decrease and eventually, for $k_{NN} = 0.75$, become smaller than those of C-2 and -3. This value still leads to qualitative agreement with the trend of a_H splittings reported in the literature for other radicals containing the NN bond; however, it cannot be used in the radical II since, owing to the orbital crossing effect,[15] its first antibonding orbital becomes symmetric instead of antisymmetric for $k_{NN} < 0.9$, and the carbon and nitrogen spin densities are thus reversed (see Table I).

Experimental Section

The radicals were obtained by allowing solutions of I, II, and III in hexamethylphosphorictriamide (HMPTA) to react with a potassium mirror in a vacuum sealed tube; the solvent was dried on barium oxide and distilled immediately before use.

The 1,4,5,8-tetraazanaphthalene (I) was obtained by the reaction of 2,3-diaminopyrazine with glyoxal;[16] the nmr spectrum (in HMPTA) shows a single peak at 9.70 ppm. The 2,3,6,7-tetraazanaphthalene (II) resulted from the reduction of ethyl pyridazine-4,5-dicarboxylate with $LiAlH_4$, followed by condensation with hydrazine.[17] The 1,4,6,7-tetraazanaphthalene (III) was obtained by condensation of 4,5-diaminopyridazine with glyoxal;[18] the nmr spectrum (in $CDCl_3$) shows two peaks of equal intensity at 9.25 and 10.0 ppm, which are shifted, in HMPTA, at 10.1 and 10.4. The deuterated derivatives of III were obtained by flowing gaseous deuterium into a CH_3OD solution of the corresponding 5,8-dichloro derivative,[18] using palladium-on-carbon as a catalyst. The nmr spectrum of the resulting compound has two peaks in the same positions of III but with a 5:1 intensity ratio, thus indicating a noncomplete deuteration at the 5 and 8 positions. The esr spectrum of the corresponding radical is in fact a 60–40% mixture, respectively, of the di- and monodeuterated tetraazanaphthalene (III) at positions 5 and 8.

The esr spectra were recorded on Varian 4502 spectrometer (100 kHz, modulation) and the simulated spectra were plotted on a IBM 7094 computer by assuming a Lorentzian line shape with a 0.12-G width.

(15) S. F. Nelsen, B. M. Trost, and D. H. Evans, *J. Amer. Chem. Soc.*, **89**, 3034 (1967).
(16) W. L. F. Armarego, *J. Chem. Soc.*, 4304 (1963).
(17) G. Adembri, F. De Sio, R. Nesi, and M. Scotton, *Chem. Commun.* 1006 (1967).
(18) N. T. Patel and N. Eastle, *J. Heterocycl. Chem.*, **3**, 512 (1966).

The Reaction of Amines with Pyridoxal Azomethines. The Question of Transaldimation and Its Role in the Mechanism of Vitamin B_6 Enzymes[1]

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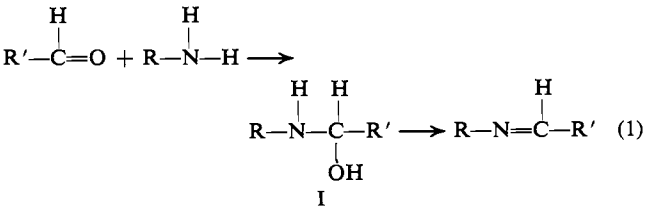
Abstract: The nuclear magnetic resonance (nmr) spectra of D_2O solutions of pyridoxal with 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, ornithine, and lysine are discussed. In all systems, two Schiff bases are observed, both of which contain one amino acid and one pyridoxal moiety. No evidence is found for the intraconversion of these Schiff bases (transaldimation) *via* a geminal diamine species such as has been proposed to arise from the condensation of the free amino group with the azomethine linkage. In the 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, and ornithine cases, slow appearance of the internal geminal diamines is observed; however, their formation is much slower than are Schiff base formation and dissociation. Schiff bases containing one pyridoxal and one amino acid moiety are observed in the ornithine and lysine cases. Evidence is reported indicating that one of the Schiff bases in the 2,3-diaminopropionic acid–pyridoxal system is in rapid equilibrium with another species. Various possibilities are considered and it is concluded that the second species is a carbinolamine. The relevance of these reactions to the initial steps of substrate–vitamin B_6 enzyme interactions is discussed.

