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Spectra of 3-Hydroxypyridines. Band-Shape Analysis and Evaluation of Tautomeric Equilibria†

David E. Metzler,* Carol M. Harris, Robert J. Johnson, Donald B. Siano, and J. A. Thomson

ABSTRACT: Absorption spectra of individual ionic and nonionic forms of pyridine, pyrazine, phenol, and 19 3-hydroxypyridines have been measured and have been described as sums of log normal curves. The log normal parameters are tabulated and are discussed. In favorable cases band positions are located to $\pm 10\ cm^{-1}$ (± 0.1 nm at 316 nm) and band widths are measured to $\pm 0.5\%$. It is concluded that band width is a highly reproducible quantity that can be used as an index of homogeneity and as an indicator of alterations in chemical environment. The data obtained provide the basis for precise resolution of overlapping spectral bands. Limits of error are considered as are the effects of minor "buried" absorption bands. The latter have been identified in spectra of highly purified 3-hydroxypyridines in uncharged and anionic forms. They are absent in cationic and dipolar ionic forms, however. Their origin is uncertain. A new method for evaluating tautomeric equilibria depends upon the observation that for many pure substances, areas of absorption bands are constant with changes in solvent composition or temperature. Precise resolution of overlapping absorption bands at two or more temperatures or in two or more solvent mixtures permits evaluation of the tautomerization constant. The latter, together with stepwise pK_a values, allows evaluation of microscopic dissociation constants. The analysis has been extended to a system of three tautomers with closely overlapping bands (5-deoxypyridoxamine). Band-shape analysis often reveals hidden vibronic fine structure in absorption bands. We show that distinct changes in the fine structure of the pyridoxamine phosphate spectrum take place upon binding to the apoenzyme of aspartate aminotransferase.

The use of log normal distribution curves, fitted by a computer-assisted iterative process to experimental data (plotted against wave number), permits a precise mathematical description of electronic absorption spectra. This *band-shape analysis* has been applied to spectra of 3-hydroxypyridines (Siano and Metzler, 1969; Johnson and Metzler, 1970), proteins (Metzler *et al.*, 1972), potassium iodide (Siano and Metzler, 1972), purines, pyrimidines, and other substances (Metzler *et al.*, 1973). The method is in general superior to the use of gaussian curves to resolve spectra plotted against wavelength. It provides information about band shape as well as intensity and position. It also provides a convenient way to visualize fine structure in absorption bands, *e.g.*, those of proteins (Metzler *et al.*, 1972).

The present paper documents the utility of the log normal distribution curve in the quantitative description of spectra of substituted pyridines and of free and enzyme-bound derivatives of vitamin B_6. A new method of evaluating tautomeric equilibria is described.

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Experimental Procedures

Sources of compounds (for numbering see tables) studied are as follows: compounds **2** and **4–8** were purchased from Aldrich Chemical Co; **9**, **11**, **13**, **15**; **19**, and **22** were from Sigma Chemical Co.; **1** was from Baker; and **3** was from Mallinckrodt. The following were gifts: 6-methylpyridoxine, from M. Karpeisky; isopyridoxamine phosphate from A. Pocker; 3-hydroxy-4-pyridinecarboxaldehyde from M. O'Leary. Other compounds were prepared in this laboratory using published procedures (see Korytnyk and Ikawa, 1970). Samples of 3-methoxypyridine (compound **6**) were prepared from 3-hydroxypyridine by treatment with diazomethane in anhydrous methanol by T. Fisher and by T. D. Bolden in this laboratory. The solution was acidified with aqueous HCl and extracted with ether; then the aqueous phase was made basic. The product was extracted with ether and crystallized from methanol and ether. Deoxypyridoxamine was synthesized by T. Fisher by reduction of the oxime of 5-deoxypyridoxal (Fisher, 1971; Testa and Fava, 1957). In every case we have attempted to obtain highly pure samples and have recrystallized several compounds to constant spectrum. Usually little change was observed except at the high-energy end in which improvement was often obtained by recrystallization from "Spectroquality" methanol and water. For some compounds

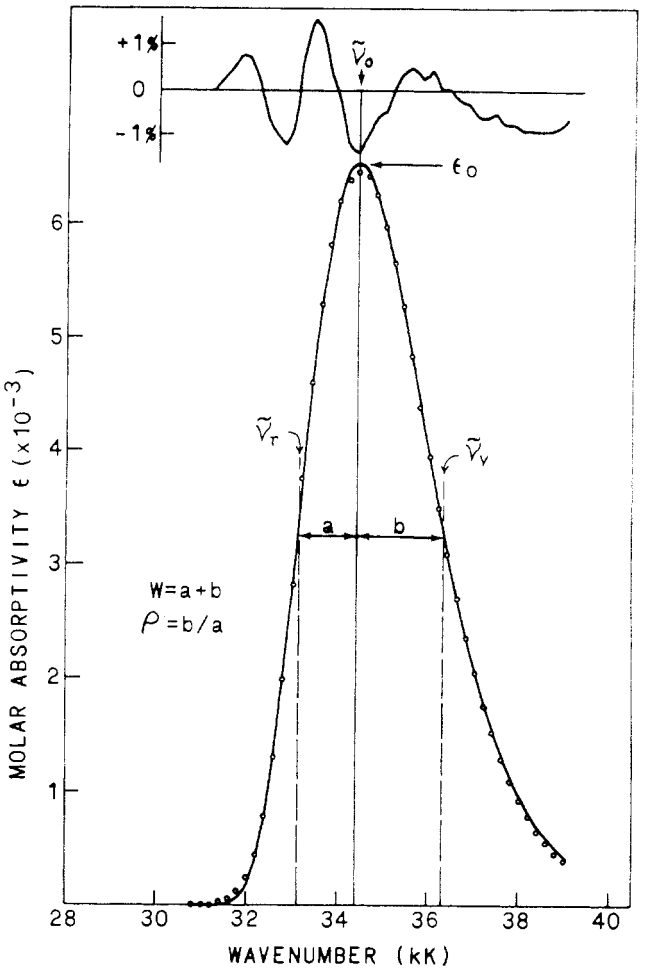


FIGURE 1: Spectrum of the cation of 5-deoxypyridoxine with definitions of band parameters. The band position is $\tilde{\nu}_0$ and the peak height ϵ_0. W is the width at the height $\epsilon_0/2$ and the skewness ρ is the ratio of the segments b/a. The points are experimental and the solid line is a log normal curve fitted to the points. At the top of the figure the differences between the experimental points and the corresponding points on the fitted curve are plotted as a percentage of the peak height.

(*e.g.*, 4-deoxypyridoxine) repeated crystallization did not remove a small amount of impurity contributing a weak absorption band. Phenol was sublimed before use.

"Spectroquality" methanol and dioxane (Matheson Coleman and Bell) and redistilled water were used. Reagent grade HCl, NaOH, and buffer salts were employed.

Spectra were recorded with a Cary Model 1501 spectrophotometer equipped with a Cary-Datex digital output system and an IBM keypunch. Absorbances from 0 to 2.0 were recorded to the nearest 0.001 unit at regular intervals of wave number (either 0.2 or 0.05 kK; 1 Kayser (K) = 1 cm^{-1}). The wave-number scale of the spectrophotometer was periodically calibrated against benzene vapor and against a solution of neodymium chloride as follows: the highest peak of the spectrum of benzene vapor, at 39.53 kK [lit. 39.53 (Garforth *et al.*, 1948; Lang, 1961)], was scanned and recorded with the dynode voltage setting at 3, sensitivity 1, and a sampling interval of 0.01 kK. To avoid error in the starting point a 1-kK range from 38.4 to 39.4 was scanned at slow speed with absorbances recorded at intervals of 0.2 kK. Immediately after the absorbance at 39.4 kK was punched, the recording was stopped, the sampling interval was changed to 0.01 kK, and a new card was injected into the system. This ensured that the first absorbance recorded would be at 39.41 kK. The peak position could easily be determined to the nearest 0.01 kK. Within this limit no change in wave-number calibration occurred during the course of the present work. The spectrum of neodymium chloride was recorded in a similar fashion at a dynode voltage setting of 7. Peak positions of 13.53, 13.68, 17.39, 19.19, 19.23, 28.25, 28.52, and 28.85 were reproducible to ± 0.01 kK over a period of 1 year.

