Pyridoxine and Pyridoxal Analogs. XIII. A Nuclear Magnetic Resonance Study of the Condensation of Polyfunctional Amino Acids with Pyridoxal[1]

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Abstract: The interactions of serine, homoserine, threonine, cysteine, homocysteine, homocystine, histidine, imidazole, and 4-amino-3-isoxazolidone ("cycloserine") with pyridoxal have been investigated by measurement of nmr spectra in D_2O solution and the resonances assigned. Serine, homoserine, and threonine form Schiff bases and no interaction between the hydroxyl groups of the amino acids with Schiff base azomethine linkage is observed; cysteine forms a five-membered thiazolidine; homocysteine forms the thiolactone Schiff base and then the six-membered thiazolidine; homocystine forms two Schiff bases; histidine condenses as the tetrahydropyridine. However, imidazole does not react with pyridoxylidinealanine; and 4-amino-3-isoxazolidinone forms only the simple Schiff base. Chemical shifts, coupling constants, and biological implications are discussed.

Most amino acids react with pyridoxal to give Schiff bases which can be catalytically hydrogenated. Cysteine, histidine, and several others, however, give products which lack the typical Schiff base absorption maxima and cannot be catalytically hydrogenated. Accordingly, it has been suggested that these compounds form Schiff bases which further cyclize to give the thiazolidine and tetrahydropyridine, respectively.[2] Subsequent kinetic studies of the reaction of pyridoxal or pyridoxal phosphate with histidine,[3,4] histamine,[5] cysteine,[3,4] and some histidine derivatives[4] have confirmed the suggestion by Folkers[2] that cyclization follows Schiff base formation.

These reactions are particularly interesting from a biological point of view because the postulated products lack unsaturation beyond the heterocyclic ring and therefore cannot participate directly in vitamin B_6 reactions by the currently accepted mechanism[6] which requires unsaturation beyond the ring. Such species should be inhibitors of vitamin B_6 action and, in fact, this is well documented;[3,4,7,8] however, structural evidence for the various participating species has been lacking for the species formed in solution.

In general, nonenzymic reactions have been followed by product analysis. Such techniques are unsatisfactory because of the rapid, mobile equilibria in pyridoxal amino acid systems. Ultraviolet spectrophotometry has also been used but in aqueous media the formation constants for the Schiff base intermediates are small while the extinction coefficients are large and so considerable excess of amino acid is necessary if the Schiff base is to be observed. The use of methanol as a solvent overcomes this difficulty and makes some vitamin B_6 reactions both rapid and complete;[9,10] however, the breadth of the absorption bands limits the number of components which can be followed and severely restricts structural analysis of the species in solution.

Nuclear magnetic resonance (nmr) has been applied to vitamin B_6 systems in various media, particularly methanol, and considerable structural information has been obtained.[11-14] Recently, however, it has been demonstrated by this group[15] and by Gansow and Holm[16,17] that pyridoxal–amino acid Schiff base systems give sharp, assignable resonances in aqueous media from which information on structure and reactivity is readily obtainable.

In this paper, the nmr technique is applied to several pyridoxal–amino acid systems in order to determine the structures of species in solution and to determine the reactivity of the azomethine linkage toward several common functional groups.

Experimental Section

L-Cysteine, DL-serine, and L-histidine were from Matheson Coleman and Bell. DL-Homocystine, DL-homoserine, DL-homocysteine thiolactone, DL-threonine, D-4-amino-3-isoxazolidone ("cycloserine"), and pyridoxal hydrochloride were purchased from Mann Laboratories. These reagents were used without further purification. The purity of D_2O was 99.8 mol % from Matheson Coleman and Bell and from Diaprep. The NaOD was prepared by dropping D_2O onto sodium under dry nitrogen. The D_2SO_4 was obtained from Matheson Coleman and Bell. Nmr spectra were recorded with a Varian HA 100 nuclear magnetic resonance spectrometer. Chemical shifts are reported in hertz with respect to the resonance of tetramethylsilane (TMS) in an internal capillary. Unless other-

(1) This work was supported by the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service under research Grant No. AM 11694.

