Pyridoxal Analogs. IX. Electron Absorption Spectra and Molecular Species in Methanol Solution[1]

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Abstract: The electron absorption spectra of pyridoxal, pyridoxal phosphate, pyridoxamine, their Schiff bases with valine and α-ketoisovaleric acid, and metal chelates of the Schiff bases and of pyridoxamine have been measured at various concentrations of added acid or alkali in methanol solution. Assignments of the absorption bands are made and the equilibria between the various molecular species in methanol are established. The over-all ionization constants are calculated from the variation of absorbancy with concentration of acid or alkali. From the absorption band assignments made for pyridoxal, pyridoxal phosphate, and pyridoxamine, it is shown that in neutral methanol both polar and nonpolar forms are present in appreciable amounts. Aldimine Schiff bases are proved to be in enol-imine and keto-enamine forms in neutral methanol. The Cu(II), Ni(II), and Zn(II) chelates of aldimine Schiff bases and pyridoxamine were fairly stable, whereas spectra of Co(II) and Mn(II) chelates underwent gradual changes with time in neutral methanol. The solvation effects of pyridoxal in methanol are also described.

Electronic and infrared absorption spectra and solution equilibria of 3-hydroxypyridinecarboxaldehydes, their derivatives, and their Schiff bases with amino acids have recently been reported.[3-10] Information derived from these studies has made it possible to relate solid-state and solution spectra to structural variations resulting from tautomeric and solvation effects. The present work deals with an analysis of the electronic absorption spectra in methanol solution of pyridoxal, pyridoxal phosphate, pyridoxamine, their Schiff bases with amino acids or keto acids, and the corresponding Schiff base metal chelates, for the purpose of obtaining information on the molecular species present in solution. A study of this nature is an essential preliminary to the understanding of detailed kinetic studies on these systems, which will be treated in the following paper.

Methanol was selected as the solvent for these studies since it is the solvent most similar to water that gives relatively simple molecular species derived from pyridoxal, amino acid, and metal ion. In aqueous solution Schiff base formation is incomplete even when the concentration of amino acid is as much as 100 times the concentration of pyridoxal. Under these conditions metal chelate formation of the Schiff base would be greatly hindered by competing metal binding with the excess amino acid. Thus aqueous systems containing metal chelates of Schiff bases of pyridoxal would be complicated by equilibria with additional free ligands and metal complexes. The assignment of electronic absorption bands and the following of kinetics of individual species would thus encounter difficulties in water solution. These difficulties are largely avoided in methanol, in which Schiff base formation of pyridoxal is complete in relatively dilute solution in the presence of a two-or threefold concentration of amino acid.

The most extensive equilibrium studies made thus far on similar systems were those of Metzler, *et al.*,[11-13] who reported the aqueous spectra of the Schiff bases of pyridoxal, and of their metal chelates, but did not make complete assignments of the electronic spectral bands. Matsuo[14] measured the spectra in ethanol of the aldimine and ketimine formed from pyridoxal hydrochloride and α-aminobutyric acid, and from pyridoxamine dihydrochloride and α-ketobutyric acid. The nature of the molecular species in solution was not determined, however, and band assignments were not made.

In some cases methanol may be considered as good as water as a solvent for the examination of biochemical models. The active site of transaminase is such as to strongly favor Schiff base formation, a situation which is quite different from the reactions of aqueous pyridoxal with amino acids but quite similar to the reactions that occur in methanol. Hence this solvent may provide a better environment for the study of vitamin B_6 model systems than one would find in pure water solutions.

Experimental Section

Measurements. The electronic absorption spectra in the visible and ultraviolet regions were recorded at 27° with a Cary Model 14 PM spectrophotometer. All solutions were measured in a pair of calibrated silica cells. All spectral studies were carried out immediately after preparing samples, unless otherwise stated.

Materials. Spectrograde methanol was used directly as solvent and proved to give reproducible results. Pyridoxal hydrochloride, pyridoxal phosphate, DL-valine, and pyridoxamine dihydrochloride were obtained from commercial sources and used without further purification after verification of their purity by analysis. α-Ketoisovaleric acid was synthesized by Dr. E. Loeser of these laboratories. All inorganic compounds and metal acetates used were certified reagent grade chemicals.

Preparation of Solutions. Methanol solutions for spectral measurements were prepared so as to contain 1.0×10^{-4} *M* concentration of the species under investigation. The desired acid–base

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Table I. Electronic Absorption Spectra of Methanol Solutions of Pyridoxal and Related Compounds[a-c]

Compound	Conditions		λ_{max}		
Pyridoxal	Acidic	1×10^{-2} *M* HCl	290 (0.91)	230[s] (0.36)	
	Neutral		328 (0.10)	280 (0.46)	220[s] (0.67)
	Alkaline	5×10^{-2} *M* KOH	389 (0.42)	303 (0.41)	234 (1.04)
		2×10^{-2} *M* KOH	(0.46)	(0.35)	(1.12)
Pyridoxal phosphate	Acidic	1×10^{-2} *M* HCl	295 (0.79)	230[s] (0.30)	
	Neutral		340 (0.19)	289 (0.32)	249 (0.25)
	Alkaline	1×10^{-2} *M* KOH	390 (0.46)	306 (0.30)	232 (1.05)
Pyridoxylidene-valine	Neutral		418 (0.28)	337 (0.35)	255[s] (0.80)
	Alkaline	2×10^{-2} *M* KOH	373 (0.53)		
(5′-Phosphate)-pyridoxylidene-valine	Neutral		418 (0.34)	340 (0.28)	260[s] (0.66)
	Alkaline	2×10^{-2} *M* KOH	350 (0.53)		
Pyridoxamine	Acidic	1×10^{-2} *M* HCl	296 (0.82)		260[s] (0.21)
		1.5×10^{-4} *M* HCl	333 (0.23)	287 (0.44)	220 (0.78)
	Neutral		310[s] (0.24)	289 (0.41)	244[s] (0.27)
	Alkaline	1×10^{-2} *M* KOH	311 (0.69)	248 (0.67)	
Pyridoxamine metal chelates					
Zn(II)	Neutral		301 (0.59)	240 (0.43)	
Cu(II)	Neutral		302 (0.78)	244 (0.72)	
Ni(II)	Neutral		311 (0.51)	248 (0.54)	
Co(II)	Neutral		302 (0.53)	246 (0.64)	
Mn(II)	Neutral		291 (0.55)	245 (0.44)	
Pyridoxamine Schiff bases	Neutral	Plus 1.0×10^{-3} *M* K α-ketoisovalerate	310 (0.14)	286 (0.47)	
	Neutral	Plus 2.5×10^{-3} *M* K α-ketoisovalerate	310 (0.10)	285 (0.52)	

[a] Wavelengths are given in mμ. [b] Numbers in parentheses give the absorbance for a 10-mm light path and 10^{-4} *M*. [c] Superscript s indicates shoulder.

species were obtained in solution by adding standard methanol solutions of HCl or KOH, prepared by dissolving potassium hydroxide or gaseous hydrogen chloride in spectrograde methanol. Since pyridoxal phosphate is only sparingly soluble in acidic or neutral methanol, the desired pyridoxal phosphate solutions were prepared by suspending weighed amounts of pyridoxal phosphate with excess alkali in methanol, with occasional shaking, until a clear solution had been obtained. Aliquots of the solution were neutralized or acidified with HCl–methanol to give solutions which did not precipitate on standing and were thus suitable for spectral measurements. For the preparation of solutions of aldimine Schiff bases, DL-valine was dissolved in methanol by adding an equimolar amount of KOH, and the appropriate amount of pyridoxal hydrochloride or pyridoxal phosphate was then added. To aliquots of the Schiff base solutions thus obtained, calculated amounts of acid or alkaline methanol solution were added to obtain the desired Schiff base species.