Jencks and Cordes[3] and Koehler, *et al.*,[4] found by kinetic analysis that the reaction of certain Schiff bases with amines to form a second Schiff base (transaldimation) was too rapid to proceed through dissociation to the aldehyde and amine. These authors[5] had previously demonstrated the importance of carbinolamines as intermediates in the Schiff base formation reaction 1. They therefore suggested that the transaldimation reaction passes through a geminal diamine type structure II analogous to the carbinolamine (I) as represented in eq 2.



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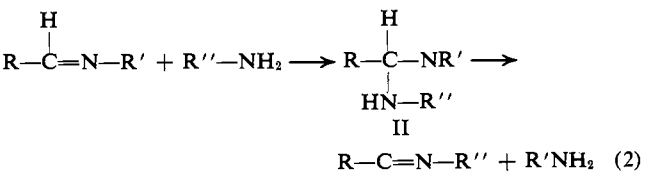

(2)

(1) This work was supported by a Research grant, No. AM11694, from the National Institute of Arthritis and Metabolic Diseases, Public Health Service.
(2) To whom inquiries may be sent.
(3) W. P. Jencks and E. H. Cordes, Proceedings of the Symposium on Chemical and Biological Aspects of Pyridoxal Catalysis, Rome, 1962, Pergamon Press, Elmsford, N. Y., 1963, p 57; E. H. Cordes and W. P. Jencks, *Biochemistry*, **1**, 773 (1962); E. H. Cordes and W. P. Jencks, *J. Amer. Chem. Soc.*, **84**, 826 (1962).
(4) K. Koehler, W. Sandstrom, and E. H. Cordes, *ibid.*, **86**, 2413 (1964).
(5) E. H. Cordes and W. P. Jencks, *ibid.*, **84**, 832 (1962).

Earlier it had been found that there was a lysyl residue near the active site of the vitamin B_6 enzyme transaminase and this led to the suggestion that the aldehyde form of the vitamin was bound to the enzyme as the Schiff base of the ϵ-amino group of the lysine.[6-8] The amino acid substrate then displaced the lysine by a reaction similar to reaction 2 above. Kinetic evidence has been reported[9] to support this type of reaction as the first step in enzymic transamination.

Since no direct physical evidence could be found by Jencks and Cordes for the existence of geminal diamines of type II, we have endeavored to investigate the possibilities of their detection and study by nuclear magnetic resonance (nmr). Nmr has been shown to be an excellent tool for investigating vitamin B_6 systems.[10,11] The nmr technique has some special diagnostic advantages with reactions of type 2. If one considers the resonance of the hydrogen bound to the azomethine carbon, it should be in the range of 910 Hz from hexamethyldisiloxane (HMDS) but the resonance of this hydrogen would be closer to 600 Hz for the geminal diamine. If an azomethine is in rapid equilibrium with a geminal diamine, its azomethine proton will appear as a single resonance whose chemical shift is a weighted average of those of its contributors. Alternatively, if the equilibrium is slow, separate resonances will be seen.

The amines of choice are those for which geminal diamine formation will be most extensive and most rapid. Such species are the amino acids having both an α and a terminal amino functional group. After Schiff base formation, condensation of the second amino group with the azomethine nitrogen will proceed by the statistically favored intramolecular pathway. We have recently reported an investigation which utilized this technique to investigate the reactivity of azomethines toward other functional groups.[11]

Experimental Section

2,3-Diaminopropionic acid hydrobromide was purchased from the Aldrich Chemical Co. The other amino acids and the pyridoxal hydrochloride were obtained from Mann Laboratories, and were of sufficient purity to be used without further purification. D_2O was 99.8% from Diaprep. The standard solution of zinc(II) was prepared by dissolving $ZnCl_2$ in D_2O and adding 1 drop of DCl. Its concentration was determined by potentiometric titration with ethylenediaminetetraacetic acid (EDTA).

The experimental techniques employed in this research have been described in detail in a previous paper.[11] The proton resonance of hexamethyldisiloxane (Peninsular Chemical Co.) in an internal capillary was used as a reference so that temperatures as high as 95° could be investigated without danger to the nmr probe. The resonance of HMDS is 10 Hz downfield from that of tetramethylsilane (TMS) at room temperature.