Absorption spectra of individual ionic forms of the 3-hydroxypyridines together with the apparent pK_a values were evaluated from experimental spectra at various pH values in buffered solutions: pH 0–2.0, $HClO_4$ or HCl; pH 3.5–5.1, acetate; pH 5.1–8.4, KH_2PO_4 + K_2HPO_4; pH 8.6–8.9, phosphate–carbonate; pH 9.5–10.6, bicarbonate; pH 10.5–12.9, NaOH. An ionic strength of ~0.2 was maintained. The apparent acid dissociation constants are defined as $K_a = a_{H^+} \cdot [A^-]/[HA]$ where $a_{H^+} = 10^{-pH}$, as calculated from a pH meter reading, and $[A^-]$ and [HA] are molar concentrations. Computations were done either by the "swing method" of Nagano and Metzler (1967) or by a method of steepest descent.[1]

Spectra of 3-hydroxypyridine and pyridoxine in solvent mixtures were prepared by dilution of a stock solution of the crystalline free bases into mixtures of spectroquality solvent plus redistilled, boiled, neutral water.

Resolution of overlapping spectral bands was accomplished with a weighted least-squares minimization computer program developed in this laboratory. Several versions of this program have been developed. One uses all data points and adjusts parameters for all the bands at once. Another employs a "sliding window" and moves from low energy to high adjusting parameters on two peaks at a time. A third version, used extensively in the present work, examines data for a given band only up to some truncation point (selected by the user) on the high-energy side of the curve. After the first band has been fitted the parameters for that band are held constant and data are added up to the second truncation limit. Parameters are then evaluated for the second band, etc.

The possibility that small amounts of hard-to-remove impurities remain in some samples must be considered. To minimize the effect of such impurities we find it desirable to truncate the data on the low-energy side of the first band. In the program used the data were truncated at $\tilde{\nu} = (\tilde{\nu}_0 - a - 0.5)$ kK where a is as defined in Figure 1. The truncation has little effect on the parameters found but tends to result in a slight increase in skewness.

An important feature of the program is the option of fixing any of the parameters at preselected values. As explained later this possibility is particularly important in resolution of spectra containing "buried" or heavily overlapping bands. Computations were performed at the Iowa State Computation Center on an IBM 360-60 computer. Programs written in Fortran IV are available on request.

Results

Quantitative Descriptions of Spectra with Log Normal Curves. Curve fitting may be done on plots of molar absorptivity against either wavelength or wave number. The latter is preferable (Wald, 1965) because wave number is proportional to energy. Bandwidths in wave numbers are often nearly constant from compound to compound and a regular spacing between vibronic fine structure bands may be observed.

[1] Written by William A. Baldwin.

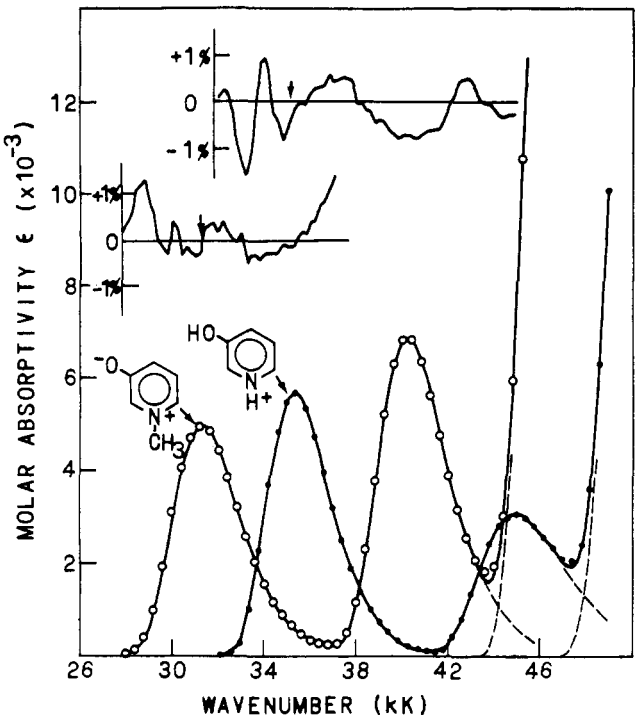


FIGURE 2: Spectra of the cation of 3-hydroxypyridine and of the dipolar ion of 3-hydroxypyridine *N*-methochloride. Each is fitted by the sum of three log normal curves. To avoid crowding on the graph every second experimental point has been omitted. Portions of the difference plots are shown; the small arrows above the difference plots locate the positions of $\tilde{\nu}_0$.

Absorption bands of most organic substances are skewed toward the high-energy end. The overall band shape can be described by four parameters: position $\tilde{\nu}_0$, height ϵ_0, width W measured at $\epsilon_0/2$, and an index of skewness. We use for the latter the ratio $\rho = b/a$ where b and a are the segments at half-height as indicated in Figure 1. The log normal (eq 1)

$$\epsilon(\tilde{\nu}) = \epsilon_0 \exp - \left\{\frac{\ln 2}{(\ln \rho)^2}\left[\ln\left(\frac{(\tilde{\nu} - \tilde{\nu}_0)}{W}\frac{(\rho^2 - 1)}{\rho} + 1\right)\right]^2\right\} \quad (1)$$

$$\tilde{\nu} > \tilde{\nu}_0 - \frac{W\rho}{(\rho^2 - 1)}$$

$$\epsilon(\tilde{\nu}) = 0; \qquad \tilde{\nu} < \tilde{\nu}_0 - [W\rho/(\rho^2 - 1)]$$

is a very suitable fitting function which approaches the gaussian in the limit as ρ approaches 1.0 (Siano and Metzler, 1969). A convenient computer program evaluates the four band parameters and also computes the area (𝒜) of each band (see Experimental Procedures).[2]

Typical good fits are presented in Figures 1–3. A single log normal curve serves to describe the well-isolated low-energy band of the cationic form of 5-deoxypyridoxine (Figure 1). Most of the cations and dipolar ions of the substances studied can be described nearly quantitatively by summing three log normals as shown in Figure 2 for the cation of 3-hydroxypyridine and for the dipolar ion of *N*-methyl-3-hydroxypyridinium chloride. The three bands correspond to the three transitions (^1L_b, ^1L_a, and B_a) predicted by molecular orbital calculations.[3] They will be designated here simply as bands I, II, and III in order of increasing energy.

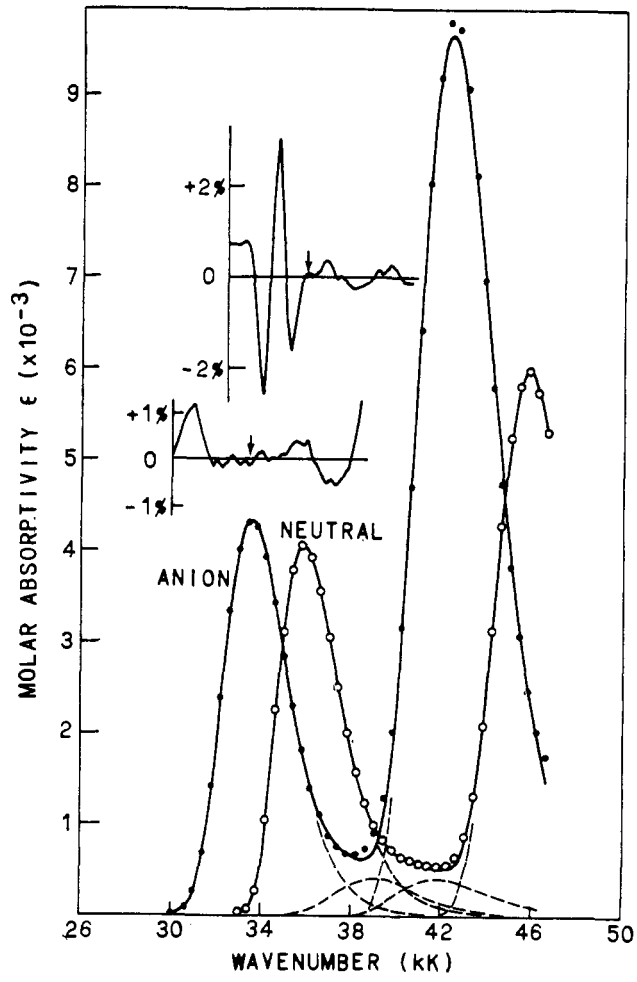


FIGURE 3: Spectra of neutral (in methanol) and anion forms of 3-hydroxypyridine. To obtain a good fit it was necessary to add minor bands in the valleys. Note how clearly the vibronic fine structure in the spectrum of the neutral form is displayed in the difference plot. Every second experimental point has been omitted.