Results and Discussion

Because of space limitations, it is not possible to present the many series of spectral curves measured in this investigation. Instead the values of λ_{max} and intensities of the main absorption bands of the systems studied are presented in Tables I–III. The quality of the spectra obtained may be judged from the sample curves presented in Figures 1–3. Figures 1 and 2 show the influence of acid and base, respectively, on the absorption characteristics of pyridoxamine and show the profound differences in absorption characteristics that result from the conversion of a relatively simple compound to its acid and base forms. The absorption spectrum of a typical Schiff base, pyridoxylidenevaline, is illustrated by Figure 3, and indicates the change of the spectrum that occurs when the Schiff base is converted to its alkaline form.

Table II. Electronic Absorption Spectra of Pyridoxal and Pyridoxamine in Neutral Methanol–Water Mixtures[a]

H_2O, %	λ_{max}, mμ		
	Pyridoxal		
0	328 (0.10)	280 (0.46)	
20	322 (0.23)	280 (0.37)	
40	320 (0.41)	280 (0.30)	256 (0.37)
60	319 (0.59)		255 (0.45)
80	318 (0.72)		254 (0.50)
	Pyridoxamine		
0	310 (0.24)	289 (0.41)	244 (0.27)
25	313 (0.38)	292 (0.34)	245 (0.37)
50	317 (0.46)		246 (0.39)
75	320 (0.54)		247 (0.42)
93.7	320 (0.70)		247 (0.43)

[a] Numbers in parentheses indicate absorbances.

The absorption characteristics of the neutral, as well as of the base, form (and the acid form, if any) of pyridoxal, pyridoxal phosphate, their valine Schiff bases, and of pyridoxamine, are given in Table I. Since it was essential to know the influence of water on the spectra, and since the direction of the band shifts with changing dielectric constant is useful in assigning spectral bands, the absorption characteristics of pyridoxal and pyridoxamine were measured as a function of solvent composition. These results are presented in Table II.

In Table III are given the absorption characteristics of various transition metal chelates of pyridoxamine, pyridoxylidenevaline, and pyridoxal phosphate valine. Information on the absorption characteristics of these three types of metal chelates is necessary to provide the basis for the kinetic studies to be reported in the subsequent paper.

Table III. Assignments of Absorption Bands of Schiff Bases

Schiff base	Metal	Ligand: metal	π_1 band[a,b]	π_2 band[c]	Band at 230 mμ	Changes of spectra with time	Conformity to Beer's law
Pyridoxylidene-valine	Zn(II)	2:1	385 (0.70)	271 (0.60)	230	None	+
	Cu(II)	2:1	390 (0.60)	271 (0.67)	227	None	+
	Ni(II)	2:1	393 (0.70)	270[s] (0.43)	233	None	
	Co(II)	2:1	383 (0.46)	270 (0.56)	231	Slowly changed	
	Mn(II)	1:1	382 (0.71)	271 (0.53)	233	Rapidly changed	+
Pyridoxal phosphate valine	Zn(II)	1:1	392 (0.57)	275 (0.63)	232	None	
	Cu(II)	2:1	385 (0.42)	270 (0.65)	229	None	
	Ni(II)	2:1	392 (0.51)	270[s] (0.38)	232	None	
	Co(II)	2:1	381 (0.37)		231	Rapidly changed	
	Mn(II)	1:1	388 (0.51)	275[s] (0.72)	234	Rapidly changed	+

[a] Wavelength for band is given in mμ. [b] Numbers in parentheses indicate absorbances. [c] Superscript s indicates shoulder.

It has been shown[3,9] that electronic absorption spectra of analogs of vitamin B_6 are characterized by two absorption bands, ascribed to the $\pi-\pi_1^*$ and the $\pi-\pi_2^*$ transitions (hereafter abbreviated as π_1 and π_2 bands, respectively) of the species present in solution. The hemiacetal cationic form IA, shown in Chart I. In neutral solution, absorption peaks and a shoulder were observed at 328, 280, and 220 mμ, and a very weak absorption was also observed at 400 mμ. The 328-mμ band is assigned to the π_1 band of dipolar

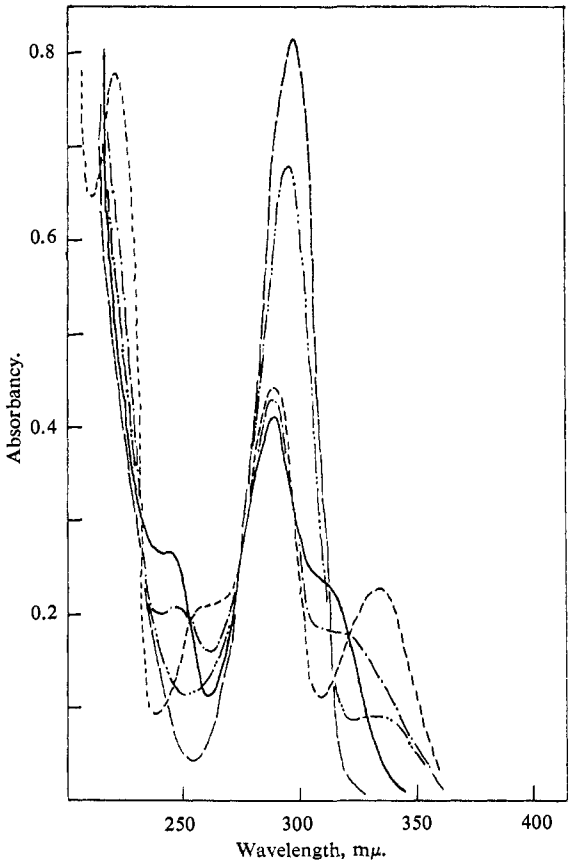


Figure 1. Electronic absorption spectra of 1.0×10^{-4} *M* pyridoxamine in acidic and neutral methanol: —·—·—, 1.0×10^{-4} *M* HCl; ----, 1.5×10^{-4} *M* HCl; —··—··—, 5.0×10^{-4} *M* HCl; — — —, 1.0×10^{-2} *M* HCl; ———, neutral solution.