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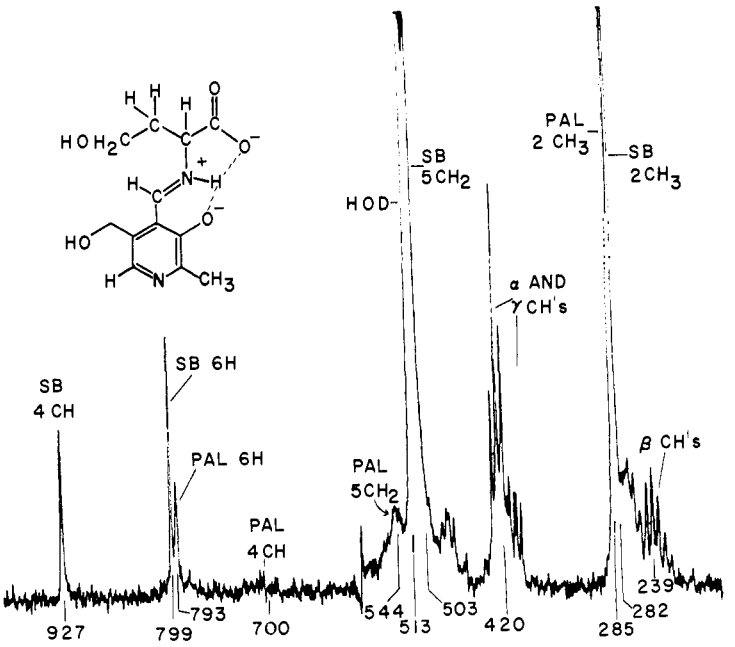

Figure 1. The 100-MHz nmr spectrum of 0.10 *M* pyridoxal and 0.10 *M* homoserine at pD 8.4 showing the formation of pyridoxylidenehomoserine.

wise noted, solutions were initially 0.10 *M* in pyridoxal and in amino acid, ionic strength was controlled at 1.0 *M* with NaCl, and sample temperature was 30 ± 2°.

In a typical experiment, 0.0010 mol of pyridoxal hydrochloride and of the amino acid were weighed into a dry 5-ml volumetric flask and the solution brought to volume with D_2O (in the cases of histidine and homocystine, enough D_2SO_4 was also added to dissolve the amino acid suspension). A pipet was used to transfer 0.50-ml aliquots of this stock solution to 1-ml volumetric flasks which contained enough NaCl to maintain the ionic strength at 1, NaOD was added, the contents of the flask brought to volume, and transfer was made to the nmr sample tube. After recording the spectra, 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 arrived at by adding 0.41 to the observed reading on the meter.[18] 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.[19] Stable values of pD were achieved within a few minutes of mixing. Measurements for all systems were made from pD 3 to 11. In the case of 4-amino-3-isoxazolidinone, the stock solution was made up at pD 4 and was neutralized to the various pD's within a few minutes to minimize hydrolysis; however, no changes were observed after 24 hr at room temperature in the nmr spectra of the acidic stock solutions of other amino acids and so these solutions were, on occasion, stored in a refrigerator for as long as several days prior to use.

The spectral features reported here are those observed within a few hours of neutralization. In all samples further changes were observed to occur over the time scale of several days. These are most probably due to vitamin B_6 catalyzed reactions of the amino acid such as decarboxylation, transamination, etc. and will not be considered further in this paper.