The apparent pD was measured using a Beckman Model G pH meter with one-drop electrodes. The pH meter was standardized with buffers and the apparent pD calculated by adding 0.41 to the observed reading on the meter.[12] In this paper pD is $-\log [D^+]$ and it was computed from the apparent pD by means of the activity coefficients for hydrogen ion in NaCl solutions.[13]

(6) W. T. Jenkins and I. W. Sizer, *J. Amer. Chem. Soc.*, **79**, 2655 (1957).
(7) W. T. Jenkins, D. A. Yphantis, and I. W. Sizer, *J. Biol. Chem.*, **234**, 51 (1959).
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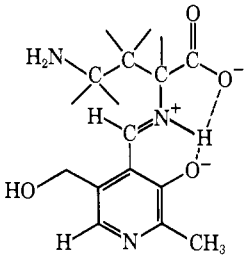
(10) O. A. Gansow and R. H. Holm, *ibid.*, **91**, 573 (1969).
(11) E. H. Abbott and A. E. Martell, *ibid.*, **92**, 1754 (1970).
(12) A. K. Covington, M. Paabo, R. A. Robinson, and R. G. Bates, *Anal. Chem.*, **40**, 700 (1968).

Results and Discussion

2,4-Diaminobutyric Acid. At pD values less than 5, the nmr spectrum of an equimolar solution of pyridoxal and 2,4-diaminobutyric acid (DABA) consists of resonances attributable entirely to the components. The pyridoxal resonances have been assigned and discussed in some detail previously.[14] The DABA resonances consist of a triplet near 430 Hz (the α proton), a multiplet near 260 Hz (the β protons), and a distorted triplet near 360 Hz (the γ protons). As the pD is raised above 6, resonances diagnostic of Schiff base formation become apparent. These are labeled SB in Figure 1 and are assigned to Schiff bases III and IV. The 5-CH_2 resonances of these Schiff bases are obscured by the intense resonance of the isotopic impurity, HOD. The reasoning upon which the Schiff base assignments are made has already been presented.[10,11] Within a half hour the resonances labeled DM in Figure 1 begin to appear and to increase steadily in intensity at the expense of the resonances of the Schiff bases and their components until after 24 hr they comprise the complete nmr spectrum. The DM resonances are readily attributable to the cyclic diamine V as assigned in Figure 1. The 4-CH resonances at 522 Hz are indicative of a pyridoxal derivative in which



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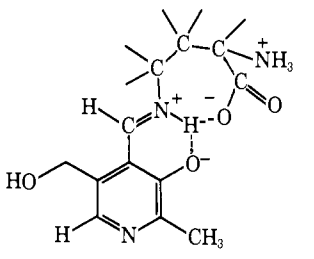

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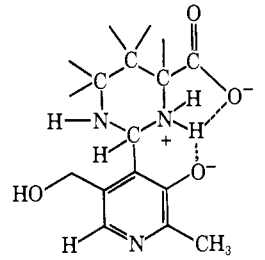

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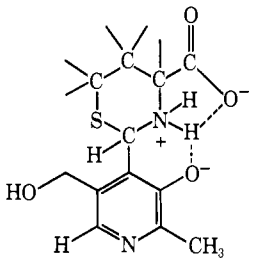


VI

the 4-C is part of a saturated system, as, for example, in the thiazolidene derivative VI resulting from the condensation of pyridoxal with cysteine. The 5-CH_2 protons are magnetically nonequivalent because of the nearby asymmetric 4-C site. Thus, they appear as an AB pattern whose outer wings are lost in the noise.

The importance of these observations and assignments is that they clearly demonstrate that at least in this system, the geminal diamine is not an intermediate in transaldimation. Rather, it is a stable, inert species wholly off the reaction pathway introconverting Schiff bases III and IV. This is emphasized by noting that although the rate of appearance of DM resonances is base catalyzed, the Schiff base resonances appear with a half-life of less than 1 min, while even at pD 10 the

(13) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," Reinhold, New York, N. Y., 1958.
(14) O. A. Gansow and R. H. Holm, *Tetrahedron*, **24**, 4477 (1968).

