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Reaction of Pyridoxal and Amino Acids in Methanol

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Reactions of amino acids and related amines with pyridoxal in methanol were studied by the changes of electronic absorption spectra. Most amino acids and amines formed Schiff bases, which existed in keto-enamine and enol-imine forms. Equilibrium between the two tautomeric forms was affected by the nature of amino acids or amine residues. Amino acids having a secondary amino group formed carbinolamine derivatives with pyridoxal. Cysteine and cysteamine gave products which had thiazolidine rings, while histidine, and 5-substituted tryptophans formed tetrahydropyridine derivatives asultimate products. The spectral properties, solution equilibria in methanol and band assignments of the Schiff bases and other products formed were described.

It is well known that enyzmes containing pyridoxal play important roles in amino acid metabolism. The first step of the enzyme catalyzed reactions is believed to be the formation of azomethine (Schiff base) between aldehyde group of pyridoxal and amino group of substrate amino acid.[2)] Although many works have been done on enzymatic and nonenzymatic reactions catalyzed by pyridoxal, little has been known about the first step of the reactions between pyridoxal and amino acids and the chemical properties of the products.

In the former work,[3)] Schiff bases of valine with pyridoxal and pyridoxal phosphate and their metal chelates in methanol were studied by means of electronic absorption spectroscopy. Unlike aqueous media, Schiff base formation was complete in low concentration of valine in methanol. For this reason, equilibria involved were very much simplified and molecular species present could clearly be determined. Each molecular species of pyridoxal and related substances were found to have two intense absorption bands in the visible and ultraviolet region. These two bands have been interpreted as derived from two π-π^* transition bands of pyridine, which were found at 256 mμ and 194 mμ. Martell and coworkers[3,4)] have studied the π-π^* bands of pyridoxal analogs and established several empirical rules concerning the wave length shifts with the changes of chemical structure of species and solvents.

In the present work, reactions of various amino acids and related amines with pyridoxal in methanol were examined by the changes of electronic absorption spectra. The spectral changes were analyzed with the aid of the empirical rules. Most amino acids and amines gave the spectra similar to that of pyridoxylidenevaline, showing the formation of Schiff bases. But some amino acids and amines did not give Schiff bases as ultimate products. The spectral properties of the Schiff bases and other products thus formed in methanol are discussed.

Experimental

Materials——Dotite spectrosol "Methanol" was used as a solvent and proved to give reproducible results. Pyridoxal hydrochloride was obtained from California Biochemical Co. Amino acids and amines were also obtained from commercial sources and used after verification of their purities.

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1) Location: *Anagawa, Chiba-shi*; Present address: *Faculty of Pharmaceutical Sciences, Kyushu University, Katakasu, Fukuoka.*
2) E.E. Snell, P.M. Fasella, A. Braunstein and A. Rossi Fanelli eds, "Chemical and Biological Aspects of Pyridoxal Catalysis," the Macmillan Co., New York, N.Y., 1963.
3) Y. Matsushima and A.E. Martell, *J. Am. Chem. Soc.*, **89**, 1322 (1967).
4) K. Nakamoto and A.E. Martell, *J. Am. Chem. Soc.*, **81**, 5857, 5863 (1959); D. Heinert and A.E. Martell, *ibid.*, **85**, 183, 188 (1963).

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Preparation of Solution——Amino acids were dissolved in methanol containing equimolar amount of KOH to acidic groups so as to exist in carboxylate forms. Amines which do not have acidic groups were dissolved in pure methanol. Pyridoxal hydrochloride was also dissolved in methanol containing equimolar KOH immediately before the measurements in order to minimize reaction with solvent.[3]

Calculated volumes of methanol solutions of pyridoxal and amino acid or amine were mixed in a volumetric flask and methanol was added to a definite volume. The solutions were so prepared to contain finally 1×10^{-4} M of pyridoxal and 1×10^{-3} M of amino group. Aliquots of the solution were transferred to glass stoppered silica cells and submitted to absorption measurements.

Measurements——The electronic absorption spectra of the sample solutions were recorded with a Cary Model 14 spectrophotometer, with pure methanol as a reference. The cell compartment was thermostated to 25° throughout the measurements.

Results

When methanol solutions of pyridoxal and amino acid or amine were mixed, the electronic absorption spectra of the mixture showed gradual changes with time. Initial spectra were identical to that of pyridoxal in slightly alkaline methanol. The spectral changes indicated slow conversion of pyridoxal to various reaction products according to the nature of amino acids or amines as follows.