Results and Discussion

Pyridoxal. The results of this, and other nmr studies[11,13,20] of pyridoxal and related forms of vitamin B_6, confirm the conclusion from other spectral[21] studies that pyridoxal exists primarily in the hemiacetal form I. Through the intermediate pH range, no resonance is observed for the free aldehyde form II. The 2-CH_3 group is a sharp singlet at about 300 Hz and the 6-H is also a sharp singlet near 800. The proton on the 4 carbon gives a sharp doublet at 700; thus, it is coupled to one of the nonequivalent protons at the 5 position. The methylene group at the 5 position appears as an AB pattern half of which shows coupling to the proton at the 4 position. All resonances shift to higher field with increasing basicity. Between pD's 7.5 and 10.5, the pyridoxal 4-CH doublet and 5-CH_2 quartet are broadened. This effect is particularly noticeable in several of the figures of spectra at the higher pD's.

Serine, Homoserine, and Threonine. The nmr spectra of serine, homoserine, or threonine with pyridoxal contain only the spectra of the components at pD's below 7. Above 7, new resonances become important. This is shown in Figure 1[22] for the case of homoserine. As the pD is raised, the pyridoxal resonances at 793 and 285 are gradually replaced by resonances at 799 and 282. The 4-CH doublet disappears as does the 5-CH_2 quartet and new singlets grow in at 927 and 503. All the resonances assigned to the various species shift to higher field with increasing pD as their various functional groups are deprotonated. These changes are consistent with the progressive formation of the pyridoxylidenehomoserine Schiff base, III, and are analogous to the pyridoxylidenealanine case.[17] The protons of the methylene group at the 5 position are equivalent in the Schiff base with the breaking of the internal hemiacetal ring and the 4-CH resonance is shifted to low field because the proton is in the plane of both the aromatic ring and the azomethine double bond.

Both *cis* and *trans* isomers of the azomethine would be anticipated; however, only one is observed. This is most probably the conformation shown in III and is most stable because of hydrogen bonding of the pro-

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(22) Abbreviations in the figures are: PAL = pyridoxal; TH = thiazolidine derivative; SB = Schiff base.

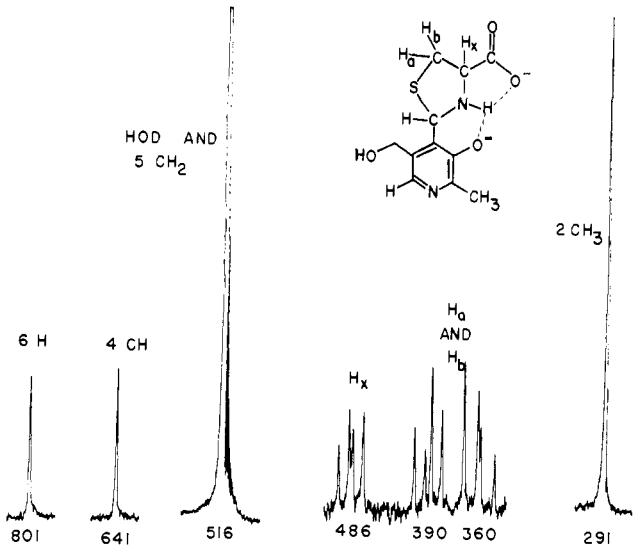
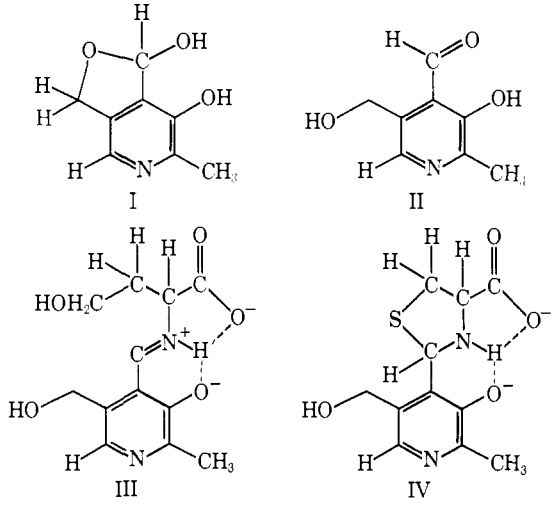