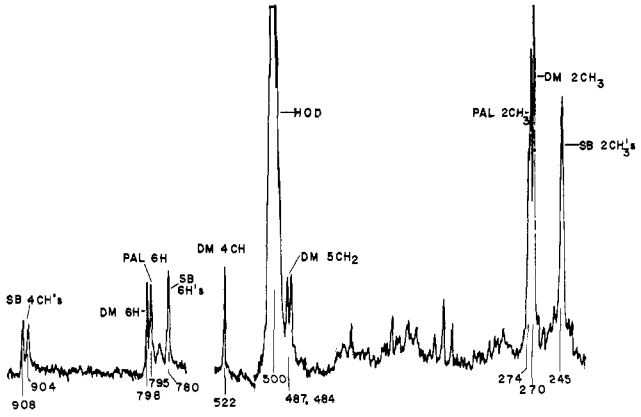


Figure 1. Nmr spectrum of a D_2O solution equimolar in pyridoxal and 2,4-diaminobutyric acid at pD > 6: SB = Schiff base; PAL = pyridoxal; numbering refers to position on the pyridine ring; frequencies given in hertz *vs.* hexamethyldisiloxane.

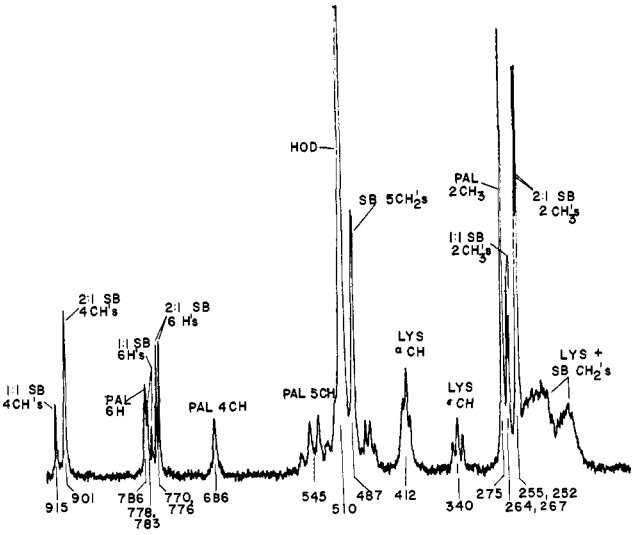


Figure 2. Nmr spectrum of a D_2O solution containing a 2:1 *M* ratio of pyridoxal to lysine at pD > 6: 1:1 SB = 1:1 Schiff base; 2:1 SB = Schiff base formed by 2 mol of pyridoxal/mol of diamino acid; numbering corresponds to position on pyridine ring; Greek letters correspond to position on amino acid residue; frequencies given in hertz *vs.* hexamethyldisiloxane.

half-life for the appearance of DM resonances is of the order of 1 hr.

Ornithine and Lysine. The composition of solutions of lysine and of ornithine at equilibrium with pyridoxal is demonstrated by the nmr spectrum of lysine shown in Figure 2. Three different Schiff bases are formed. Two contain one pyridoxal and one amino acid and are labeled 1:1 SB in the figure. They have structures represented by VII and VIII. The third Schiff base contains two pyridoxals to one amino acid and has structure IX. Its resonances are labeled 2:1 SB and were assigned by varying the pyridoxal concentration. At the highest levels of pyridoxal, resonances labeled 2:1 SB predominate. No evidence could be found for the formation of a cyclic diamine species in the lysine system; however, in the ornithine system slow and incomplete cyclic diamine formation takes place. Resonances assignable to the cyclic geminal diamine structure X appear after 1 day at room temperature. The assignments are reported in Table I and are quite similar to those of V discussed above.