The misfit between the experimental points and the fitted curve is conveniently displayed as a percentage of the height of one of the peaks, usually peak I. Portions of these difference plots are shown in Figures 1–4 and 7–9. There is sometimes a small amount of systematic misfit near the bases of the peaks and there are, for the cations and neutral forms, characteristic peaks in the difference plots which represent buried vibronic fine structure. It should be noted that the

[2] Although best results are obtained by taking data directly in digital form and using a least-squares procedure to fit spectra, band parameters can also be determined quite accurately by hand from a carefully recorded spectrum against wavelength. Locate the peak wavelength λ_0 by finding the band center at two or three values of ϵ in the upper quarter of the band and drawing a line (slightly inclined to the perpendicular) through them. The intersection with the peak gives λ_0 and ϵ_0. Wavelengths λ_r and λ_v corresponding to $\tilde{\nu}_r$ and $\tilde{\nu}_v$ of Figure 1 are easily located. If overlapping with a higher energy band occurs the position of $\tilde{\nu}_v$ may have to be corrected a little to take this into account. From λ_0, λ_r, and λ_v the corresponding values of $\tilde{\nu}$ are computed as $\tilde{\nu}$(kK) = $1/\lambda$(nm) × 10^4.

[3] Using P–P–P molecular orbital calculations, P. S. Song (private communication) has predicted energies for the $^1L_b \leftarrow {}^1A$, $^1L_a \leftarrow {}^1A$, and $^1B_a \leftarrow {}^1A$ transitions (bands I, II, and III) of the 3-hydroxypyridine cation as 36.8, 45.1, and 52.4 kK, respectively (observed $\tilde{\nu}_0$ in water 35.3, 44.9, >50). For the neutral, uncharged molecule two transitions were predicted at 36.5 and 46.3 kK (observed $\tilde{\nu}_0$ in methanol, 35.8, 45.8). Predictions for dipolar ion and anion forms were not as close, *e.g.*, dipolar ion, predicted, 27.5, 41.3, 49.0 kK; observed $\tilde{\nu}_0$ in H_2O, 31.8, 40.65, 46.7 kK.

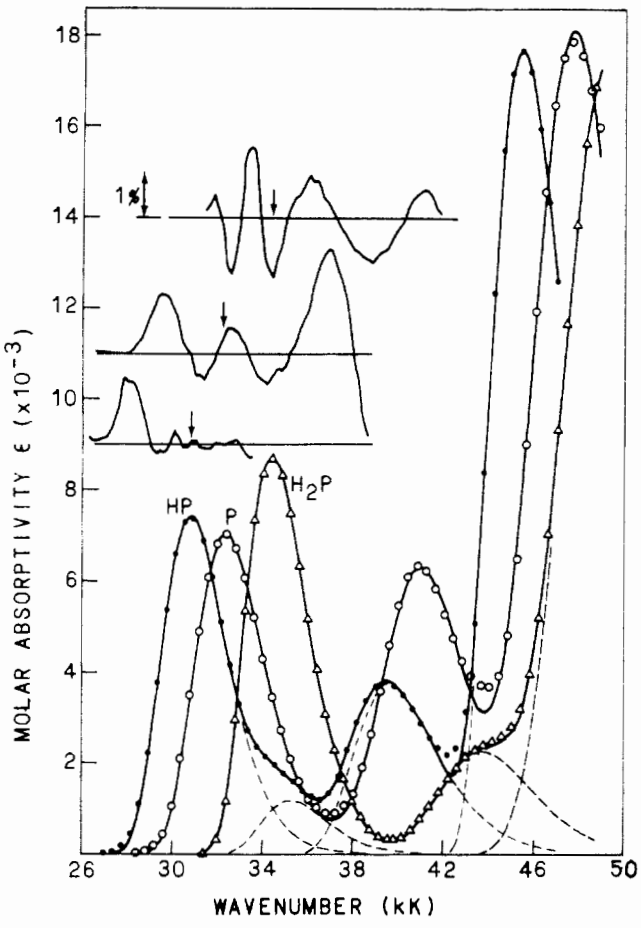


FIGURE 4: Spectra of three ionic forms of pyridoxine represented as sums of log normal curves: H_2P, cation; HP, neutral form (dipolar ion plus small amount of uncharged form); P, anion.

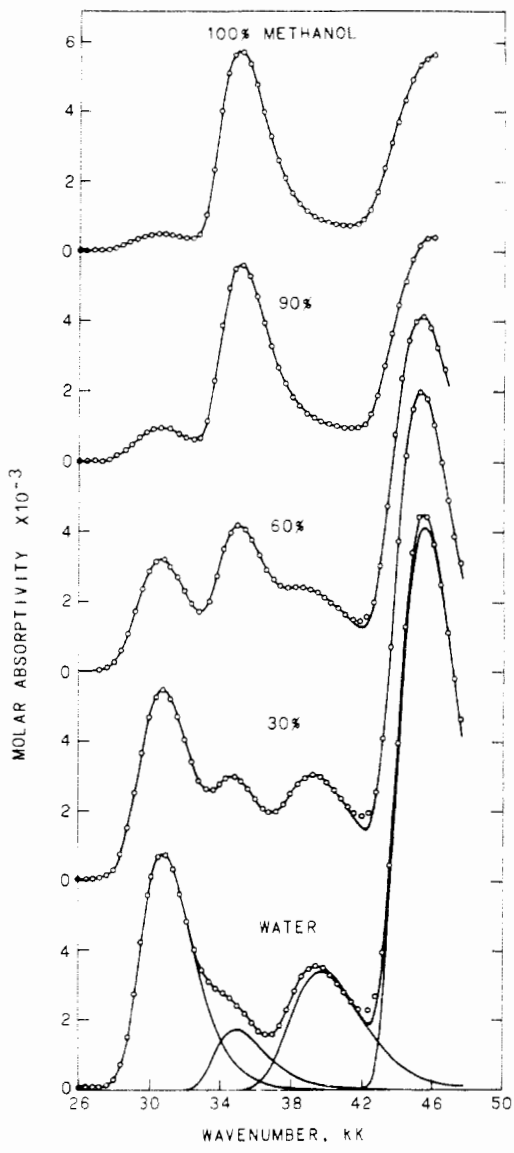


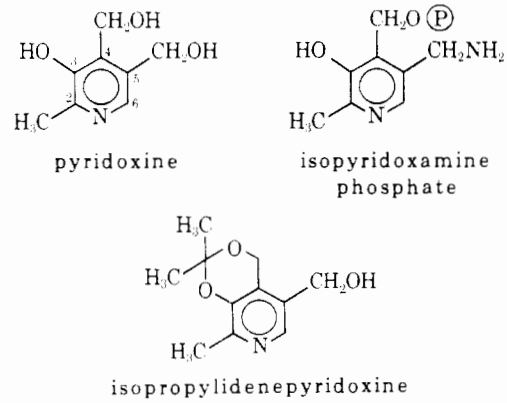
FIGURE 5: Spectra of pyridoxine in water–methanol mixtures together with the fitted curves (solid lines). Each fitted curve is the sum of four log normals. Apparent molar absorptivities are plotted and each curve is displaced from the next by 6000 units of molar absorptivity. The per cent methanol by volume is indicated beside each curve.

difference plot ("fine-structure plot") together with the band parameters provides a complete description of a spectrum.

Figure 3 shows typical spectra of uncharged neutral species and of anions. An excellent fit is obtained around the two peaks of the neutral forms and around the three peaks of the anion. However, unless one or more additional "buried" bands are placed in the valleys the fitted curves fall below the experimental points in the valleys. This is most clearly seen for the neutral, uncharged forms for which at least one minor band is distinctly present between bands I and II. To obtain accurate band parameters for the major π–π^* transitions it is desirable to fit the small bands into the valleys. However, good parameters for band I can be obtained by truncating the data at a wave number at which the contribution of the buried band is insignificant.

Figure 4 shows complete spectra of the three ionic forms of pyridoxine resolved with log normal curves. These are typical of the spectra of more highly substituted 3-hydroxypyridines. The molar absorptivities are higher than those of the compounds in Figures 2 and 3 and the presence of three bands in each of the three major forms can be seen. A small amount of the uncharged neutral tautomer of the compound is present in equilibrium with the larger amount of dipolar ion.