Schiff Base Formation

Electronic absorption spectra of pyridoxylidenevaline in methanol studied before[3] were characterized by the bands at 418, 337, 285 and 255 mμ. This fact was supposed to indicate that the Schiff base existed in two tautomeric forms in methanol; the bands at 418 mμ and 285 mμ were assigned to π_1 and π_2 bands of *keto*-enamine form (I), whereas the bands at 337 mμ and 255 mμ to π_1 and π_2 bands of *enol*-imine form (II), respectively.[5]

Spectral change, when pyridoxal and potassium glycinate were mixed in methanol, is shown in Fig. 1. Final spectrum was very much alike to that of pyridoxylidenevaline, having absorption bands at 418, 335, 285 and 252 mμ. Therefore, the spectral change should indicate the formation of Schiff base, *i.e.* pyridoxylideneglycine, which existed in keto–enamine and enol–imine forms. Although the presence of neutral dipolar form (III) may also be possible, the fact that intense band was not observed at 380—390 mμ region, where π_1 band of this form was expected, showed to be of negligible amount. Information from NMR data, which will be reported, was also in favor of the absence of this form. On deprotonation, three neutral forms were converted to the common anionic form (IV), π_1 band of which was observed at 375 mμ in alkaline methanol solutions. The structures which contribute to these forms of pyridoxylidene amino acid are shown in Chart 1 with the band assignments in pyridoxylideneglycine.

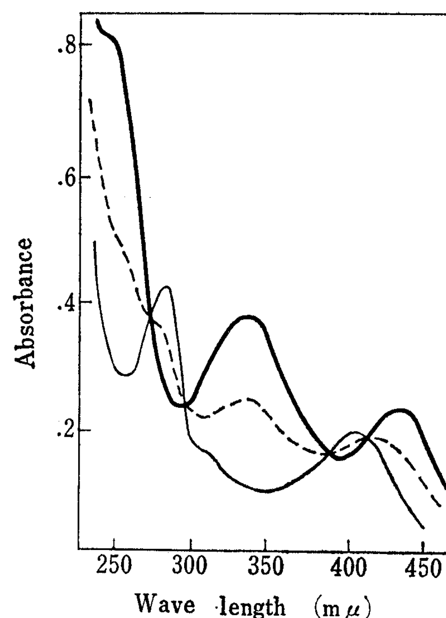

Fig. 1. Change of Electronic Absorption Spectra with Time for Methanol Solution containing 1×10^{-4} M Pyridoxal and 1×10^{-3} M K Glycinate

——— initial spectrum
- - - - intermediate spectrum
━━━ final spectrum

Most amino acids and amines having a primary amino group, when reacted with pyridoxal in methanol, were shown to form Schiff bases as

5) Of the two $\pi-\pi^*$ bands of pyridine derivatives, the longer wavelength band have been named as π_1 band, whereas the shorter one as π_2 band.

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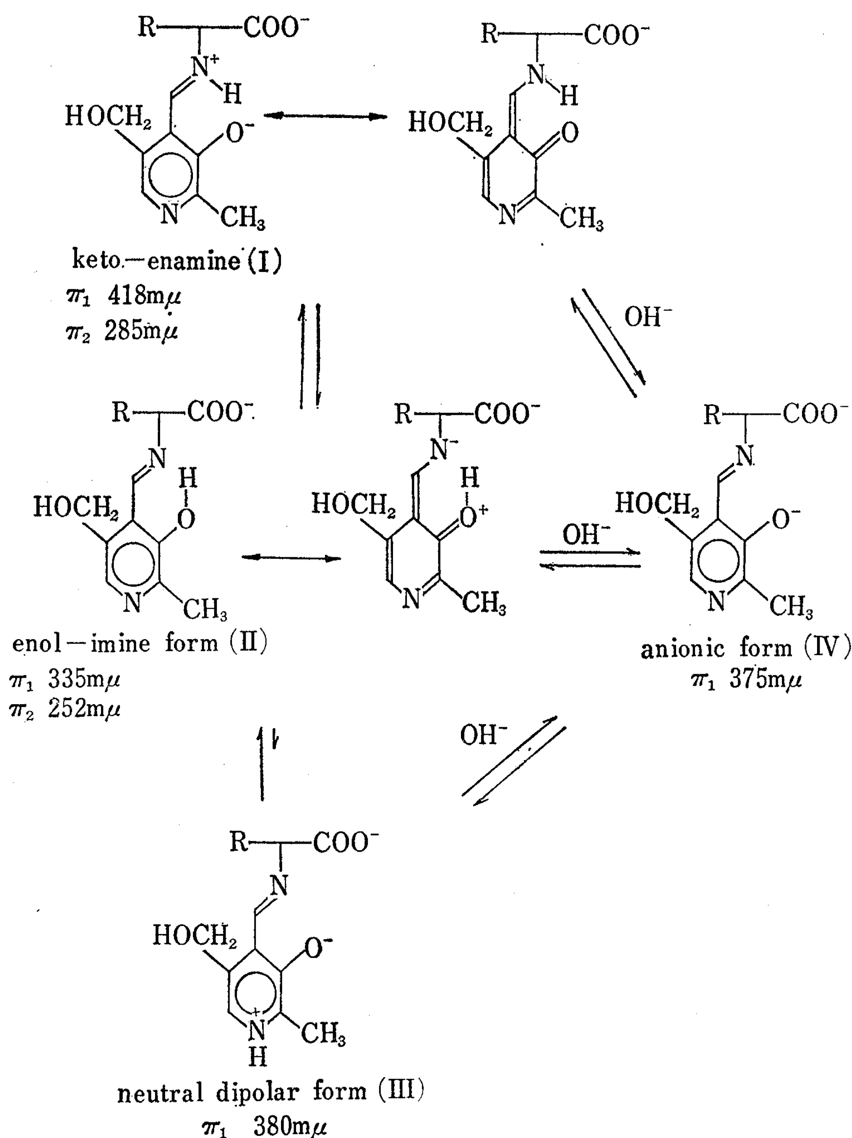