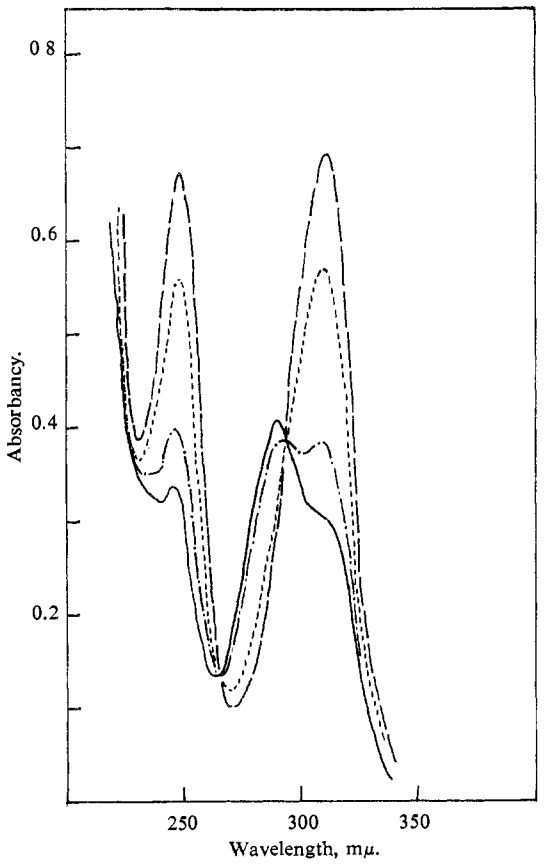
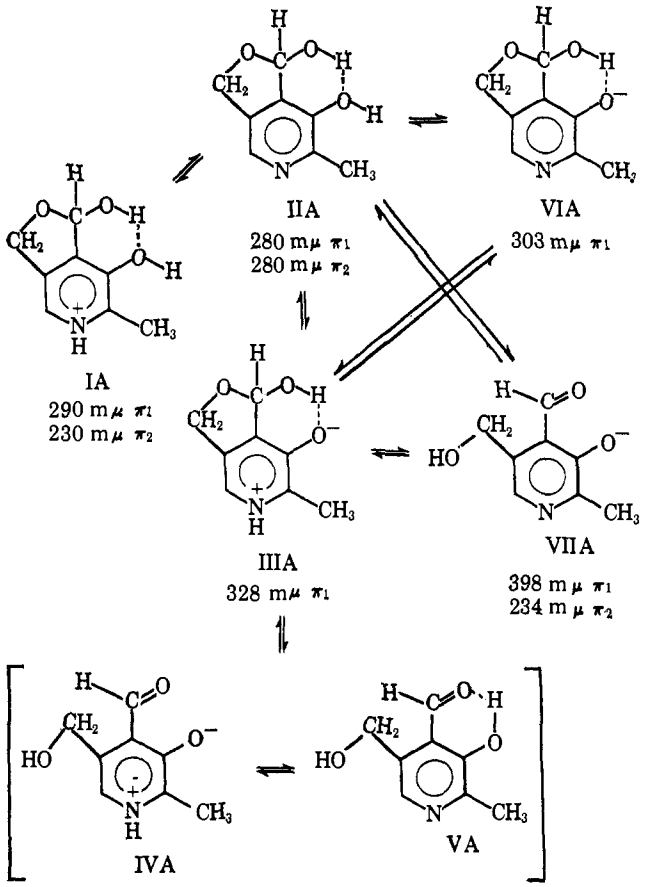


Figure 2. Electronic absorption spectra of 1.0×10^{-4} *M* pyridoxamine in alkaline methanol: ———, 1.0×10^{-4} *M* KOH; —·—·—, 2.0×10^{-4} *M* KOH; ----, 1.0×10^{-3} *M* KOH; — — —, 1.0×10^{-2} *M* KOH.

forbidden n–π transitions due to the pyridine nitrogen, carbonyl, or imine groups gave only very weak absorption bands, which were sometimes obscured by the strong π bands. Therefore the n–π transitionbands will be disregarded in the following band assignments.

Pyridoxodal. In acidic methanol solutions (Table I) there is a strong band at 290 mμ, and a shoulder at 230 mμ, which are assigned to the π_1 and π_2 bands of the hemiacetal form IIIA, the π_2 band of which is probably hidden by the lower wavelength side of the 280-mμ absorption. The 280- and 220-mμ peaks are assigned to the π_1 and π_2 bands of the nonpolar hemiacetal form IIA. The absorption band at 400 mμ indicated the presence of free aldehyde forms (*i.e.*, IVA and VA) in neutral solution, but the low intensity of the ab-

Chart I. Pyridoxal in Methanol



sorption indicated the existence of only small amounts of these species.

Spectral changes of pyridoxal in neutral methanol–water solutions that occur with a change of solvent composition are shown in Table II. As the water content increases, the intensity of the 280-mμ band, assigned to the nonpolar form IIA, decreases showing that the nonpolar form does not exist in appreciable concentrations in aqueous media. The considerable increase in absorbancy and the blue shift of the 328-mμ band provide support for the assignment of this band to the dipolar form, in accordance with the general behavior of band shifts with changes of solvent dielectric constant described in a previous paper.[3] The π_2 band of the dipolar hemiacetal form, not detectable in pure methanol, becomes observable as the aqueous content of the solvent increases.

In alkaline methanol solution, three absorption bands at 389, 303, and 234 mμ were observed. The 389-mμ band is assigned to the π_1 band of the aldehyde anion form VIIIA, and the 303-mμ band is assigned to the π_1 band of the hemiacetal anion VIA. The 234-mμ absorption is probably associated with the π_2 band of the aldehyde form rather than with the hemiacetal, because the general features of the observed changes in absorbancy resemble those of the 398-mμ band. On the addition of excess alkali, the equilibrium between aldehyde and hemiacetal shifts toward the formation of the aldehyde form. Thus the absorbancy of the 398-mμ band increases with an increase in concentration of alkali, while that of the 303-mμ band showed a slight decrease, in the strongly alkaline region. The solution equilibria and the corresponding band assignments are summarized in Chart I. Plots of the absorbancies of the bands *vs.* acid or base concentrations shown in Figure 4 were used to determine the over-all neutralization constants of the pyridoxal species in methanol.