Tables of Band Parameters. Band parameters and band areas were measured for the spectra of the various ionic forms of the pyridine, pyrazine, phenol, and a series of 3-hydroxypyridines. The latter include derivatives of pyridoxine, pyridoxamine (4-CH_2NH_2), isopyridoxamine phosphate, isopropylidenepyridoxine, and 4-pyridinecarboxaldehydes (only

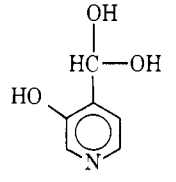


absorption bands of the covalent hydrates and hemiacetal forms of the latter were considered). The results are summarized in Tables I–VI. Apparent pK_a values are shown in Table VII. Several regularities can be seen, especially in the

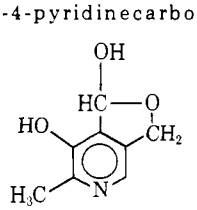
TABLE I: Widths and Values of Skewness (ρ) for Band I.[a]

No.	Compd	Cations		Neutral (uncharged)		Dipolar Ions		Anions	
		W (kK)	ρ	W (kK)	ρ	W (kK)	ρ	W (kK)	ρ
1	Pyridine	3.10†	1.44	3.8	1.7				
		M 3.11	1.46						
2	Pyrazine			3.23	1.60				
				H 3.20	1.42				
3	Phenol			2.91	1.60			3.18†	1.43†
4	3-Hydroxypyridine	3.26	1.41	3.63	1.52*	3.45	1.35	3.44	1.42
				M 3.22	1.52				
5	*N*-methochloride	3.24	1.46			3.45	1.37		
6	*O*-methyl-	3.09†	1.46	3.27	1.56				
				M90 3.16	1.60				
7	2-CH_2OH-	3.29	1.43	3.41	1.52*	3.44*	1.36*	3.57	1.34
8	6-CH_3-	3.34	1.45			3.54	1.40	3.43	1.38
9	Pyridoxine	3.20	1.43	M 3.23	1.52	3.45	1.36	3.57	1.35
10	5-deoxy-	3.21	1.45			3.40	1.32	3.59	1.33
11	4-deoxy-	3.30	1.42			3.17	1.34	3.35*	1.45
12	6-methyl-	3.28	1.44			3.40	1.37	3.58	1.34
13	Pyridoxamine	3.27	1.45			3.37	1.35	3.30	1.45
14	5-deoxy-	3.30	1.50			3.34	1.36	3.34	1.44
15	5′-phosphate	3.27	1.44			3.37	1.38	3.31	1.41
16	Isopyridoxaminephosphate	H_4P 3.50†	1.38			H_3P 3.72†	1.36*	3.40†	1.39†
						H_2P 3.55†	1.36*		
17	Isopropylidenepyridoxine	3.01†	1.49	3.09	1.53				
				M 3.02	1.59				
18	5-deoxy-	3.02†	1.50	3.17	1.56				
19	Pyridoxal (hemiacetal)	3.22	1.48			3.32	1.39		
20	3-Hydroxy-4-pyridinecarboxaldehyde (hydrate)	3.36	1.48						
21	5-Deoxypyridoxal (hydrate)	3.14	1.45			3.32	1.35		
22	Pyridoxal 5′-phosphate (hydrate)	3.25	1.46*						

[a] All values are for aqueous solutions at 25° unless otherwise indicated: H, in hexane; M, in methanol; M90, in 90% methanol. Parameters indicated by an asterisk were fixed at preselected values. Those indicated by a dagger were omitted in compiling averages. For pyridoxamine derivatives the cations are the dications and the dipolar ions are the monocation–dipolar ions. For isopyridoxamine phosphate H_3P refers to the form with a protonated phosphate, while H_4P and H_2P refer to the forms bearing four and two dissociable protons, respectively.



hydrate of
3-hydroxy-4-pyridinecarboxaldehyde



hemiacetal form
of pyridoxal

parameters for band I. For example, the bandwidths and skewnesses are remarkably uniform for given ion types. The widths for 15 selected 3-hydroxypyridine cations averaged 3.26 ± 0.06 kK with a range of 3.14–3.36 kK (Table I). That of the pyridine cation is only slightly less (3.10 kK). Methylation of the phenolic group narrows the band to 3.09 kK and formation of an isopropylidene ring involving the same OH group causes even more narrowing to 3.01–3.02 kK. The only unusually wide cation band is that of isopyridoxamine phosphate (3.49 kK). The average width for all 20 cations of Table I is 3.23 ± 0.12 kK.

Dissociation of the phenolic hydrogen to form a dipolar ion leads to a marked (3–6%) broadening of band I to 3.39 ± 0.09 kK (for 13 compounds). Further dissociation of a proton from the ring nitrogen causes an additional smaller amount of band broadening (3–4%). Thus, for seven anions the average width was 3.53 ± 0.14 kK. On the other hand anions of pyridoxamine derivatives have even narrower bands (3.22 ± 0.02 kK) than do the dipolar ions.

Spectra for a few neutral, uncharged forms were measured, some in organic solvents. In all instances the band was broadened somewhat from that of the cation, a result consistent with the loss of a proton from the ring nitrogen. Note

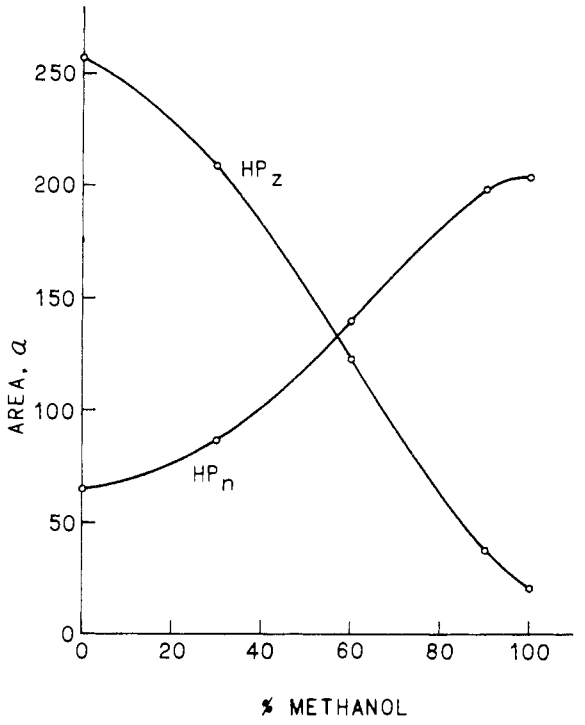


FIGURE 6: The areas (km mol^{-1}) of the first (dipolar, HP_z) and the second (neutral, HP_n) peaks of pyridoxine in methanol–water mixtures.

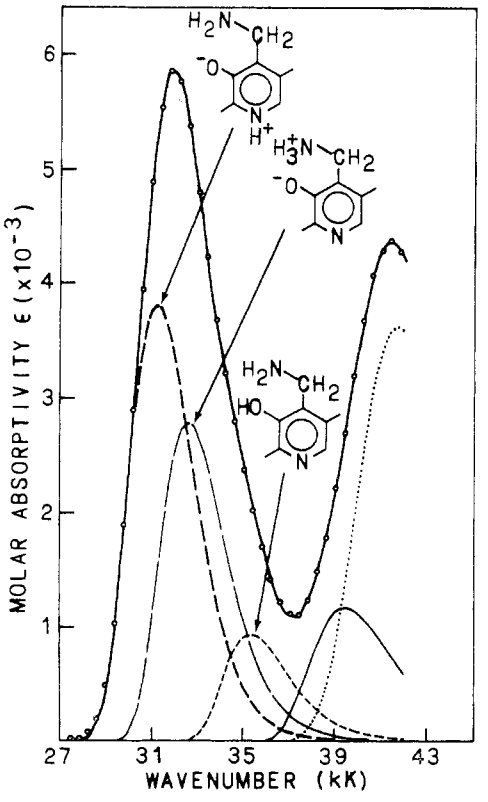


FIGURE 7: Spectrum of the HP form of 5-deoxypyridoxamine resolved into components corresponding to the three tautomeric forms whose structures are shown. Bandwidths and skewness values were fixed as explained in the text.