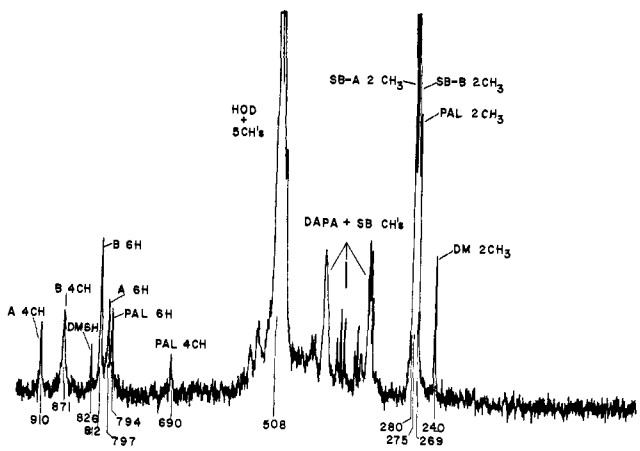
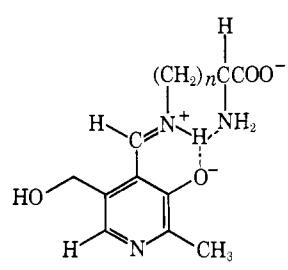
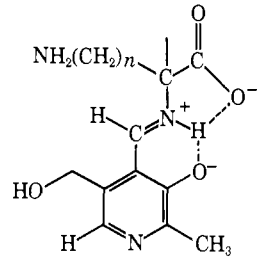


Figure 3. Nmr spectrum of a D_2O solution 0.10 *M* in 1,2-diaminopropionic acid (DAPA) and 0.10 *M* in pyridoxal (PAL) after 1.0 hr at pD 6 and above: SB-A = Schiff base formed with the terminal amino group; SB-B = Schiff base formed with the α-amino group; numbers refer to positions on the pyridine ring; frequencies are given in hertz relative to hexamethyldisiloxane.

2,3-Diaminopropionic Acid. The resonances of Schiff bases of diaminopropionic acid (DAPA) with pyridoxal are different in one significant way from those of the



VII



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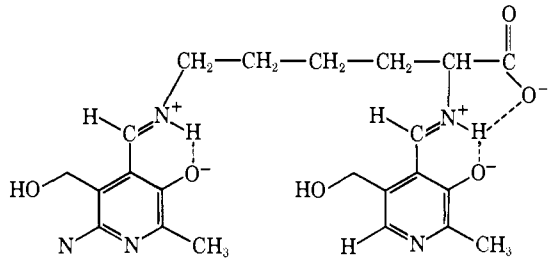
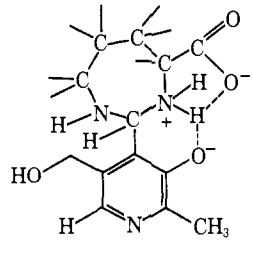


IX



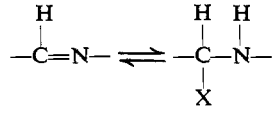
X

other amino acids discussed in this paper. Figure 3 shows the nmr spectrum of a D_2O solution initially 0.10 *M* in DAPA and in pyridoxal after about 1 hr. The resonances are assignable to two Schiff bases, SB-A and SB-B, compounds XI and XIII, as was done in the DABA case. However, variation of pD in the region 8–10 shows that the dependences of chemical

Table I. Resonances Arising from the Cyclic Diamine Formed by Internal Condensation of the Second Amino Group of Pyridoxylideneornithine with Its Azomethine Carbon

Resonance	Chem shift, Hz
2-CH_3	276
4-CH	481
5-CH_2	511
6-H	804

shifts of the 4-CH resonances upon pD are completely different. This is demonstrated in Figure 4. The change in chemical shift for resonance B is nearly 100 Hz in this pD interval—far too great to result from the deprotonation of an adjacent group. Such a pD dependence is best attributed to a rapid equilibrium such as is illustrated in eq 3, in which the observed proton resonance will be a weighted average of those of the two



(3)

components. The azomethine proton is in the deshielding region of the aromatic system while the proton of the saturated species is not and so a spread of 200–300 Hz might be anticipated as the composition changes. Such an equilibrium may be conceived of as arising from rapid interconversion of the azomethine with either a carbinolamine or an internal diamine. No such pD dependence of a 4-CH resonance has been observed for any pyridoxal Schiff base hitherto investigated and so it is tempting to attribute it to very rapid cyclic diamine formation. However, this is unlikely both because no such phenomenon is observed in the DABA case and because the two Schiff base 4-CH resonances of XI and XII are not averaged with one another. That is, if one Schiff base were in rapid equilibrium with a cyclic geminal diamine form, it might be expected that the other one would also be. This would result in the averaging of two observed 4-CH resonances into a single one.