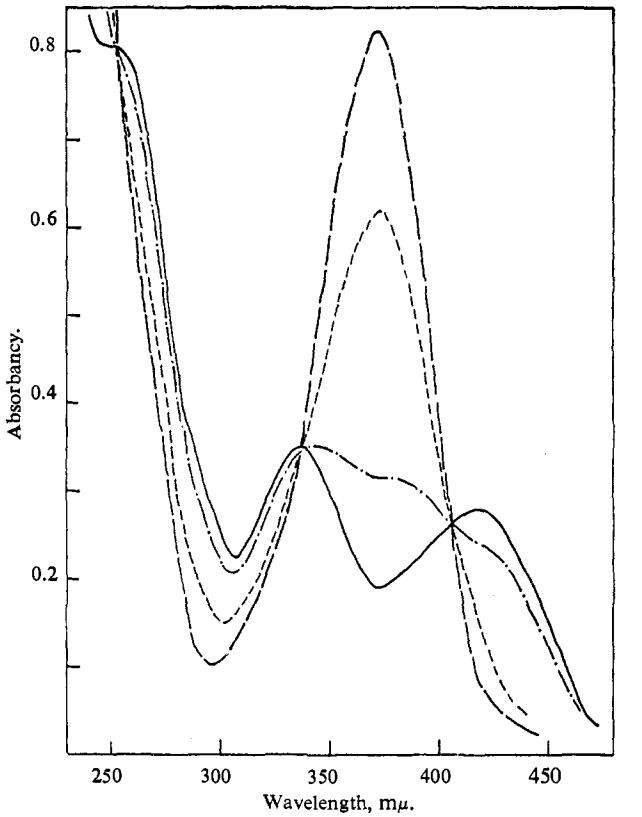


Figure 3. Electronic absorption spectra of 1×10^{-4} *M* pyridoxylidenevaline in methanol: ———, neutral and 1.0×10^{-3} *M* HCl; —·—·—, 1.0×10^{-3} *M* KOH; ----, 5.0×10^{-3} *M* KOH; — — —, 2.0×10^{-2} *M* KOH.

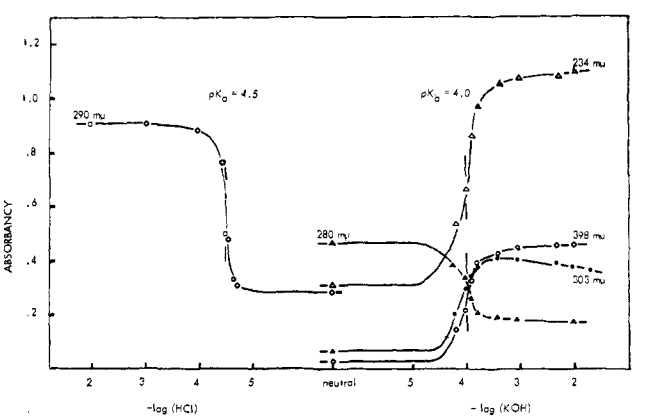
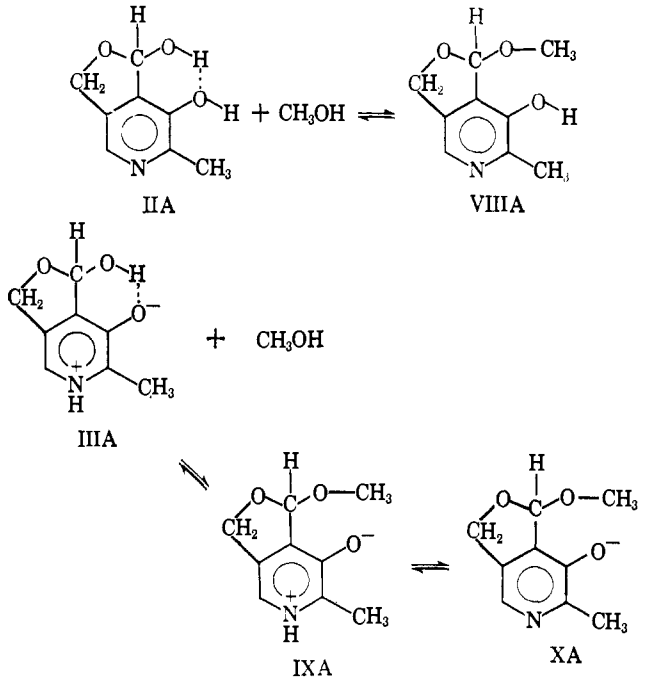


Figure 4. Variation of absorbancy for various absorption bands of 1×10^{-4} *M* pyridoxal as a function of HCl and KOH concentrations in methanol.

Solvation of Pyridoxal in Methanol. The electronic absorption spectra of pyridoxal in methanol showed gradual changes with time. Although the changes were slow, and not very noticeable in acidic or neutral solutions, the absorption maxima increased in intensity but the frequency did not change noticeably. These changes can be interpreted as probably involving acetal formation with the solvent as shown below.



It is a reasonable assumption that acetals VIIIA and IXA have approximately the same absorption characteristics as the parent hemiacetals such as IIA and IIIA, respectively. When KOH–methanol was added to this solvated acidic or neutral pyridoxal solution, a marked difference was observed from that of freshly prepared alkaline pyridoxal solutions. When solvated the 398-mμ band gave a smaller increase in absorbancy. A similar solvation effect was observed for ethanol solutions by Matsuo.[14] For more concentrated alkaline solutions, even a decrease in the absorbancy was observed. A decrease of absorbancy at 398 mμ in excess alkali was also observed by Metzler and Snell[12] for aqueous solutions.

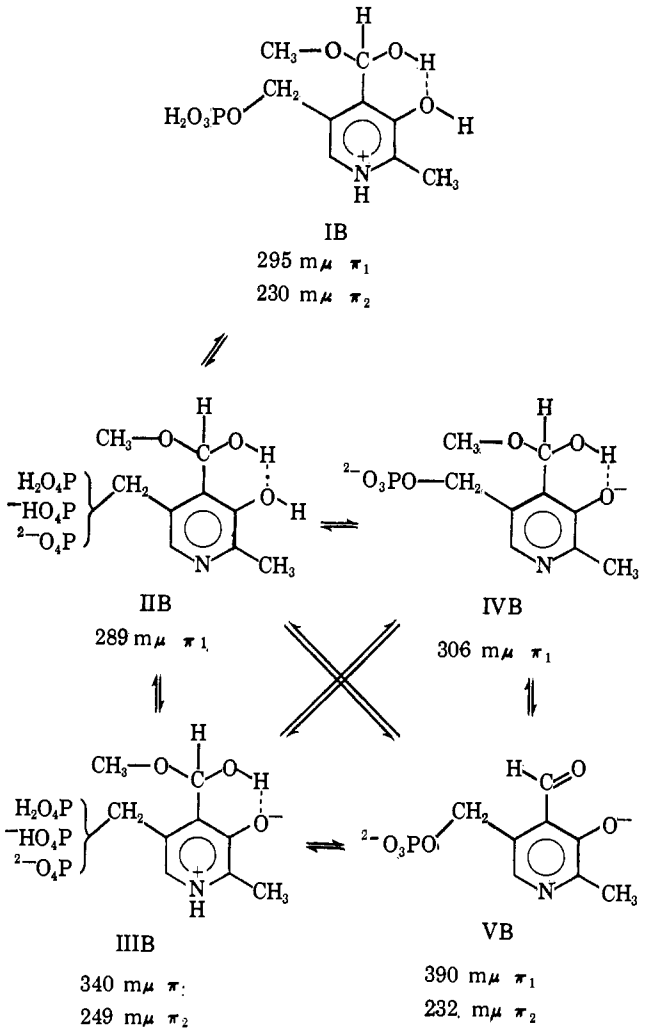
These spectral changes can be explained on the basis of reduction in intensity of the 398-mμ band of the aldehyde through the formation of solvated hemiacetal forms (VIIIA, IXA), followed by dissociation in excess alkali, thus producing a further decrease in the 398-mμ band through the formation of dissociated forms such as XA. It is noted that acetals of the type represented by XA could not undergo a second acid dissociation in excess alkali of the type described by Heinert and Martell[3] for the hydrated aldehyde species in aqueous solution. Such dissociation, however, is possible for the analogous hemiacetal VIA, and it is thus seen that appreciable acetal formation in pure methanol and ethanol would make the spectra (and species present) considerably different from that observed for aqueous solution at high pH.