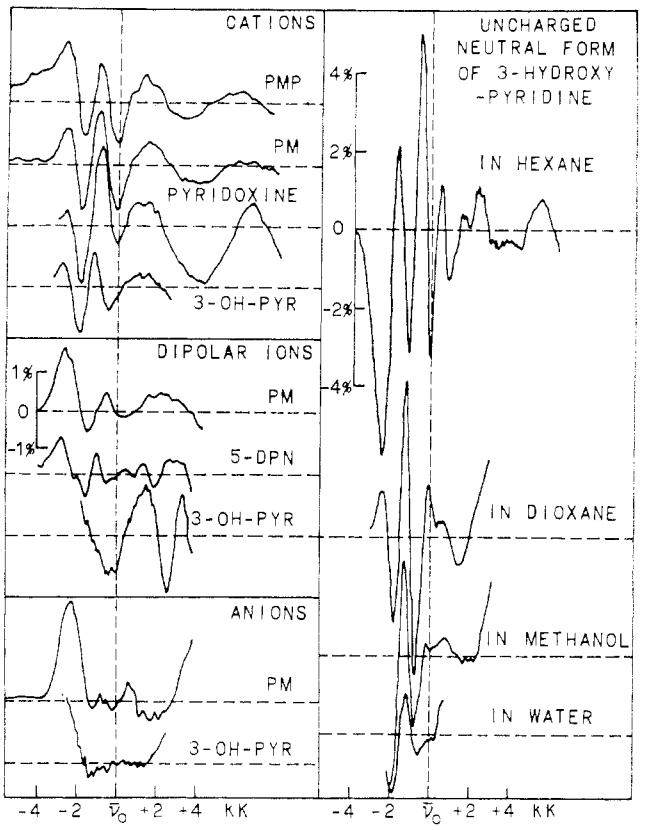


FIGURE 8: Fine structure plots for a series of 3-hydroxypyridines: PMP, pyridoxamine phosphate; PM, pyridoxamine; 3-OH-PYR, 3-hydroxypyridine; 5-DPN, 5-deoxypyridoxine. All plots are to the same scale. Spectra were recorded at 0.05-kK intervals.

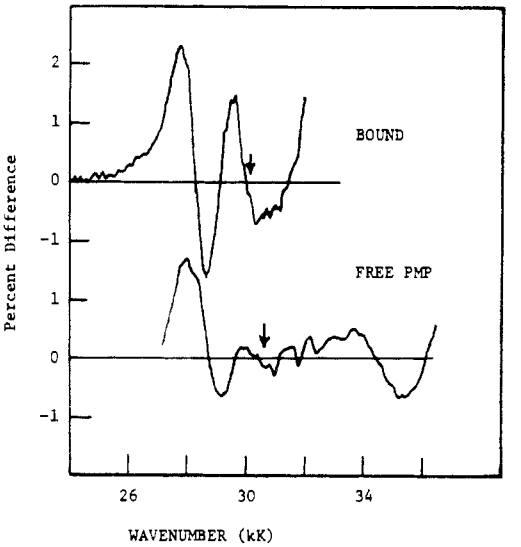


FIGURE 9: Fine structure plots for free pyridoxamine phosphate (H_2P form) and for the same coenzyme bound to apoaspartate aminotransferase (cytoplasmic, α subform from pig heart). The spectrum of the apoenzymes plus pyridoxamine phosphate was recorded at 0.05-kK intervals. The absorption of the protein component was subtracted and a single log normal curve was fitted to the difference. Band parameters are: free pyridoxamine phosphate, $\bar{\nu}_0 = 30.59$ kK, $\epsilon_0 = 8.55 \times 10^3$, $W = 3.37$ kK, $\rho = 1.38$; bound pyridoxamine phosphate, $\bar{\nu}_0 = 30.13$, $\epsilon_0 = 8.45$, $W = 3.30$, $\rho = 1.54$.

that band I of undissociated phenol is especially narrow (2.91 kK), the narrowest we have obtained for any compound.

Skewness values are even more uniform than is W. For band I of 14 cations $\rho = 1.45 \pm 0.02$; for 13 dipolar ions $\rho = 1.36 \pm 0.02$. For the anions ρ is essentially the same (1.37 ± 0.05 for seven compounds). Values for pyridoxamine derivatives are not significantly different than those for the other

TABLE II: Widths and Values of Skewness for Bands II and III.[a]

No.	Compd	Cations		Neutral (Uncharged)		Dipolar Ions		Anions	
		W (kK)	ρ	W (kK)	ρ	W (kK)	ρ	W (kK)	ρ
				Band II					
3	Phenol			7.06*	1.85			4.00†	1.41†
4	3-Hydroxypyridine	4.64 ± 0.2	1.55 ± 0.1			3.52	1.44	3.89	1.36
5	*N*-methochloride	4.50	1.45			3.55	1.40		
6	*O*-methyl-	4.43	1.66	4.10	1.31				
				M 4.02	1.43				
7	2-CH_2OH-					3.24†	1.34†	4.13	1.21
8	6-CH_3-	4.85	1.65			3.63	1.43	4.20	1.20
9	Pyridoxine					4.4	1.4	4.27	1.25
11	4-deoxy-					4.9	1.7	4.29	1.16
12	6-methyl-					4.18	1.53		
13	Pyridoxamine	4.21†	1.12†			4.05	1.51	4.43	1.21
14	5-deoxy-					4.29	1.49	4.42	1.08
15	5′-phosphate					4.07	1.54		
	Av	4.60 ±0.14	1.58 ±0.08			4.06 ±0.34	1.49 ±0.06	4.23 ±0.14	1.21 ±0.05
				Band III					
5	3-Hydroxypyridine *N*-methochloride					3.63	1.43		
9	Pyridoxine	4.72	1.21	M 3.96	1.58	4.38	1.40	4.48	1.23
13	Pyridoxamine	4.02	1.08			4.02	1.62		

[a] Abbreviations are explained in footnote *a* of Table I. An asterisk indicates that two bands are probably closely overlapped; a dagger indicates that the entry was omitted in compiling averages.

compounds except in the anion forms where $\rho = 1.43 \pm 0.03$ for the pyridoxamine compounds.

The skewness of neutral, uncharged forms is always markedly higher (1.52–1.60) than that of cations.

Precision and Reproducibility of Band Parameters. How good are the numbers given in the accompanying tables? In most instances band I is well resolved. In such cases, $\tilde{\nu}_0$ is reproducible to ±0.01 kK (= ±10 cm^{-1} = ±0.1 nm at 316 nm), W to ±0.015 kK (about ±0.5% relative error), and ρ to ±0.03 (±2%) in repeated measurements on the same compound.

When a band is overlapped by a higher energy band (for example, in a tautomeric mixture) not all parameters can be obtained with precision unless at least one parameter of the overlapping pair is "fixed" at a preselected value during the fitting procedure. It is most often desirable to fix ρ and in most cases a suitable value for that parameter can be selected from Table I or Table II. Since ρ tends to stay nearly constant with changes in solvent and temperature, fixing the value of this parameter tends to provide maximum sensitivity in observing small changes in W accompanying change of environment of the chromophore. Shifts in peak position can also be established more reliably. In the resolution of tautomeric equilibria (discussed later) the fixing of one or more values of ρ is absolutely essential to the accurate measurement of the band areas. In fact, when a minor tautomer is present and provides a "buried" band in the spectrum it is necessary to fix both ρ and W for such a band. When this is done tautomerization constants can be evaluated precisely.

Parameters for Peaks II and III. Because of overlap by bands on both sides the parameters of band II are difficult to obtain precisely. The values shown in Table II are for those spectra for which an excellent fit[4] was obtained. Nevertheless the variation is markedly greater than for band I. Parameters for the higher energy band III are even more uncertain and have been obtained for only a few compounds.

It has been particularly difficult to evaluate W and ρ of band II of cations because of the low intensity of these bands. Another difficulty arose because our earlier measurements were made in HCl-containing solutions and because KCl was often added to maintain an ionic strength of 0.2. Chloride ion apparently induces a charge-transfer band near the peak of band II increasing the intensity in that region and blurring the spectrum. The presence of chloride even has a small effect on the band parameters of peak I. As little as 0.2 M chloride causes a small shift to lower energy (~0.02 kK), a broadening of 1–2%, and a slight increase in skewness. While the effect of chloride on band I is barely significant it is essential to avoid chloride-containing solutions for evaluation of parameters of bands II and III. We have found mixtures of perchloric acid and sodium perchlorate entirely satisfactory.