Chart 1. Equilibria of Pyridoxylidene Amino Acid in Methanol

glycine. However, wave lengths and relative intensities of absorption bands and rate of formation were affected by the nature of amino acids and amines. Amino acids and amines can be classified by these properties in the following three groups.

(1) Glycine type: Most amino acids belonged to this group. Spectral properties as well as the approximate rate of formation of Schiff bases of this group are compiled in Table I.

Schiff bases of this group of amino acids had intense bands at both 418 mμ and 335 mμ regions, indicating the presence of keto–enamine and enol–imine forms. Compared in different amino acids, intensities of the two bands were found to be negatively correlated, showing equilibria between two tautomeric forms were affected by amino acid residues. The δ value in Table I, which is the ratio of absorbances of the two bands, may be of a certain measure of the equilibria.

(2) Cystamine type: Spectral changes with time, when pyridoxal solution was mixed with cystamine is shown in Fig. 2. Benzylamine showed almost the same spectral change. These changes were more rapid than in the cases of glycine type. Final spectra had strong bands at 335 mμ and 255 mμ, but lacked intense band at 418 mμ and 285 mμ regions. This indicates that the Schiff bases of these amines exist almost only in enol–imine form.

(3) Glycinamide type: Spectral change with time when glycinamide was mixed with pyridoxal is shown in Fig. 3. Methyl glycinate, glycylglycine and methyl glycylglycinate

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Table I. Spectral Properties of Schiff Bases derived from Pyridoxal and Glycine Type Amino Acids

Amino acids	Rate of formation[a]	$\varepsilon_{max} \times 10^{-1}$ (418 mμ band)	$\varepsilon_{max} \times 10^{-1}$ (335 mμ band)	λ_{max} (250 mμ band)	δ[b]
β-Alanine	m.	132	422	252	0.31
Butylamine	rapid	131	389	251	0.34
Homocysteine	slow	122	347	257	0.35
Lysine	rapid	138	359	251	0.38
S-Methylcysteine	m.	159	386	252	0.41
Histidine	m.	122	378	253	0.44
2-Phenylglycine	m.	165	326	252	0.51
3,4-Methylene-dioxyphenylalanine	slow	210[c]	380	—	0.55
Glycine	m.	200	353	252	0.57
Tyrosine	slow	261[c]	379	—	0.61
Glutamic acid	slow	210	340	255	0.64
α-Alanine	slow	211	320	254	0.66
Serine	m.	221	363	255	0.68
Valine	m.	280	350	255	0.80
Aspartic acid	slow	302	310	260	0.97
5-Methoxytryptophan	slow	477[c]	472	—	1.01
5-Hydroxytryptophan	slow	547[c]	541	—	1.01
Tryptophan	slow	360[c]	353	—	1.02

a) Rate of formation of Schiff bases are expressed as compared with the case of glycine. m. indicates comparable rate as glycine.
b) $\delta = \varepsilon_{max}$ (418 mμ band)/ε_{max} (335 mμ band)
c) λ_{max} of 418 mμ bands were 422—424 mμ.

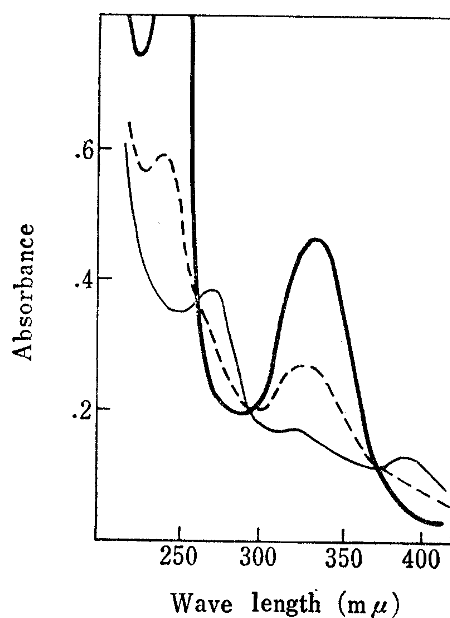

Fig. 2. Change of Electronic Absorption Spectra with Time for Methanol Solution containing 1×10^{-4}M Pyridoxal and 5×10^{-4}M Cystamine