Figure 2. The 100-MHz nmr spectrum of 0.10 M pyridoxal and 0.10 M cysteine at pD 9.05 showing the complete formation of the pyridoxal–cysteine thiazolidine. J_{ax} = 7.8 Hz, J_{bx} = 6.8 Hz, J_{ab} = 10.0 Hz, H_a = 399.5 Hz, H_b = 370 Hz, H_x = 446 Hz.

ton of the azomethine nitrogen to the carboxylate and/or phenolate oxygens. The resonances at 239 and at 420



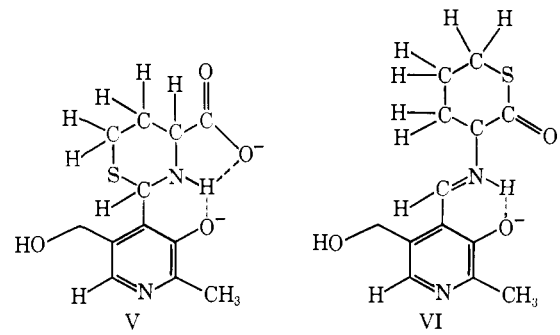
arise from the amino acid and from the amino acid portion of the Schiff base. In the case of homoserine, as well as serine and threonine, the resonances from the amino acid portion of the Schiff base are somewhat broader than those of the free amino acid. The reason for this broadening is not understood.

It might be anticipated that the amino acid hydroxyl group of the Schiff base would condense with the azomethine double bond as the 5-CH_2 hydroxyl group does with the pyridoxal carbonyl group. Derivative structures of the Schiff base, analogous to the internal hemicacetal of pyridoxal, would not have a 4-CH resonance above 900 Hz but rather would be expected near 700. Thus, the spectra show that internal amino acetal species are not formed and the azomethine linkage is relatively inert to attack by the alkyl hydroxyl groups of either the pyridoxal 5 position or of the amino acid chain.

Cysteine. The nmr spectrum in Figure 2 shows that, as originally suggested,[2] cysteine forms a thiazolidine derivative with pyridoxal with the structure IV. No resonance is observed to lower field than the 6-H resonance, thus precluding the existence of Schiff base. The 5-CH_2 group masked by the HOD resonance in Figure 2, can be seen as a sharp singlet very close to the HOD resonance at lower pD, showing that the protons of the methylene group are equivalent as in the Schiff base spectra. Evidently the asymmetric site generated at the 4-C position by thiazolidine formation is too remote to cause the 5-CH_2 protons to be observed as magnetically nonequivalent. The resonance at 641 Hz is close to the 4-CH of the pyridoxal hemiacetal and is therefore assigned to the thiazolidine 4-CH. No spin–spin coupling is observed between the β methylene group of the cysteine moiety of the thiazolidine and the 4-CH through the sulfur, in contrast to the observation of coupling of the 4-CH proton of the pyridoxal to one of the 5-CH_2 protons.

The strong inhibitory action of cysteine is reflected in the observable formation of the thiazolidine at pD 5 as opposed to the first observation of Schiff base in other amino acid systems only above pD 7. In addition, at pD 9, the thiazolidine is nearly completely formed whereas the Schiff bases investigated were less than half formed at any pD for solutions initially 0.1 M in the components.

Homocysteine. Homocysteine presents an interesting case in that it exists as the thiolactone in solution and thus presents an opportunity to determine whether the azomethine linkage or the carboxyl group is more reactive toward the sulfhydryl group. When base is added to the acidic solutions of pyridoxal hydrochloride and homocysteine thiolactone hydrochloride, a red color immediately develops and then fades slowly away until the solution takes on the more typical yellow color of the pyridoxal–amino acid condensation products. The fading of the red color is most rapid in the more basic solutions. Figure 3 is an nmr spectrum taken within a few minutes of mixing. The resonances at 813, 616, 522, and 295 are similar to the cysteine thiazolidine resonances and are assigned to the homocysteine thiazolidine, V. In contrast to the cysteine case, some broadening of the 4-CH resonance is observed, probably as a result of coupling to the γ methylene group of the amino acid portion of the Schiff base through the sulfur. The resonances at 936, 832, 525, and 291 are typical of Schiff bases of pyridoxal and amino acids and are assigned to the Schiff base of the thiolactone, VI. The nmr spectrum of this Schiff base



would be expected to be very similar to that of the homocysteine Schiff base. However, in view of the stability of the thioazolidines and their rapid rate of formation, the concentration of Schiff base with a free sulfhydryl group is probably too low to be detected.

