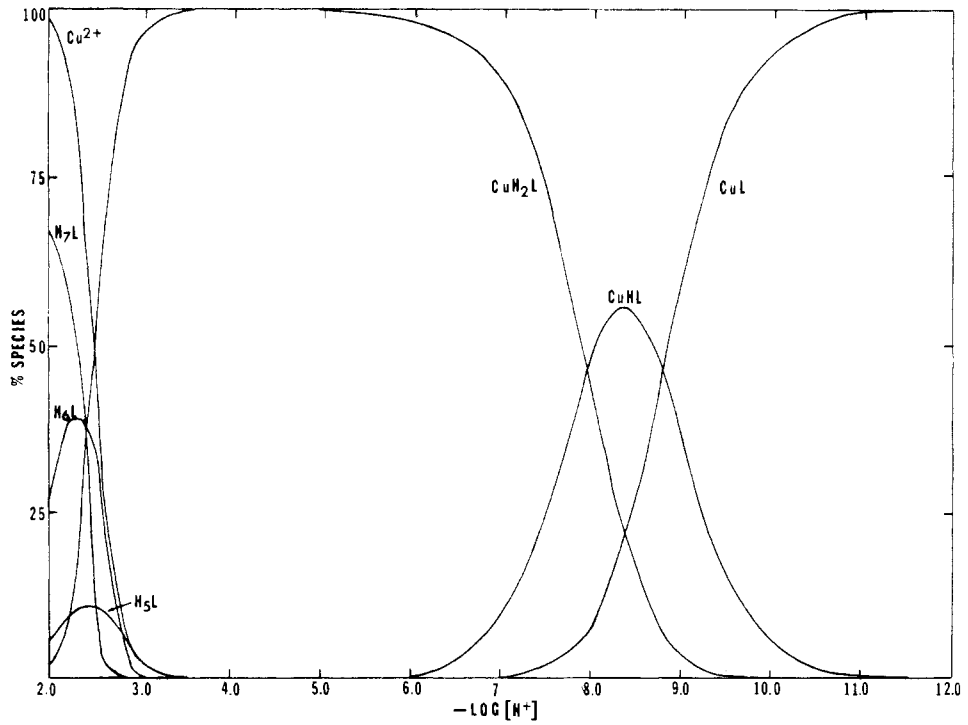
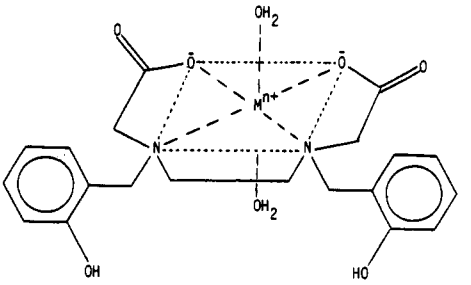


Figure 9. Distribution of species as a function of −log [H^+] in a system containing a 1:1 molar ratio of PLED to Cu(II) (μ = 0.100 M (KCl); t = 25.0 °C; concentration of ligand and metal ion 2.00 × 10^{-3} M).

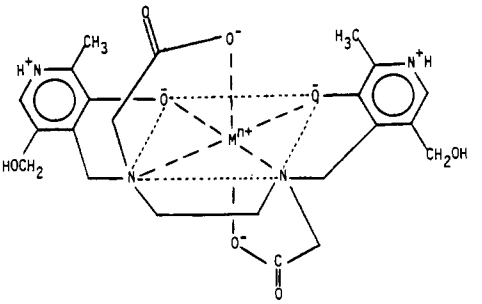
namic stabilities of the chelates are of course reduced considerably by protonation even though coordinate bonds are not broken. Protonation reactions of this nature would certainly strongly influence the effectiveness of this ligand in complexing metal ions in biological systems. However, it is seen that the adjustment for this effect through the calculation of the formation constants of the protonated chelates would still give reasonably high metal ion affinities. Thus the stability constants for Ga(III), In(III), and Fe(III) with the diprotonated ligand (log $K^{M}_{MH_2L}$) are 28.23, 29.73, and 29.17, respectively.

As seen in Figures 3–8, the curves representing the Zn(II) (○) and Ga(III) (△) chelates of PLED show marked upfield chemical shifts for all ring substituents in the pD range corresponding to the calculated protonation constants of the metal chelates (8.22 and 8.85 for Zn(II); 6.38 and 7.31 for Ga(III)). These upfield chemical shifts correspond to the deprotonation of the pyridine nitrogens in the chelate. Figures 3–8 also show that the Ga–PLED chelate is completely formed at low pD, whereas the Zn–PLED chelate shows a marked chemical shift upfield at low pD due to its gradual formation as the pD is increased. In all cases, the proton magnetic resonance peaks of the Ga–PLED chelate are located downfield relative to those of the Zn–PLED chelate, because of the higher charge of the Ga(II) ion.

The species distribution curves for the 1:1 Cu(II)–PLED system shown in Figure 9 provide a typical picture of the succession of complexes formed by all of the metal ions listed in Table I. On the basis of the properties of HBED previously described,[2,21] one would consider it logical to assign the protonation sites of the complexes to uncoordinated phenolic donor groups (formula **8**) and to consider the stepwise displacement of these protons to be due to stepwise involvement of the phenolate donors in coordination. However, the NMR spectra of Zn(II) and Ga(III) chelates of PLED show that for this ligand the situation is entirely different. Since the first coordination step in the Zn(II)–PLED system involves displacement of four protons from the hexaprotonated, dipositive ligand to give a neutral species having the formulation ZnH_2L (with the protons residing on the pyridinium nitrogens), it is evidence that, in contrast to the behavior of HBED, both aliphatic amino nitrogens and the phenolate groups are



$MH_2L.2H_2O^{(n-2)}$ (HBED), **8**



$MH_2L^{(n-2)}$ (PLED), **9**

involved initially in Zn(II) ion coordination. The easier access to the phenolate groups in this case is due to their much lower proton affinity, as compared to that of HBED, because of the inductive electron-withdrawal effects of the protonated pyridinium groups. Thus the coordinate bonding arrangement in formula **9** is suggested for the 6-coordinate diprotonated complexes of PLED, and an analogous arrangement is suggested for its 4-coordinate complexes.

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