initial spectrum
intermediate spectrum
final spectrum

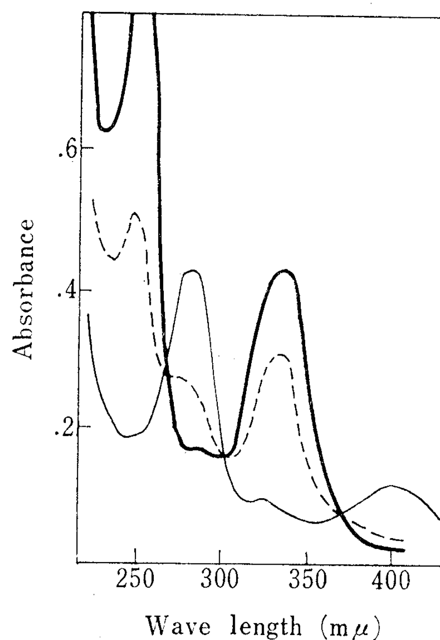

Fig. 3. Change of Electronic Absorption Spectra with Time for Methanol Solution containing 1×10^{-4}M Pyridoxal and 1×10^{-3}M Glycinamide

initial spectrum
intermediate spectrum
final spectrum

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showed almost the same changes. These changes were slower than the cases of the preceding two types. Final spectra showed only small intensities at 418 mμ and 285 mμ regions, but had intense bands at 335 mμ and at around 255 mμ, indicating the equilibria were in favor for enol–imine form.

Carbinolamine Formation

In Fig. 4 is shown the spectral change with time observed in methanol solution of 1×10^{-4} M pyridoxal and 1×10^{-3} M potassium sarcosinate at room temperature. Absorption bands at 398 mμ and 328 mμ which were assigned to pyridoxal species decreased the intensities and band at 280 mμ increased with slight red shift. Amino acids having a secondary amino group such as proline, N-phenylglycine showed analogous spectral changes. These changes were rapid compared to Schiff base formation.

Final spectra had a single peak at 282 mμ, which could be understood as a result of carbinolamine derivative formation as shown in Chart 2. It is reasonable

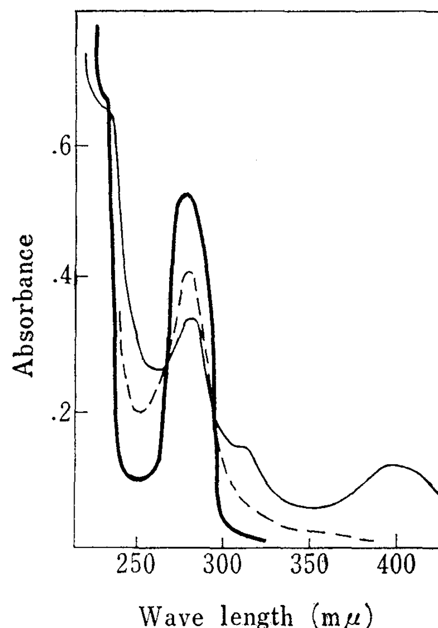

Fig. 4. Change of Electronic Absorption Spectra with Time for Methanol Solution containing 1×10^{-4} M Pyridoxal and 1×10^{-3}M K Sarcosinate