For neutral and alkaline solutions, slow acetal formation was found to make a considerable difference in the amount of Schiff base formed if the pyridoxal solution was allowed to stand for more than a few hours. The noticeably lowered yield of Schiff base under these conditions (which was lowered further by continued standing over a period of days) indicated that acetal formation was irreversible (or nearly so) in alkaline solution and should be avoided if pure solutions of hemiacetal or Schiff base are desired.

When the spectra of neutral and alkaline pyridoxal–methanol solutions were observed over a period of days, the absorption bands changed in character and generally increased in intensity, presumably because of side reactions such as oxidation.

Pyridoxal Phosphate. The characteristics of the electronic absorption spectra of pyridoxal phosphate in methanol at various concentrations of acid and alkali are shown in Table I. The strong band at 295 mμ and the shoulder at 230 mμ, observed in acidic solution, are assigned to the π_1 and the π_2 bands, respectively, of the solvated cation (IB), shown in Chart II. In neutral solution three absorption peaks appear

Chart II. Pyridoxal Phosphate in Methanol



at 340, 289, and 249 mμ. The 340- and 249-mμ bands are assigned to the π_1 and π_2 bands of the dipolar solvated form IIIB, in accordance with pyridoxal band assignments. Dissociation of phosphate hydrogens would be expected to have little influence on these spectra. At 1.0×10^{-4} *M* concentrations of pyridoxal phosphate, the absorbancy at 340 mμ increased as the KOH concentration was increased to 1.0×10^{-4} *M*, and then decreased with further increase of KOH concentration. The 289-mμ band was assigned to the π_1 band of the nonpolar solvated form IIB, the π_2 band of which should appear around 215 mμ. In weakly alkaline solution (KOH was $0.5–3.0 \times 10^{-4}$ *M* at 1.0×10^{-4} *M* pyridoxal phosphate), the 289-mμ peak shifted toward longer wavelength and then decreased in absorbancy with an increase in KOH concentration. The slight red shift is probably due to the dissociation of phos-

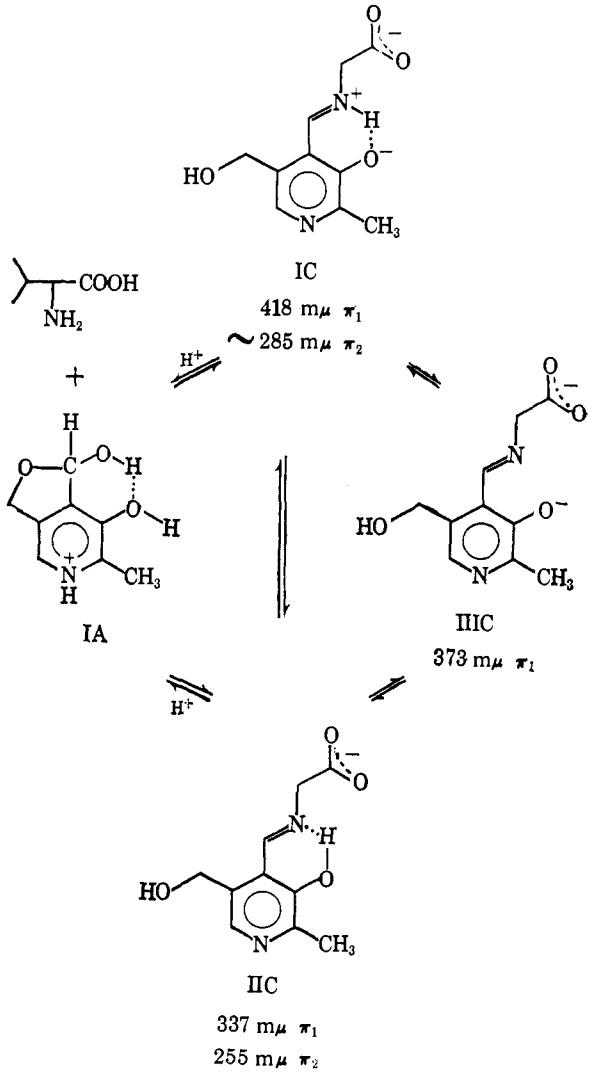
phate hydrogens. The total concentration of nonpolar solvated forms IIB has its maximum value in solutions containing 0.5–1.9 equiv of KOH (provided that dissociation of the phosphate hydrogens has little effect on the absorption coefficients of the nonpolar forms). Since there was no appreciable absorption at wavelength greater than 340 mμ, no significant amount of the free aldehyde form exists in acidic and neutral solution.

For strongly alkaline solutions, three absorption peaks are observed at 390, 306, and 232 mμ. The 390- and 232-mμ bands are assigned to the π_1 and π_2 bands of the unsolvated anion VB, while the 306-mμ band is assigned to the π_1 band of the solvated anion IVB.

The band assignments are summarized in Chart II, and changes of absorbancies with acid and alkaline concentrations along with the dissociation constants are shown in Figure 5, from which it is seen that the unsolvated anionic form VB is more stable than the solvated anion IVB in very strongly alkaline solutions.

Pyridoxylidenevaline. When pyridoxal and valine were dissolved together in methanol, the spectra obtained were completely different from the spectra of either compound alone. Valine has no appreciable absorption in the 240–450-mμ region at the concentration employed. Absorption spectra of the solutions in which 1.0×10^{-4} *M* pyridoxal was dissolved in ten times its concentration of valine are shown in Figure 3. Complete formation of the Schiff base of pyridoxal was demonstrated by the fact that exactly the same spectrum was obtained at higher valine concentrations. Clear isosbestic points resulted when the KOH concentration was changed.