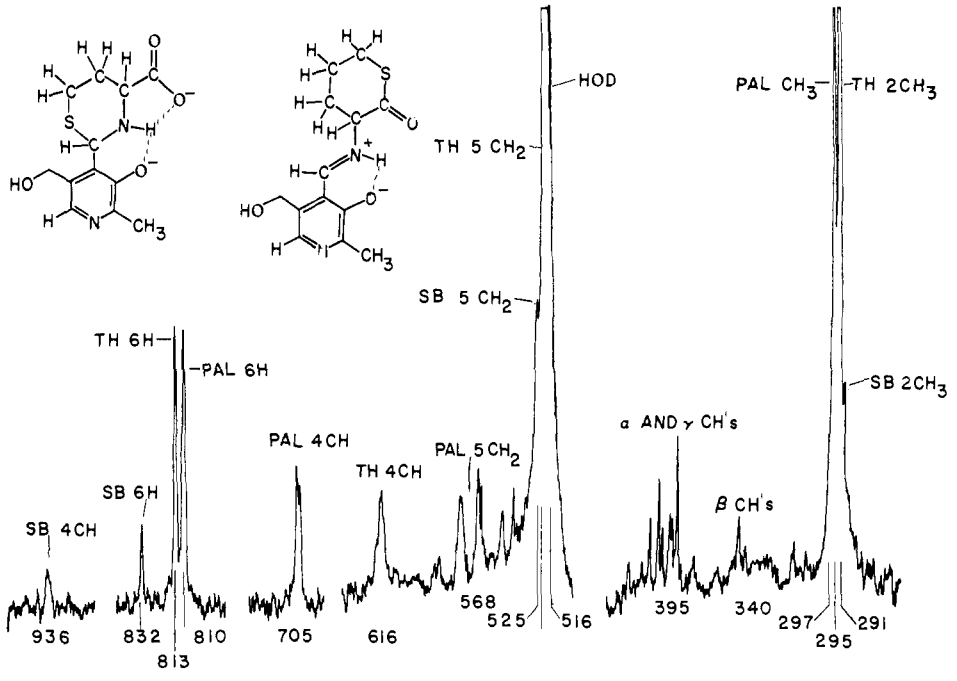


Figure 3. The 100-MHz nmr spectrum of 0.10 *M* pyridoxal and 0.10 *M* homocysteine thiolactone at pD 7.1 showing the formation of the homocysteine–pyridoxal thiazolidine and the transient formation of the homocysteine thiolactone–pyridoxal Schiff base.