initial spectrum
intermediate spectrum
final spectrum

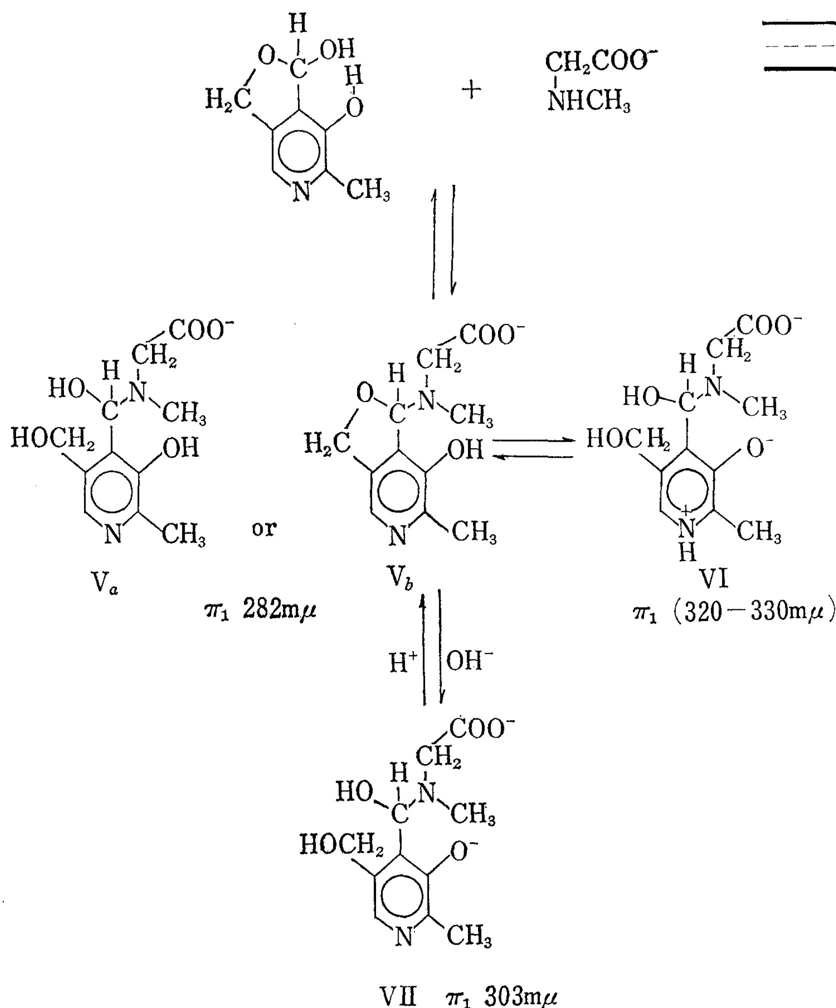

Chart 2. Formation and Equilibria of Carbinolamine Derivative

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to assume that species as V_a and V_b has π band at the same region as neutralhemi acetal species of pyridoxal.[3] However, to distinguish between V_a and V_b is impossible by electronic absorption data alone. Information from NMR data suggested the presence of V_a species.

Dissociation of phenol proton and protonation on pyridine nitrogen were known to produce red shifts to π bands. Neutral dipolar species (VI), then, is expected to have π_1 band at around 320—330 mμ. Inexistence of strong band at this region indicated the absence of this species in methanol. Addition of KOH to the methanol solution caused the decrease of 282 mμ band and the increase of new band at 303 mμ, which was assigned to π_1 band of anionic species (VII). In acidic methanol rapid decomposition to the components was observed.

Thiazolidine Derivatives Formation

When cysteamine (2–mercaptoethylamine) was brought to mix with pyridoxal, spectral change shown in Fig. 5 was observed. Final spectrum had a broad intense band at 295 mμ and a weak band at 330 mμ. Cysteine showed almost the same change except that the change was slower.

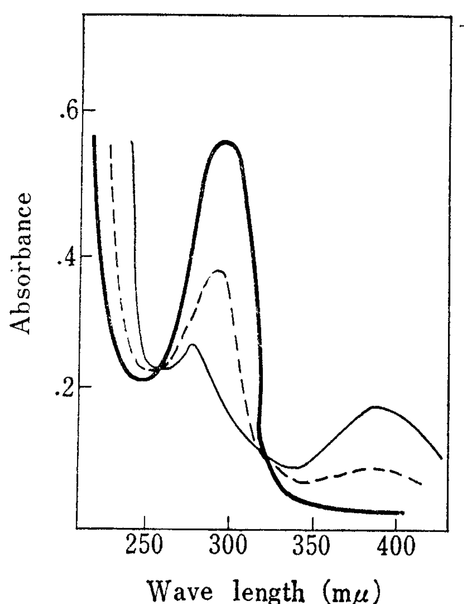

Fig. 5. Change of ElectronicAbsorption Spectra with Time for Methanol Solution containing 1×10^{-4}M Pyridoxal and 1×10^{-3}M Cysteamine

——— initial spectrum
– – – – intermediate spectrum
━━━ final spectrum

Heyl, *et al.*[6] prepared 4–thiazolidine–carboxylic acid derivatives from cysteine and penicillamine with pyridoxal in 80% ethanol and Buell and Hansen[7] observed the formation of thiazolidine derivative with λ_{max} at 325 mμ in aqueous solution containing pyridoxal phosphate and cysteine. From these facts, the spectral changes in methanol can be interpreted as thiazolidine ring formation as shown in Chart 3.

Bands at 295 mμ and 330 mμ are assigned to π_1 bands of neutral nonpolar and dipolar species, respectively. From the intensities, nonpolar species was supposed to predominate in neutral methanol. In acidic methanol only one intense band was observed at 305 mμ, which was assigned to π_1 band of cationic form. Here again observed the conformation to the empirical rules that dissociation of phenolic proton causes red shift while that of pyridinium proton does blue shift to π bands of hydroxypyridine derivatives. In alkaline methanol, it was observed that anionic species of the thiazolidine derivatives had π_1 band at 318 mμ and π_2 band at 245 mμ. These band assignments are summerized in Chart 4. 2–Thiazolidinyl group on 4 position of pyridine ring is seen to cause additional red shift of 15 mμ to hemiacetal of pyridoxal and of 5 mμ to aminomethyl group of pyridoxamine in each species.[3]

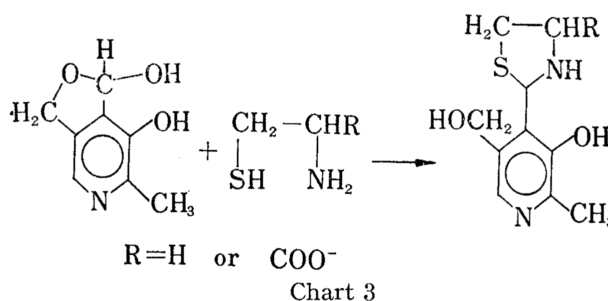

Chart 3

6) D. Heyl, S.A. Harris and K. Folkers, *J. Am. Chem. Soc.*, **70**, 3429 (1948).
7) M.V. Buell and R.E. Hansen, *J. Am. Chem. Soc.*, **82**, 6042 (1960).