Thus, the possibility must be considered that it is carbinolamine formation which is responsible for the large pD dependence of chemical shift. Perhaps the most likely way for this to happen is for the second amine group or the carboxyl group to hydrogen bond to the hydroxyl group of the carbinolamine stabilizing it. The pD's for deprotonation of the two amine groups of DAPA are 6.79 and 9.51, as reported by Hay, *et al.*[15] As in the valine–pyridoxal or glycine–pyridoxal cases[16,17] one may predict that the pK's of the species with monoprotonated amino acid moieties are increased somewhat by Schiff base formation (probably because of internal "chelate" hydrogen bond formation). On this basis the principal species present in the pD region of 9.0 and above would be the monoprotonated forms, as indicated by XI and XII, respectively. The principal structures indicated for the monoprotonated forms suggested by XI and XII, with the protons principally bound to the amino group rather than to the azomethine nitrogen, are based on the idea that the latter are probably less basic since they are conjugated with the electron-withdrawing pyridine ring. On the other hand one must allow for the possibility that forms XI and XII are in equilibrium with appreciable concentrations of trifurcated hydrogen-bonded species in which the protons are covalently bound to the azomethine nitrogens. The species in which the diamino acid moieties are diprotonated would be expected to have pK's of about 2.0–2.5 log units below the pK's of the monoprotonated forms. The difference of 3.0 log units usually observed in the pK's of 1,2-diamines because of electrostatic repulsions is expected to be reduced somewhat in these Schiff base systems because of the influence of hydrogen bonding in reducing electrostatic interactions.

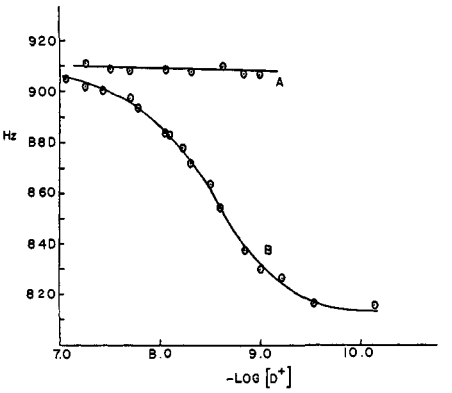


Figure 4. Dependence of chemical shift of 4-CH proton resonances of Schiff bases of 2,3-diaminopropionic acid and pyridoxal as a function of pD in D_2O solution: A = Schiff base formed by the terminal amino group (XI, XIII); B = Schiff base formed by the α-amino group (XII, XIV); hertz measured *vs.* hexamethyldisiloxane.

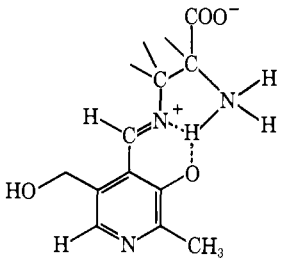
Addition of the elements of water to the monoprotonated species represented by XI and XII would give the carbinolamines XIII and XIV, respectively. Models indicate that XIV, as shown, can more easily form a hydrogen bond involving the hydroxyl and carboxylate groups. This would stabilize XIV with respect to XIII.

Resonances in the nmr spectrum of the pyridoxal–alanine system above pD 10 have been interpreted as arising from a carbinolamine which must have a neutral nitrogen.[10] Such evidence provides further support for the molecular species indicated by XIII and XIV.

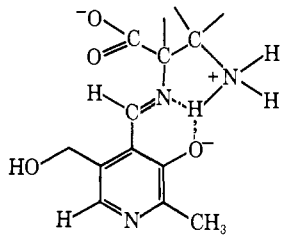
To summarize, the differences in the variations of chemical shift shown in Figure 4 are ascribed to the stabilization of the carbinolamine XIV of the α-amino Schiff base XII relative to the carbinolamine XIII of the β-amino acid Schiff base XI. In the pH region of the observed pH shift for the XII–XIV system, the diamine moieties are being converted from the diprotonated to the monoprotonated forms, thus favoring carbinolamine formation. On the basis of the arguments given above, it appears that carbinolamine formation in the XII–XIV system could be much more extensive than in the XI–XIII system, thus explaining the differences in behavior indicated by Figure 4.