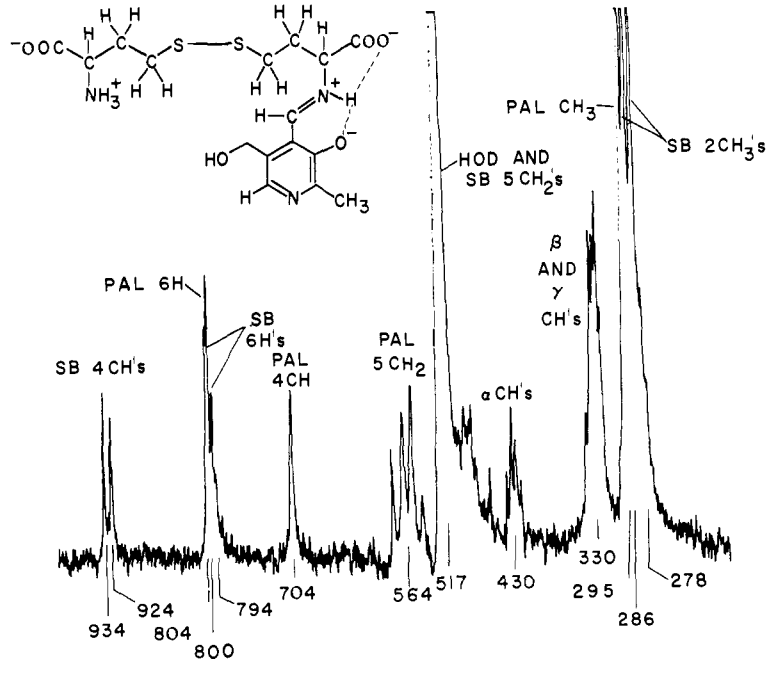


Figure 4. The 100-MHz nmr spectrum of 0.10 *M* pyridoxal and 0.050 *M* homocystine at pD 7.4 showing the formation of the two Schiff bases.

Within 15 min, the Schiff base resonances have vanished as the equilibrium concentration of the homocysteine thiazolidine is reached. Since the rate of intramolecular condensation is expected to be very much higher than intermolecular condensation and since the thiolactone Schiff base is present in detectable concentrations, the mechanism of thiazolidine formation probably consists of Schiff base formation of the thiolactone, hydrolysis of the thiolactone portion of the Schiff base, and condensation of the sulfhydryl group with the azomethine carbon to form the thiazolidine.

Homocystine. This amino acid forms normal Schiff bases with pyridoxal, as shown in Figure 4. These experiments were performed with 0.05 *M* homocysteine, and it is possible to resolve separate nmr spectra for each of the two Schiff bases formed, VII and VIII. Due to the insolubility of the amino acid, measurements could only be carried out above pD 7.

No resonances could be found which would be attributable to further reaction of the second amino group of VII or of the disulfide linkage in either species with the azomethine bond. Thus, the disulfide bond is, as