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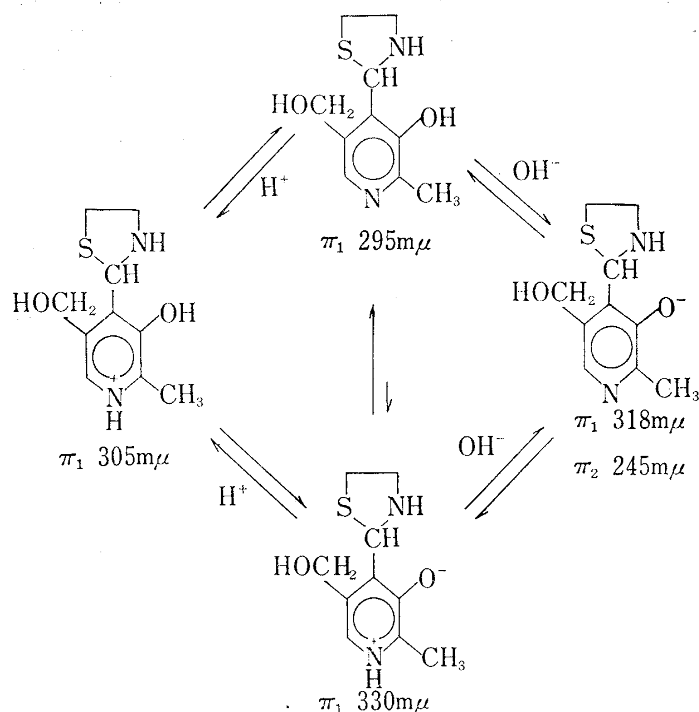

Chart 4. Solution Equilibria and Band Assignments of Thiazolidine Derivative in Methanol

From the absorbance at 295 mμ of solutions in which initial concentration of cysteamine varied, apparent formation constant of thiazolidine derivative could be calculated. The log value of the constant along with the analogous values of Schiff bases and carbinolamine is listed in Table II. While carbinolamine has fairly low value, that of the thiazolidine derivative is comparable to Schiff bases.

Table II. Apparent Formation Constants of Products between Pyridoxal and Amino Acids or Amines in Methanol

Amino acids or amines	Products	log K[a)]
Glycine	Schiff base	5.35
Valine	Schiff base	5.21[b)]
Cystamine	Schiff base	5.11
Sarcosine	carbinolamine	3.80
Cysteamine	thiazolidine	5.19

a) K=[Product]/[Pyridoxal][R–NH_2]
where [ ] indicates total concentration of the substances involved.
b) reference 3

In aqueous solution, formation of thiazolidine derivatives were also observed by π_1 band of dipolar species at 325 mμ, although formation constants were fairly small. The bands, however, were overlapped with the strong band of pyridoxal, which unabled the estimation of the formation constants.

Tetrahydropyridine Derivatives Formation

Spectral change with time when pyridoxal and histidine were mixed in methanol showed two step reaction. The first step (0—20 min), shown in Fig. 6A, consisted of red shift of 398 mμ band to 418 mμ and the increase of 335 mμ absorption, which indicated the formation of Schiff base. In the second step (20 min—6 hr) shown in Fig. 6B, bands at 418 mμ and 335 mμ began to decrease and final spectrum had a single peak at 290 mμ.

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Heyl, *et al.*[6] isolated colorless crystal from 80% ethanol containing pyridoxal and histidine and found to be a substance having an imidazotetrahydropyridine ring. Mackay and Shephard[8] found that pyridoxal phosphate and histidine condensed to a cyclic compound having an absorption at 320 mμ. Then, the spectral changes in methanol can be attributed to Schiff base formation and followed intramolecular cyclization.