Experiments were performed in which Zn(II) ion was present in concentrations from 0.10 to 0.050 *M*. Throughout the pD range the 4-CH resonance labeled

(15) R. W. Hay, P. J. Morriss, and D. D. Perrin, *Aust. J. Chem.*, 21, 1073 (1968).
(16) D. L. Leussing and J. Huq, *Anal. Chem.*, 38, 1389 (1966).
(17) D. E. Metzler, *J. Amer. Chem. Soc.*, 79, 485 (1957).



XI



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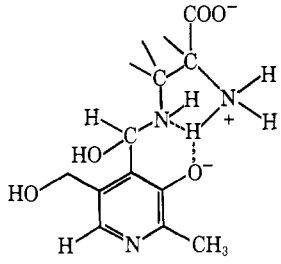
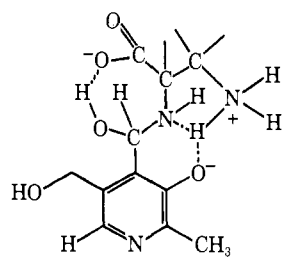


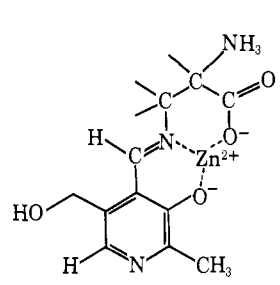
XIII



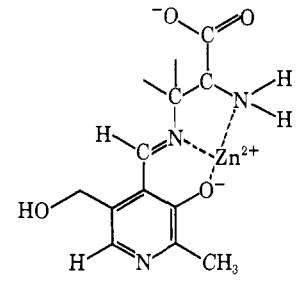
XIV

B was shifted and broadened much more than the A resonance. Since both amino nitrogens and carboxylate groups are available for coordination of Zn(II), there are four possible complex species, XV–XVIII, depending on the degree of protonation of the ligand and the site at which Schiff base formation occurs. On the basis of molecular models, and first principles of metal chelate formation, it is apparent that for the monoprotonated species, XVII is more stable than XV, while there is no appreciable difference in the stabilities of XVI and XVIII. Since both Zn(II) chelates were protonated up to pH 10, the compounds of concern in the pH range 7–10 are XV and XVII. The greater interaction between Zn(II) and the ligand in XVII, as detected by nmr, is thus in accord with the relative stabilities of these chelate compounds.

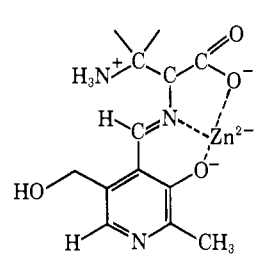
The last point to be noted which argues against kinetically labile geminal diamines in these systems is the slow appearance of resonances assigned to kinetically inert geminal diamines in the DAPA–pyridoxal systems. These resonances are labeled DM in Figure 3. The ring closure reaction is slow and does proceed to completion in 2 days at room temperature over a variety of pD values which were investigated. During this time other reactions also occur and, in particular, all 4-CH resonances gradually vanish, the result of deuterium exchange.



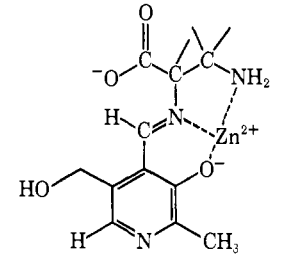
XV



XVI



XVII



XVIII

Conclusions

The significant conclusions to be drawn from this work concern the role of transaldimation in enzymes and in model systems. If amines in model systems do in fact react more readily with azomethines than with carbonyl groups, then one would certainly expect amino groups held five or six bonds away from an azomethine linkage to react at a maximum rate. The results discussed above indicate, however, that this reaction is relatively slow and leads to rather inert products. These experimental results do not contradict the work of Jencks and Cordes, since the reaction, *e.g.*, of semicarbazide, with an aldimine is expected to be quite different chemically from the reaction of the free amino group of a diamino acid with the aldimine derived from the same amino acid moiety. The kinetic pathway suggested by Jencks and Cordes seems to be a logical and reasonable one for their systems.

The results of the work described in this paper would not predict transaldimation as the first step in the reaction of transaminase-bound pyridoxal with amino acid substrates. While it cannot be concluded that this suggestion of Jencks and Cordes is incorrect, it does now seem that further kinetic work is required to establish what the intermediates are in the catalysis of Schiff base formation between pyridoxal and α-amino acids at the active site of transaminase enzymes.