Chart III. Pyridoxylidenevaline in Methanol



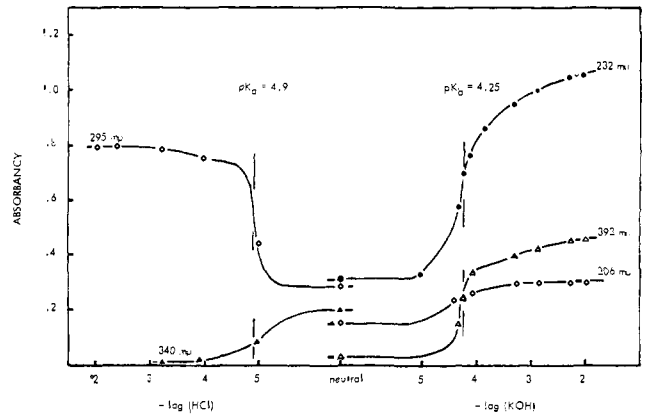


Figure 5. Variation of absorbancy for various absorption bands of 1.0×10^{-4} *M* pyridoxal phosphate as a function of HCl and KOH concentrations in methanol.

For weakly acidic and neutral solutions, four absorption bands were observed at 418, 337, 255, and 212 mμ. From the results described by Heinert and Martell,[6] the 418- and 285-mμ bands are assigned to the π_1 and π_2 bands of the ketoenamine form IC, and the 337- and 255-mμ bands to the π_1 and π_2 bands of enolimine form IIC, shown in Chart III. The single strong band observed at 373 mμ in alkaline solutions is assigned to the π_1 band of the anionic form IIIC. From the change of absorbancy at 373 mμ as a function of KOH concentration, the negative log of the dissociation constant, pK_b, of the Schiff base was calculated as 3.6.

In weakly acidic solution ([HCl] $\leq 1 \times 10^{-3}$ *M*), the absorption spectrum of pyridoxylidenevaline was the same as that observed for neutral solutions. On the other hand, the addition of more acid ([HCl] $\leq 2.5 \times 10^{-3}$ *M*) produced completely different spectra which were, however, the same as those obtained for pyridoxal in acidic methanol. This indicates rapid and complete decomposition of the Schiff base to pyridoxal and amino acid in acid solutions. In weakly acidic and neutral solutions, the Schiff base was found to be reasonably stable. In alkaline solution, however, the spectra underwent gradual changes involving both a lowering of intensity and a blue shift in the 373-mμ band. Band assignments for pyridoxylidenevaline under varying conditions are summarized in Chart III.

Spectra of solutions in which 1.0×10^{-4} *M* pyridoxal was dissolved with an equimolar amount of valine in methanol solution were similar to those obtained with excess valine, indicating the predominant species to be the Schiff base. However, the lowered absorption maximum and the lack of isosbestic points showed that Schiff base formation was incomplete in this solution. From absorption at 418 mμ, the log of the equilibrium constant for formation of the Schiff base was found to be 5.35 where

$$K = \frac{[\text{pyridoxylidenevaline}]}{[\text{pyridoxal}][\text{valine}]} = \frac{[\text{IC} + \text{IIC}]}{[\text{IIA} + \text{IIIA}][\text{valine}]}$$

Under these conditions, the Schiff base was found to dissociate by the addition of as little as 10^{-3} *M* HCl,

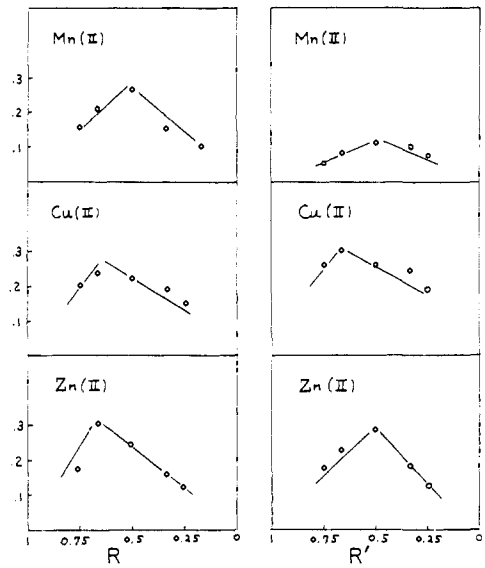


Figure 6. Application of the method of continuous variation for determining the metal chelates of Schiff bases. Optical densities at frequencies of maximum absorption of chelate are plotted against concentration of Schiff base, while the total concentration of metal salt and of Schiff base is maintained at 1.0×10^{-4} *M*; the metal ions involved are indicated on the individual figures; R = molar concentration of pyridoxylidenevaline; R' = concentration of pyridoxal phosphate valine.

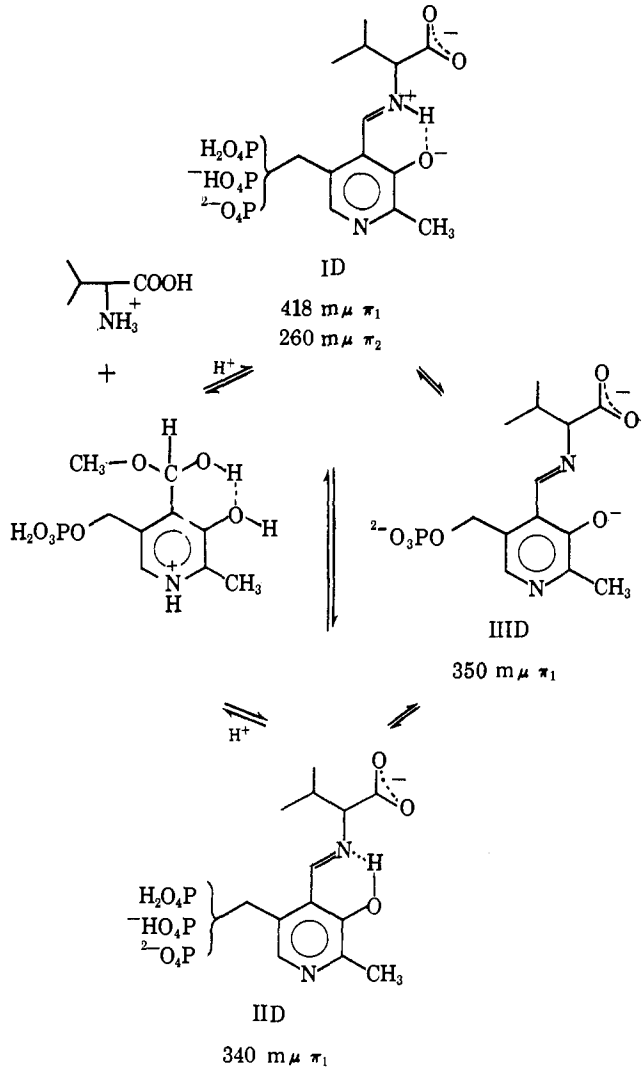
and the change of spectra in alkaline solution was more rapid than that observed for the Schiff base in the presence of a larger excess of valine. Equimolar mixtures of valine and a neutral pyridoxal–methanol solution that had been aged for a few hours or more failed to give the same spectra for the Schiff base as was obtained by the addition of pyridoxal to a methanol solution of valine, as is true of solutions containing excess valine, because of conversion of the hemiacetal of pyridoxal to the acetal.