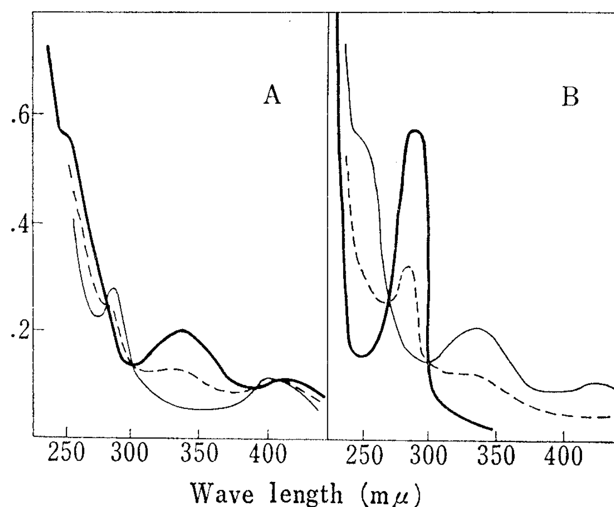

Fig. 6. Change of Electronic Absorption Spectra with Time for Methanol Solution containing 1×10^{-4}M Pyridoxal and 1×10^{-3}M K Histidinate

A; first step: B; second step
—— initial spectrum
- - - intermediate spectrum
━━ final spectrum

The cyclic product showed weak absorption at 330 mμ besides the strong band at 290 mμ. The former is assigned to π_1 band of dipolar species, while the latter to that of nonpolar species. By the addition of hydrogen chloride to methanol solution, a new absorption peak appeared at 299 mμ, which should be assigned to π_1 band of cationic species. In alkaline methanol, bands were observed at 315 mμ and 245 mμ, which were assigned to π_1 and π_2 bands of anionic species, respectively. These assignments are summerized in Chart 5.

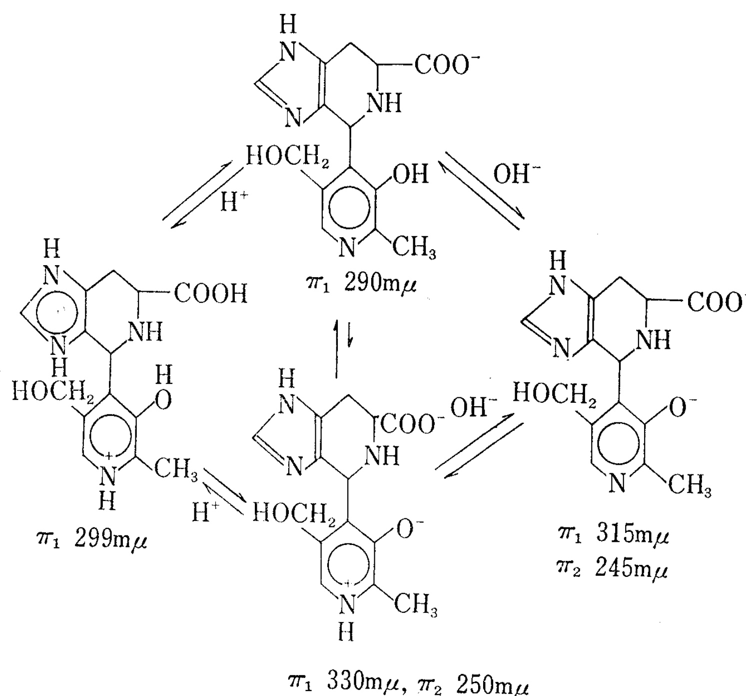

Chart 5. Solution Equilibria and Band Assignments of Cyclic Product from Pyridoxal and Histidine in Methanol

Histamine showed analogous spectral change as histidine. Methyl histidinate also underwent the same reaction with pyridoxal, though much slower. Upon standing at room temperature, methanol solution of Schiff base of pyridoxal with 5-hydroxytryptophan showed extremely slow decrease of 418 mμ and 335 mμ bands. This change was attributed to

8) D. Mackay and D.M. Shephard, *Biophys. Biochem. Acta*, **59**, 553 (1962).

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Table III. Reaction Rates of Schiff Base Formation of Pyridoxal and Followed Intramolecular Cyclization in Methanol

Compounds	Schiff base formation[a)]	Cyclization[b)]
Histamine	7 min	1 hr
Histidine	10 min	1.5 hr
Methyl histidinate	23 hr	2.5 days
Tryptamine	7 min	——
5-Methoxytryptamine	7 min	16 days
Tryptophanol	10 min	——
5-Methoxytryptophanol	10 min	10 days
Tryptophan	1.5 hr	——
5-Hydroxytryptophan	1.5 hr	4 days
5-Methoxytryptophan	1.5 hr	6 days
3,4-Methylenedioxyphenylalanine	1.5 hr	——

a) Rates of Schiff base formation were expressed by the time of completion of increase of 335 mμ band.
b) Rates of intramolecular cyclization were expressed by the half lives of decrease of 335 mμ band.

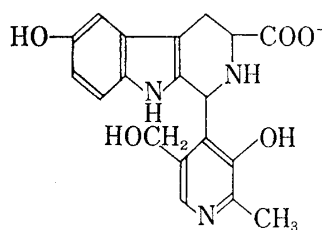

Chart 6

formation of cyclic product as VIII. 5-Methoxytryptophan showed analogous change, while tryptophan itself did not in the experimental condition employed. These were also true in tryptamine and tryptophanol series. Reaction rates of Schiff base formation and followed intramolecular cyclization in methanol are compiled in Table III.