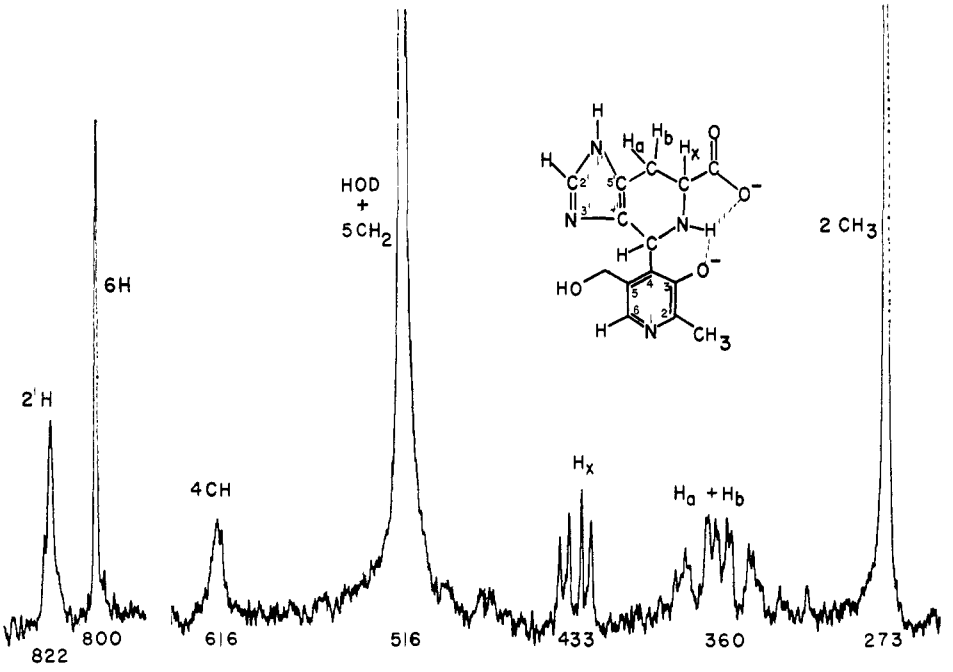


Figure 5. The 100-MHz nmr spectrum of 0.10 M pyridoxal and 0.10 M histidine at pD 8.5 showing the formation of the tetrahydropyridine. With the resonance at 616 decoupled, J_{ax} = 9.1 Hz, J_{bx} = 6.9 Hz, J_{ab} = 15.9 Hz, H_a = 370 Hz, H_b = 364 Hz, H_x = 433 Hz.

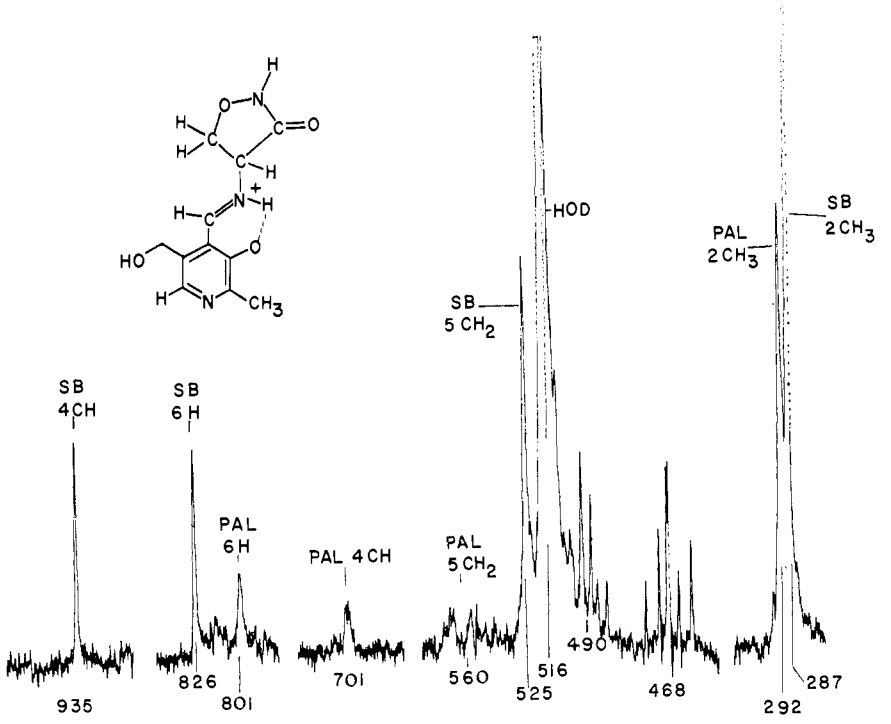
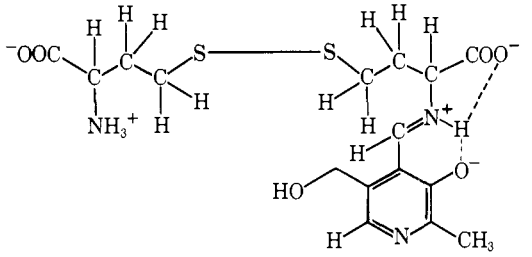


Figure 6. The 100-MHz nmr spectrum of 0.10 M pyridoxal and 0.10 M 4-amino-3-isoxazolidone at pD 8.05 showing the formation of the Schiff base.

expected, quite inert in comparison with the sulfur–carbon bond of the homocysteine thiolactone.