Pyridoxal Phosphate Valine Schiff Base. When pyridoxal phosphate was dissolved in a methanol solution containing ten times its concentration of valine, the spectra taken immediately after mixing were poorly defined and did not give clear isosbestic points. However, the spectra of the solution changed gradually with time, finally giving the absorption bands indicated in Table I. On the basis of the similarity of the final spectrum with that of pyridoxylidenevaline, and in view of the formation of sharp isosbestic points, it was concluded that the final spectrum obtained is that of the pyridoxal phosphate valine Schiff base.

For weakly acidic and neutral solution, the absorption bands at 340 and 260 mμ are assigned to the π_1 and π_2 bands, respectively, of the enolimine form IID, shown in Chart IV. The π_1 band of the ketoenamine ID is found at 418 mμ, while the corresponding π_2 band must be hidden around 290 mμ. In alkaline solution there is only one band, at 350 mμ, which is assigned to the π_1 band of the anion IIID. As in the case of pyridoxylidenevaline, the addition of acid to the Schiff base solution gave the same spectrum as that of pyridoxal phosphate in acidic solution, indicating the decomposition of the Schiff base to pyridoxal phosphate and the amino acid. Absorption band assignments for this system are shown in Chart IV.

Metal Chelates of Aldimines. Characteristics of absorption spectra of methanol solutions containing

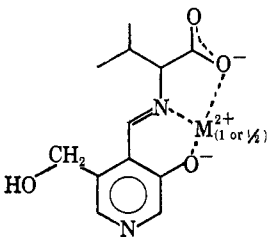
Chart IV. Pyridoxal Phosphate Valine Schiff Base



equimolar mixtures of various metal acetates and pyridoxylidenevaline are given in Table III. Studies of the variation of the spectra with a variation of the concentrations of the three components showed that chelate formation was essentially complete at 10^{-4} *M* concentrations of the components. The metal acetates employed have no appreciable absorption at the wavelengths and concentration range employed, except that the copper acetate solution has a weak but broad absorption band around 245 mμ. All the metal chelates of pyridoxylidenevaline had three absorption bands at 382–393, 270–271, and 227–233 mμ. The former two bands are assigned to the π_1 and π_2 bands, respectively, of the species X. The third band is unassigned.

The spectra of the Co(II) and Mn(II) chelates changed in frequency and intensity with time, indicating the species X to be unstable for these metal ions. For some metal chelates the method of continuous variation was employed to determine the composition of the chelate formed in solution as indicated in Figure 6. The results of these determinations are summarized in Table III, along with other information.

On the basis of the findings indicated in Table III, that the zinc (II)–pyridoxylidenevaline chelate contains a 2:1 molar ratio of ligand to metal ion, the equilibrium constant for the formation of the Zn(II) chelate was determined from the absorption spectra of equilibrium



X

382–393 mμ π_1
270–271 mμ π_2
227–233 mμ

mixtures of the components

$$K = \frac{[ZnL_2^{2-}]}{[Zn^{2+}][L^{2-}]^2} = 10^{11.5}$$

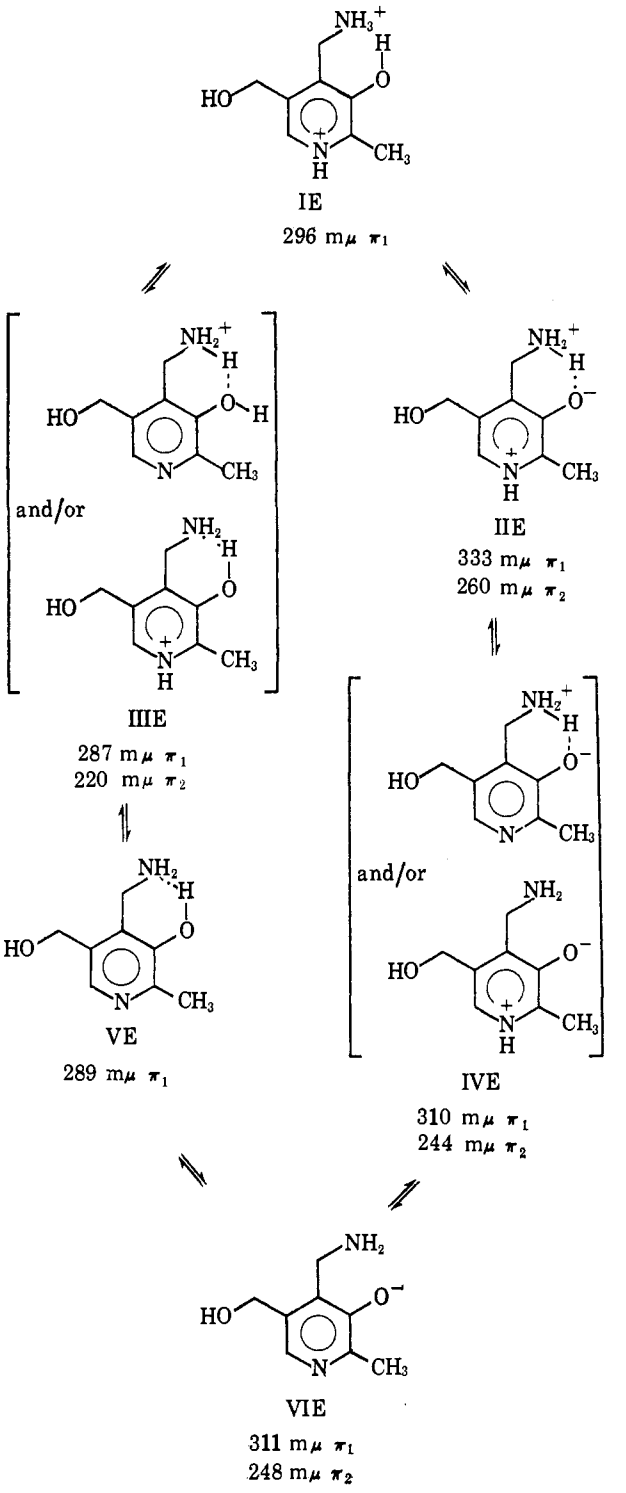
where H_2L represents the Schiff base, pyridoxylidene valine.