No Spectral Change

When mixed with pyridoxal in neutral methanol, N,N-dimethylglycine, betaine, N-acetylglycine and N-acetylcysteine did not show any spectral change. That tertiary amines, quaternary ammonium and N-acyl groups do not react with aldehyde can be reasonably understood, though the possibility that thiol group in N-acetylcysteine reacts to form thioacetal cannot be excluded.

Discussion

As shown above, Schiff bases derived from pyridoxal and amino acids exist in the two tautomeric species, which have absorption bands at 418 mμ and 335 mμ regions. Then, the ratio of absorbances of the two bands appeared in Table I as δ values should give the relative abundance of one of the species to the other, that is to say Schiff bases of high δ values are relatively keto-enamine rich in methanol.

Following relations between the structures of amino acids and δ values may be induced.

a) Carboxylate group in the vicinity of amino group increased δ values. Keto-enamine form was absent in Schiff bases from cystamine and glycinamide type substances, which do not have carboxylate group. On the other hand, dicarboxylic acids as aspartic acid and glutamic acid had high δ values. Values of α- and β-alanine also hold this relation.

b) Aromatic rings tended to increase δ values.

c) Sulfur atoms in molecule lowered δ values.

d) Rates of formation were generally slow, when Schiff bases have high δ values.

Many reports[9)] have pointed out the absorption at 420 mμ in the pyridoxal enzymes bound with substrate. This suggests the keto-enamine type structure in the enzyme substrate complexes. If this is the case, partial positive charge on imine nitrogen would attract electron

9) P. Fasella, *Ann. Rev. Biochem.*, **36**, 185 (1967).

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from vicinal carbon atoms. This would stabilize the intermediate structure shown in Chart 7 and promote the enzyme reaction smoothly. Many nonenzymatic reactions concerning pyridoxal were reported to be catalyzed by metal chelate formation.[10,11] This could attribute to the fact that by chelation enol–imine type Schiff bases are converted to keto–enamine like structure. Although it is possible to assume that in the enzymes which do not contain metals, proteine structure is so arranged to favor keto–enamine form, it is interesting to note that amino acids which are the substrate of pyridoxal enzymes have generally high δ values.

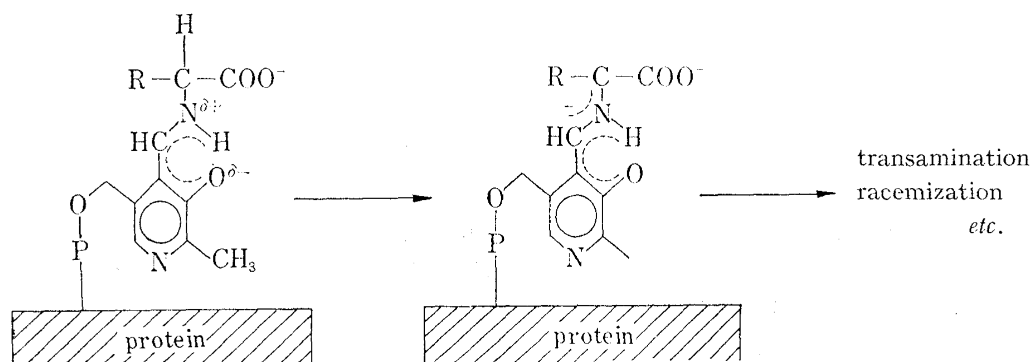

Chart 7

Schiff base from lysine was very much alike to that from butylamine both in spectral properties and in the rate of formation. This suggests that amino group which involved in Schiff base is ε– rather than α– amino group. Many workers[9] have proved that in some enzymes aldehyde group of pyridoxal form Schiff base type bond with ε–amino group of lysine in apoprotein. Formation of enzyme substrate complex would then proceed through transaldimination from pyridoxal lysine Schiff base to pyridoxal substrate amino acid Schiff base. Transaldimination is believed to proceed more rapidly than Schiff base formation from free aldehyde and amino groups.[12] This fact, with the present results, would suggest that enzyme substrate complex formation accompany the change of Schiff base from enol–imine rich type and by transaldimination, the disadvantages of slower rate of formation of Schiff base with high δ values are avoided.

Acknowledgement The author is indebted his knowledge and interests in pyridoxal to Prof. A.E. Martell, Department of Chemistry, Texas A & M University. He is also grateful to Prof. Z. Tamura, Faculty of Pharmaceutical Sciences, University of Tokyo, and to Dr. S. Akaboshi and Dr. T. Hino of this Institute for the discussion.

10) D.E. Metzler, *et al.*, *J. Am. Chem. Soc.*, **74**, 979 (1952); **76**, 648 (1954); **77**, 2431 (1955); **79**, 485 (1957).
11) Y. Matsushima and A.E. Martell, *J. Am. Chem. Soc.*, **89**, 1331 (1967).
12) E.H. Cordes and W.P. Jencks, *J. Am. Chem. Soc.*, **84**, 826 (1962).

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