Histidine. Histidine–pyridoxal solutions contain considerable precipitate between pD's 4 and 8. Above this range, the nmr spectra (Figure 5) of such solutions is identifiable as that of the tetrahydropyridine, IX. The resonance at 822 is very close to that of the 2-H resonance of the imidazole ring of free histidine and since no resonance can be found in H_2O or D_2O corresponding to the 4-H of the imidazole ring, the bridge is from its 4-carbon to the azomethine carbon. As in the cysteine thiazolidine case, the histidine protons of the tetrahydropyridine appear as an ABX pattern. In addition, however, the A and B protons are coupled to the 4-CH proton with coupling constants of 1.5 and 2.3 Hz, respectively, as can readily be determined by decoupling the 4-CH proton with an auxiliary oscillator. Coupling constants vary with the dihedral angle be-



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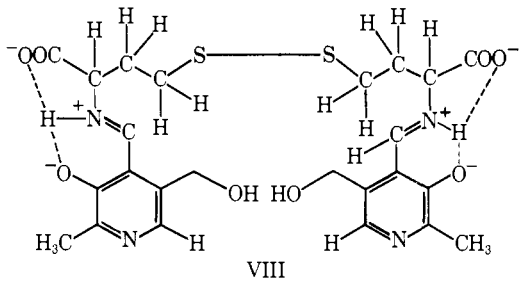

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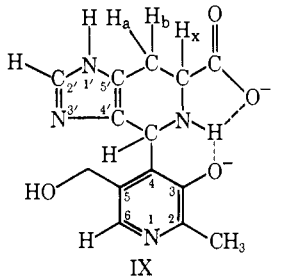
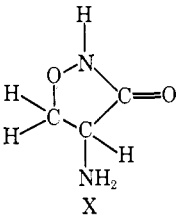


IX

tween the coupled nuclei and so the difference in these constants reflects the nonplanarity of the system.

Some experiments were performed with amino acids, pyridoxal, and imidazole to see if the same type of condensation could occur intermolecularly. Presumably, the formation of a ternary complex of these species would result in exchange of the imidazole ethylenic protons. In the case of the alanine–pyridoxal–imidazole system at several pD's between 7 and 9, exchange of these protons was not observed in 24 hr. In addition, the 4-CH resonance of the pyridoxylidenealanine was clearly detectable above 900 Hz and no resonances between 600 and 750, except that of free pyridoxal, could be found. This shows that the ternary complex is negligibly formed, perhaps because of the severe steric hindrance of the various groups near the azomethine linkage.

4-Amino-3-isoxazolidone. 4-Amino-3-isoxazolidone, X, perhaps better known by the misnomer "cycloserine," is a vitamin B_6 inhibitor[23] and has been observed to give non-Schiff base ultraviolet spectra in the presence of pyridoxal and amino acids.[24] The nmr spectra of solutions of this amine with pyridoxal are, however, fully consistent with the formation of the Schiff base XI, the resonance at 935 HZ being indicative of Schiff base formation. Therefore, it seems most probable that the inhibitory effect of 4-amino-3-isoxazolidone on enzymic reactions is the result of other of the groups of the molecule interacting with important group(s) at the active site of the enzyme rather than with the coenzyme alone.



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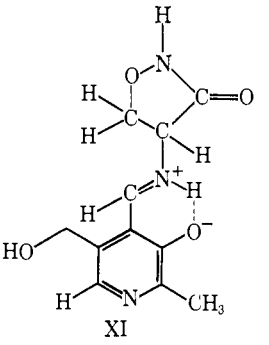

XI

Conclusions

High-resolution nmr spectroscopy provides a convenient method for examining equilibria and structure in the interaction of pyridoxal with inhibitors and for studying the reactivity of the azomethine bond toward various functional groups of polyfunctional amino acids. The results of this investigation show that groups five or six bonds away from the azomethine moiety have enhanced statistical effects favoring intramolecular condensation. It is believed that although these data are collected at relatively high coenzyme and substrate concentrations, they provide a realistic reflection of equilibria and observed reactivity at biological concentrations because the equilibria and rate constants for intramolecular condensation are not concentration dependent.

Further investigations of this type with related compounds are currently underway in these laboratories as part of a continuing research program into the structure and reactivity of vitamin B_6 systems.

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