Only four compounds (**4**, **5**, **6**, and **8**) show band II with an actual maximum in the cation form. Other spectra have only a shoulder. We selected 3-hydroxypyridine in perchloric acid for further study and made a large number of curve fittings, both with all parameters free and with the skewness fixed at selected values. On the basis of these results we estimate the

[4] "Excellent" fits are those in which all points lie close enough to the fitted curve to give a difference of less than 1.5% in the fine structure plots. However, deviations arising from characteristic vibronic fine structure may exceed this limit. "Good" fits have differences of no greater than 2% around the peaks but may have larger differences (up to 5%) in the valleys.

TABLE III: Band Positions ($\tilde{\nu}_0$) for Band I.[a]

No.	Compound	Cation	Neutral (Uncharged)	Dipolar Ion	Anion
1	Pyridine	39.29	39.22		
2	Pyrazine		38.24		
			H 38.74		
3	Phenol		36.94		34.78
4	3-Hydroxypyridine	35.31	36.31	31.84	33.52
			M 35.84		
5	*N*-methochloride	34.73		31.24	
6	*O*-methyl-	35.20	36.29		
			M90 36.06		
7	2-CH_2OH-	34.88	35.86	31.73	33.03
8	6-CH_3-	34.44	35.41	31.21	32.71
9	Pyridoxine	34.37	35.14	30.84	32.33
			M 35.05		
10	5-deoxy-	34.37	35.38	31.00	32.55
11	4-deoxy-	35.33	35.7	31.77	33.17
12	6-methyl-	33.55	34.54	30.15	31.62
13	Pyridoxamine	34.03	35.19	30.69	32.48
14	5-deoxy-	34.02	35.26	30.92	32.68
15	5′-phosphate	34.01	35.27	30.59	32.50
16	Isopyridoxamine phosphate	34.17	H_3P 34.85	H_3P 30.61	23.16
			H_2P 35.16	H_2P 30.68	
17	Isopropylidenepyridoxine	34.29	35.35		
18	5-deoxy-	34.63	35.71		
19	Pyridoxal (hemiacetal)	34.72	36.03	31.59	33.19
20	3-Hydroxy-4-pyridinecarboxaldehyde (hydrate)	35.06			
21	5-Deoxypyridoxal hydrate	34.01		31.33	
22	Pyridoxal 5′-phosphate (hydrate)	33.99			

[a] Solvent is water at 25° unless otherwise specified. Peak position $\tilde{\nu}_0$, in kK = $10^4/\lambda$(nm).

width of band II as 4.64 ± 0.2 kK and the skewness as 1.55 ± 0.1. Similar uncertainty is presumably present in W and ρ for the other compounds. This uncertainty may lead to a relative error in the area of band II of as much as 8% for cations. However, the peak positions are reliable. Thus for all of the "good" [4] fits obtained with the 3-hydroxypyridine cation, $\tilde{\nu}_0$ for band II was constant to ±0.01 kK.

While it is not possible to give a general rule we offer the tentative suggestion that the parameters of band II of cations can be approximated[5] as $W(\text{II}) = 1.4W(\text{I})$ and $\rho(\text{II}) = 1.1\rho(\text{I})$. For both dipolar ions and anions $W(\text{II}) \simeq 1.2W(\text{I})$. For dipolar ions $\rho(\text{II}) \simeq 1.1\rho(\text{I})$ while for anions $\rho(\text{II}) \simeq 0.9\rho(\text{I})$.

Band III is well resolved but lies at a high enough energy that we have few data with which to evaluate W and ρ. It appears that widths and skewnesses for band III are not greatly different from those of band II for dipolar ions and for anions. For cations the skewness of band III is distinctly less than that of bands of I and II. However, this statement must be qualified by the observations that band III has always been truncated near the peak; although the absorption appears to be dropping rapidly with increasing energy (indicating a low skewness) photometric errors make conclusions uncertain. In some cases no maximum was observed but a computer-assisted fit was obtained by assuming a reasonable value for ϵ_0. In such cases the peak position in Table IV is preceded by the symbol ~.

[5] Some doubt about the generality of these rules is raised by the excellent fit obtained for the pyridoxamine cation (Table II) with band II narrower and less skewed than predicted by the "rules."

The Problem of Minor Bands. The spectra of the anion and neutral uncharged forms of 3-hydroxypyridine can be fitted well with log normal curves only if a minor band is placed in the valley (Figure 3). The same is true for pyridine and to a lesser extent for pyrazine and for the phenolate anion. To obtain a quantitative description of spectra by band resolution it is necessary to know the approximate widths and skewnesses of these bands. To do this it is essential to understand the nature of these bands so that suitable width and skewness parameters can be selected from related bands in better resolved spectra.

Possible causes of the minor bands include the presence of impurities, dimerization, n–π^* transitions, and charge-transfer bands involving solvent molecules. Repeated recrystallization of 3-hydroxypyridine from "Spectroquality" solvents causes no change in the intensity of the minor band. The same is true of 3-methoxypyridine obtained from two different sources. We believe it highly unlikely that the minor bands we observe arise from impurities nor do they seem to be influenced by the ionic environment. When oxygen was bubbled through a solution of the anion of 3-hydroxypyridine, no decrease in the depth of the valley was noted; thus the bands cannot be attributed to interaction with oxygen. No dependence of band intensity on concentration was observed for 3-hydroxypyridine in methanol.

The appearance of the minor bands in anion and neutral uncharged forms but not in cation or dipolar ions immediately suggests n–π^* transitions. However, it is generally assumed that pyridine derivatives have only one n–π^* transition and that is buried under the first π–π^* band. The presence of a

weak minor band in the phenolate anion shows that such bands cannot always be associated directly with the pyridine nitrogen. Nevertheless, it is of interest to compare parameters of some known n–π^* transitions. The width for the n–π^* transition of acetone is 6.3 kK while for pyrazine (in which the n–π^* is well separated from the π–π^* transition) it varies from 2.5 in hexane to >3 in water. It is impossible on the basis of these data to select a single appropriate set of band parameters for n–π^* transitions but a possible range (~3–6 kK) is established. A clue as to the cause of the buried bands comes from the observation that the broad valley in the pyrazine spectrum in aqueous buffer contains a minor band, about half as intense as that of 3-hydroxypyridine. The band is virtually absent in hexane. The observation could not be verified with 3-hydroxypyridine because of significant changes in band shape of the π–π^* transition which suggest that association may occur in hexane. We tentatively conclude that the minor bands may arise from some kind of charge-transfer interaction between polar solvent molecules and the chromophore.

What effect do uncertainties in the width and skewness of the minor bands have on the parameters found for the major π–π^* transitions? The question has been pursued systematically with 3-hydroxypyridine for which repeated resolutions of the spectrum in 100% methanol have been made. Good fits[4] were obtained with widths ranging from 4.25 to 6.3 kK for the buried band and with ρ varying from 1.07 to 1.56. However, narrower bands or much wider bands gave poor fits. Significantly, for all of the *good* fits, the parameters of band I remained completely constant; thus we feel that fitting the minor bands into the valleys is a legitimate way of improving the overall fit and in obtaining reliable band parameters for the major bands. Minor bands become less of a problem in the more highly substituted 3-hydroxypyridines because the π–π^* bands are more intense whereas the minor bands tend to remain about the same as in 3-hydroxypyridine. For most of the highly substituted 3-hydroxypyridines the parameters in Tables I–VI were obtained *without* inclusion of minor bands. The fits of Figure 4 are typical. The fitting of minor bands into "valleys" in spectra is not always successful. Thus addition of a small band to the spectrum of the anion of pyridoxine (Figure 4) does little to improve the fit. No matter what parameters are used, the fit is poor unless the minor band is allowed to become several times more intense than that in 3-hydroxypyridine. Thus, some basic uncertainty remains concerning the significance of the minor bands and their influence on the shape of the major bands.

Changes in Band Parameters with Solvent and Temperature. Effects of changes in solvent composition in binary water–alcohol and water–dioxane mixtures have been investigated systematically for the cation and dipolar ion of 3-hydroxypyridine *N*-methobromide. For a number of other compounds spectra have been measured in methanol, dioxane, and hexane. Spectra have been measured in water for all forms of 3-hydroxypyridine *N*-methochloride from 7 to 70°, for 3-hydroxypyridine from 6 to 50°, and for pyridoxamine at 25 and 50°.