The results of similar studies of metal chelates of the Schiff base of pyridoxal phosphate and valine are shown in Table III. The spectra obtained were similar to those of the pyridoxylidenevaline chelates, except for an absorption band at around 340 mμ, which is probably due to unchelated Schiff base. At higher concentrations of metal acetate, some precipitation took place. However, this phenomenon was not observed in the case of pyridoxylidenevaline. The precipitate was probably due to conversion of the pyridoxal phosphate valine Schiff base to an insoluble Zn(II) salt containing a higher ratio of Zn(II) to ligand. This reaction is probably aided by the presence of the phosphate group. Further information on the metal chelates is summarized in Table III.

Pyridoxamine. Electronic absorption spectra of pyridoxamine in the presence of various concentrations of acid and alkali are shown in Figures 1 and 2. In strongly acidic solution, there is one strong absorption peak at 296 mμ, which is assigned to the π_1 band of fully protonated (*i.e.*, diprotonated) form IE, shown in Chart V. At lower HCl concentration, the intensity of the 296-mμ absorption decreased, with a shift to shorter wavelength (λ_{max} 287 mμ), and new bands appeared at 333, 260, and 220 mμ. These bands are assigned to the monoprotonated forms. Applying the general rule of the direction of frequency shift with dissociation of a phenolic group used by Heinert and Martell,[3] the 333-mμ band is assigned to the π_1 band of the monoprotonated dipolar form IIE. On the basis of the change of absorbancy with HCl concentration, the absorption band at 260 mμ is assigned to the π_2 band of the monoprotonated dipolar species. The 287- and 220-mμ bands are assigned to the π_1 and π_2 bands of the monoprotonated form, with the proton residing either on the pyridine N or amine N (IIIE), since little spectral change is to be expected for the conversion from a fully protonated to a monoprotonated "nonpolar" species.

In neutral solution, two absorption peaks and one shoulder were observed at 310, 289, and 244 mμ, respectively. The spectra in methanol–water mixtures indicated in Table II show that, with an increase of water content, the intensities of the 310- and 244-mμ bands increase, with red shifts of the absorption peaks,

Chart V. Pyridoxamine in Methanol



whereas the 289-mμ band disappears. Because of this behavior, the 310- and 244-mμ bands are assigned to the π_1 and π_2 bands of the dipolar neutral form IVE, and the 289-mμ absorption is assigned to the π_1 band of the nonpolar form VE.

For alkaline methanol solutions, the two absorptions observed at 311 and 248 mμ are assigned to π_1 and π_2 bands of the anionic form VIE. The changes of absorbancies of bands as a function of acid and alkali concentrations, along with dissociation constants calculated from these shifts, are shown in Figure 7, and the band assignments are shown in Chart V.

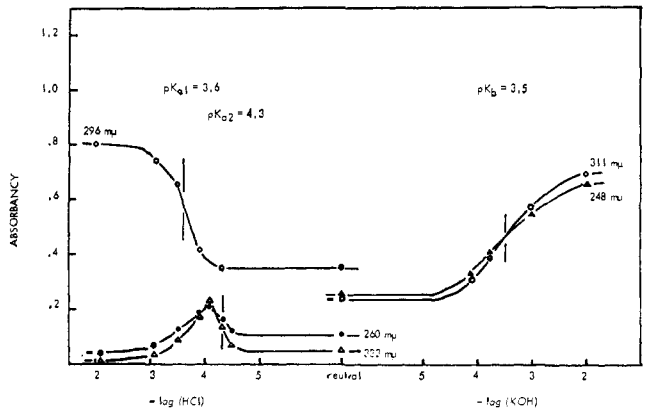
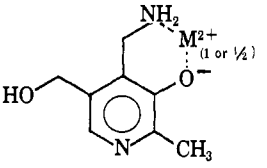


Figure 7. Variation of absorbancy of various absorption bands of 1.0×10^{-4} *M* pyridoxamine as a function of HCl and KOH concentrations in methanol.

Pyridoxamine Metal Chelates. Characteristics of the absorption spectra obtained when various metal acetates were added to pyridoxamine in neutral methanol solution are given in Table III. In the case of Zn(II), Cu(II), and Ni(II), clear, stable absorption spectra consisting of two absorption bands each were obtained. The absorption observed at 300–310 mμ is assigned to the π_1 band, and 240–248 mμ absorption is assigned to the π_2 band of metal chelate XI.

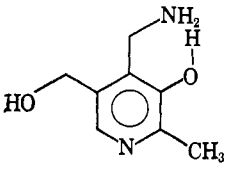


XI

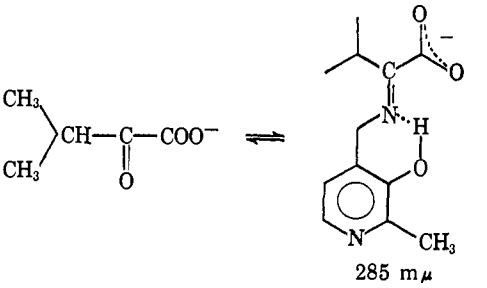
300–310 mμ π_1
240–248 mμ π_2

In the case of Mn(II) and Co(II), unstable absorption spectra which underwent gradual changes of frequency and intensity with time were obtained, presumably because of oxidation–reduction reactions involving the metal ion. The same phenomena were observed for the pyridoxylidenevaline chelates of these metal ions. In the case of metal chelate formation by both pyridoxylidenevaline and pyridoxamine, the same order of metal ions was observed for the extent of the blue shift which occurred in the absorption maxima when the proton attached to the nitrogen atom is replaced by the divalent metal. The order for the blue shift observed was Mn(II) > Co(II) ~ Zn(III) > Cu(II) > Ni(II).

Interaction of Pyridoxamine with Potassium α-Ketoisovalerate. An equimolar mixture of pyridoxamine and potassium α-ketoisovalerate in methanol solution had approximately the same spectrum as does pyridoxamine itself. However, under conditions such that the keto acid is ten times as concentrated as pyridoxamine, the spectra of the mixture showed gradual changes with time at room temperature. The shoulder at 310 mμ decreased in intensity, and the absorption peak at 289 mμ showed a slight shift toward shorter wavelength. These changes were completed in several hours. The spectra thus obtained are indicated in Table I. Potassium α-ketoisovalerate showed no appreciable absorption at 260 mμ and at the longer wavelength region over the concentration range employed. These results indicated the partial formation of Schiff base (ketimine), in accordance with the following equation.



289 mμ



285 mμ

It is a reasonable assumption that there is little difference between the electronic absorption spectrum of the ketimine and that of the nonpolar neutral form of pyridoxamine. The equilibrium constant for the formation of the ketimine Schiff base was not determined since the absorption spectra of the ketimine and of the present pyridoxamine are very similar, rendering the spectroscopic measurements relatively insensitive to the degree of ketimine formation. This situation was further aggravated by the relative instability of the ketimine solutions, as the result of transamination and possibly some side reactions.