The first generalization emerging from these experiments is that the band area $\mathcal{A}$ (at least for band I) stays constant within experimental error (±0.5%) with both changes in temperature and with solvent composition. The rule holds only for single ionic forms in which no tautomerism occurs. It has been tested only with mixtures of water and alcohols and for water–dioxane mixtures up to about 90% dioxane.

Constancy of area is predicted theoretically for transitions for which the transition matrix is independent of frequency and configuration coordinate. It has been previously noted for other compounds. For example Ito (1960) reports a constant area for chlorobenzene from −70 to 15° and cites other papers. See also Stepanov and Gribkovskii (1968). We conclude that an appropriate extension of Beer's law (Beer, 1852; Swinehart, 1962) is that *the concentration of an absorbing substance is proportional to the area under the band.* We define the *molar area*, $\mathcal{A}°$ (km mol^{-1}), for a single absorption band as the area[6] of that band in a plot of ϵ *vs.* $\tilde{\nu}$ (in kilo-Kaysers) for a single ionic form or tautomer of a substance.

Some small deviations from constancy of molar area were observed for *O*-methyl-3-hydroxypyridine in basic solution and for the uncharged neutral tautomer of 3-hydroxypyridine in dioxane mixtures (see below); hence the proposed extension of Beer's law must be applied with caution. Nevertheless, we believe that it will be widely applicable.

The band parameters all change in a regular, smooth fashion when solvent composition or temperature is varied. The effects observed for band I are summarized in Table VIII. Bands of neutral and cationic forms usually become narrower and slightly more skewed in going from water to methanol while bands of dipolar ions and of anions become broader. Bands of cations and uncharged and dipolar ionic forms usually

TABLE IV: Some Values of $\tilde{\nu}_0$ for Bands II and III.[a]

No.	Compd	Cation	Dipolar Ion	Anion
		Band II		
3	Phenol			42.68
4	3-Hydroxypyridine	44.94	40.65	42.42
5	*N*-methochloride	44.77	40.19	
6	*O*-methyl-	44.45		
7	2-hydroxymethyl-		40.33	~41.92
8	6-methyl-	44.68	40.51	42.03
9	Pyridoxine	43.67	39.62	40.9
10	5-deoxy-		40.21	41.27
11	4-deoxy-		39.41	40.92
12	6-methyl-		38.99	40.53
13	Pyridoxamine	44.38	39.66	40.85
14	5-deoxy-		40.07	41.12
15	5′-phosphate		39.58	40.87
16	Isopyridoxamine phosphate	44.1	H_3P 38.96 H_2P 39.03	40.90
17	Isopropylidene-pyridoxine	43.5		
18	5′-deoxy-	43.8		
		Band III		
4	3-Hydroxypyridine			
5	*N*-methochloride		46.72	
8	6-methyl-		46.8	
9	Pyridoxine	~49.1	45.43	47.68
11	4-deoxy-		45.95	
12	6-methyl-			
13	Pyridoxamine	~49.3	45.67	~47.0

[a] A single value for ν_0 (II) for a neutral uncharged form was obtained. That for *O*-methyl-3-hydroxypyridine (**6**) is 46.03 kK.

[6] The numerical value of the area in a plot of ϵ (in l. mol^{-1} cm^{-1}) *vs.* $\tilde{\nu}$ (kK) × 10 gives the area in km mol^{-1}.

TABLE V: Molar Areas for Band I and Estimates of K_z.[a]

No.	Compd	Cations	Neutral (Uncharged)	Dipolar Ions	Anions	K_z
1	Pyridine	351	317			
2	Pyrazine		204			
			Hexane 172			
3	Phenol		47.5		91	
4	3-Hydroxypyridine	214	143 (69.9)	220 (112.9)	162	1.05
5	*N*-methochloride	194		188		
6	*O*-methyl-	205	140			
			M90 144			
7	2-hydroxymethyl-	287	175 (60.0)	291 (191.0)	206	1.9
8	6-methyl-	232	144 (59.0)	240 (141.2)	165	1.4
9	Pyridoxine	308	198 (40.5)	348 (276.4)	272	3.9
10	5-deoxy-	303	211 (27.9)	352 (305.5)	273	6.6
11	4-deoxy-	277	184 (22.1)	307 (269.9)	237	7.3
12	6-methyl-	330	207 (34.0)	345 (288.0)	265	5.1
13	Pyridoxamine	300	186 (19.6)	309 (276.2)	243	8.4
14	5-deoxy-	303	189 (11.8)	314 (294.7)	250	15
15	5′-phosphate	323	208 (20.1)	346 (312.5)	280	9.3
16	Isopyridoxamine phosphate	334	H_3P 240 (120.3)	401 (200.1)	305	1.00
			H_2P 335 (137.0)	392 (163.6)		0.72
17	Isopropylidenepyridoxine	280	201			
18	5′-deoxy-	275	182			

[a] Areas are given in units of kilometers per mole. For the neutral tautomers the molar areas were calculated from the measured areas (values in parentheses). It was assumed that $\mathcal{A}_n = 0.64\mathcal{A}_z$ for 3-hydroxypyridine; $\mathcal{A}_n = 0.57\mathcal{A}_z$ for pyridoxine, and $\mathcal{A}_n = 0.6\mathcal{A}_z$ for all other compounds.

shift to lower energies, the shift being greatest for dipolar ions and least for uncharged forms.

Some typical shifts (kiloKaysers) in $\tilde{\nu}_0$ for the change of solvent from water to methanol are: pyridine, −0.11 (cation), −0.02 (uncharged); *O*-methyl-3-hydroxypyridine, −0.21 (uncharged); isopropylidenepyridoxine, −0.15 (uncharged); 3-hydroxypyridine *N*-methochloride, −0.43 (cation), −0.63 (dipolar ion, 80% methanol), −1.06 (dipolar ion, 80% dioxane); 3-hydroxypyridine, −0.40 (dipolar ion); pyridoxine, −0.23 (cation), −0.28 (dipolar ion). Exceptional behavior was shown by pyrazine for which $\tilde{\nu}_0$ shifted by +0.17 and +0.50 kK between water and methanol and water and hexane, respectively.

Anion bands sometimes shift to lower and sometimes to higher energies upon transfer from water to 90% methanol. Shifts for bands I and II of 3-hydroxypyridine are −0.32 and −0.56 kK, respectively; for pyridoxamine the shifts are −0.15 and −0.49 kK. On the other hand band I of pyridoxine shifts to higher energy (+0.16 kK) while band II shifts to lower energy (∼−0.10 kK).

Typical changes in band width for solvent change from water to methanol are indicated in Table VIII. Notice that W decreases for cations and neutral forms but increases for dipolar ions. Small changes in either direction have been observed for anions. It is noteworthy that in almost every instance if a band is *narrowed* upon changing solvent or temperature ρ *increases* slightly.

Increases in temperature shift cation peaks to higher energies by a small amount: 3-hydroxypyridine, +0.04 kK (6–25°), +0.07 kK (6–50°); pyridoxamine, +0.01 kK (25–50°). Bands of dipolar ions and anions shift to lower energies as T increases, *e.g.*, 3-hydroxypyridine, dipolar ion, −0.12 kK (6–25°), −0.23 kK (6–50°), anion, −0.09 kK (6–25°), −0.22 kK (6–50°). All three bands (I, II, and III) of the dipolar ion of pyridoxamine shift to lower energies (−0.10, −0.11, and −0.08 kK, respectively) when the temperature is raised from 25 to 50°. Band I of the pyridoxamine anion is shifted by −0.11 kK by the same temperature increase.

All bands are broadened by an increase in temperature. The effect is larger (by a factor of ∼1.2–1.3) on dipolar ions and anions than on cations. In all cases the rate of broadening increases with increasing temperature. A typical example is 3-hydroxypyridine *N*-methobromide. The dipolar ion band broadened from 3.55 kK at 7.4° to 3.58 and 3.70 kK at 26 and 70°, respectively. The rate of change, dW/dt, increases from ∼0.0015 kK (0.04%) per degree at 7° to ∼0.004 kK (0.1%) per degree at 70°. The rate of change at 25° is 0.0025 kK (0.07%) per degree. Limited data indicate very similar variation for other dipolar ions and anions. However, a three times greater rate of change was recorded for the anion of pyridoxamine.

While W increases with temperature ρ decreases slightly. However, to a good approximation ρ can be assumed constant when spectra at various temperatures are resolved.

Resolution of Overlapping Bands. When absorption bands are well separated by deep valleys all four band parameters can be measured with confidence. However, when bands overlap strongly no unique resolution is possible. Good fits to experimental spectra may be obtained for various values of ρ and W for the overlapping band. Our computer program permits fixing of one or more band parameters at preselected values. An instructive experiment is to fix a single parameter of an overlapping pair of bands, for example, the skewness of the lower energy band. When this is done it is found that good resolutions may be obtained for a variety of fixed values of ρ. Furthermore, the higher the value of ρ the higher the value of

W will be for the same band. On the other hand as ρ is raised for the first band ρ increases and W decreases for the second band.

Either ρ or W may be fixed for a given band to yield a more nearly unique resolution and in some cases, *e.g.*, where three bands overlap, it may be necessary to fix two or more parameters. These results suggest that precise resolution of overlapping bands is possible if enough reference compounds are studied to establish appropriate values of ρ and or W for overlapping bands in mixtures of compounds. The data of Tables I and II are based largely on well-separated bands and provide appropriate values of W and ρ for various ionic forms and structures.

Evaluation of Tautomeric Equilibria. Figure 5 shows the spectrum of pyridoxine in mixtures of methanol and water. The neutral compound is a mixture of two tautomers. In water the dipolar ion HP_z[7] (with peaks at 30.8, 39.6, and 45.5 kK) predominates. The uncharged form HP_n possesses at least two bands which, in 100% methanol (Figure 5), are located at 35.1 and ~46.2 kK (a small amount of the 30.8-kK band of HP_z is also present in methanol). In water a relatively small fraction of the molecules exist as the neutral form and the higher energy peak is completely "buried." However, the low-energy band is distinctly visible as a shoulder at ~30.8 kK.

The curves in Figure 5 were resolved using five log normal curves. In order of increasing energy they are: (1) band I of the dipolar ion, (2) band I of the neutral form, (3) band II of the dipolar ion, (4) the sum of band III of the dipolar ion and band II of the neutral form, and (5) band III of the neutral form and/or uncharacterized "end absorption." Parameters for band I of the neutral form were fixed at W = 3.34 kK and ρ = 1.50 in water (compare with W = 3.23 kK and ρ = 1.52 observed in methanol). Appropriate intermediate values of W and ρ were fixed for these same parameters in the solvent mixtures. Likewise, ρ for both bands I and II of HP_z was fixed at 1.36 for all solvent mixtures. The width (unfixed) of band I of HP_z increased from 3.50 in water to 3.76 in 90% methanol, similar to (and somewhat exceeding) the increase in width with methanol concentration seen for the dipolar ion of 3-hydroxypyridine *N*-methobromide.

The areas (for the first two bands) obtained from the curve resolutions are plotted against solvent composition in Figure 6. If, as we have demonstrated, the area of a given band is independent of solvent composition, we can compute the tautomeric ratio, K_z, of the mole fractions of dipolar ion, f_z, to neutral, f_n, forms in water as

$$K_z = \frac{f_z}{f_n} = \frac{\mathfrak{A}_z}{\mathfrak{A}_n}\frac{\mathfrak{A}_n^\circ}{\mathfrak{A}_z^\circ} \qquad (2)$$

where $\mathfrak{A}_z$ and $\mathfrak{A}_n$ are areas, *e.g.*, under the first (dipolar ion) and second (neutral form) peaks in the spectrum for aqueous pyridoxine (Figure 5), $\mathfrak{A}_z^\circ$ and $\mathfrak{A}_n^\circ$ are the corresponding molar areas, and $f_z + f_n = 1$. In general, for tautomeric mixtures, the molar areas are not directly measurable. Nevertheless, if, as we propose, the $\mathfrak{A}^\circ$ values remain constant with changes in solvent composition, we have a simple way of obtaining them. Let the solvent composition change so that $\mathfrak{A}_n$ is increased or decreased by $\Delta\mathfrak{A}_n$ and $\mathfrak{A}_z$ changes by $\Delta\mathfrak{A}_z$. Then

$$-\Delta\mathfrak{A}_n/\Delta\mathfrak{A}_z = \mathfrak{A}_n^\circ/\mathfrak{A}_z^\circ \qquad (3)$$

and

$$K_z = (-\Delta\mathfrak{A}_n/\Delta\mathfrak{A}_z)(\mathfrak{A}_z/\mathfrak{A}_n) \qquad (4)$$

This result follows directly from the constancy of $\mathfrak{A}_n^\circ$ and $\mathfrak{A}_z^\circ$ together with the assumption that only two tautomeric species are present. Equation 4 describes a new, simple method for evaluation of the tautomerization constant which will be widely applicable.

From the data of Figure 5, the ratios $(-\Delta\mathfrak{A}_n/\Delta\mathfrak{A}_z)$ for changes of solvent from 0–30, 0–60, 0–90, and 0–100% methanol are 0.50, 0.58, 0.62, and 0.60. The average ratio $-\Delta\mathfrak{A}_n/\Delta\mathfrak{A}_z = 0.57 \pm 0.04$. The area of the zwitterion band, $\mathfrak{A}_z$, can be obtained with confidence in water but $\mathfrak{A}_n$ is less certain (likewise, $\mathfrak{A}_z$ is less certain than $\mathfrak{A}_n$ in methanol). Taking account of the way in which band parameters were fixed to obtain more nearly unique resolutions we estimate the maximum error in W for HP_n as ±5%. Systematic errors in the resolution procedure could conceivably amount to ±10%. We estimate, using eq 4, that $K_z = 0.57(257/63.1) = 2.3 \pm 0.5$ in water at 25°. Also $\mathfrak{A}_n^\circ = 210$ and $\mathfrak{A}_z^\circ = 367$ km mol^{-1} for band I.

Tautomeric equilibria can also be recognized and evaluated by measuring spectra at different temperatures. Table IX shows data for the first two bands of the spectrum of neutral pyridoxine in aqueous phosphate buffers at three different temperatures. With increasing temperature the fraction of neutral, uncharged form increases and that of dipolar ion decreases. The ratio $-\Delta\mathfrak{A}_n/\Delta\mathfrak{A}_z = 0.51$. Considering the

TABLE VI: Some Molar Areas for Bands II and III.

No.	Compd	Cation	Dipolar Ions	Anions
		Band II[a]		
3	Phenol			414
4	3-Hydroxypyridine	160	(165)	419
5	*N*-CH_3-	174	269	
6	*O*-CH_3-	198		
7	2-hydroxymethyl-		(177)	379
8	6-CH_3-	242	(238)	471
9	Pyridoxine	119	(177)	291
11	4-deoxy-		(195)	281
12	6-methyl-		(246)	311
13	Pyridoxamine	119	(185)	271
14	5-deoxy-		(178)	275
15	5′-phosphate		(208)	261
16	Isopyridoxamine (phosphate)	183	H_3P (124) H_2P (103)	258
		Band III		
4	3-Hydroxypyridine		(545)	
5	*N*-methyl-		843	
9	Pyridoxine	776	(774)	~900
11	4-deoxy-		(800)	
13	Pyridoxamine		(731)	
16	Isopyridoxamine phosphate	696		

[a] The molar area of band II of neutral uncharged *O*-methyl-3-hydroxypyridine (compound **6**) is 279 km mol^{-1}.

[7] Individual ionic forms are abbreviated P, HP, H_2P, etc., according to the total number of dissociable protons present. HP_z is the dipolar ionic, monoprotonated form and HP_n is its uncharged tautomer.

TABLE VII: Apparent pK_a Values at 25°.[a]

No.	Compd						Lit.
4	3-Hydroxypyridine		5.10		8.60		*b*
			4.86		8.72 T (20°)		*c*
			4.84		8.65		*d*
			4.91		8.62		
5	*N*-methyl-		4.96 T				*e*
			4.93				
6	*O*-methyl-				4.88		*c*
7	2-CH_2OH-		5.00		9.07		*f*
			4.86		8.72		
8	6-methyl-		5.56		9.08		
9	Pyridoxine		5.00		8.97		*g*
			4.94		8.89		
10	5-deoxy-		5.48		9.46		
11	4-deoxy-		5.35		9.73		
12	6-methyl-		5.39		9.61		
13	Pyridoxamine		3.4				*g*
			3.54 T		8.21 T	10.63 T	*h*
			3.31		7.90	10.4 T	*b*
			3.14		8.19	10.28	*i*
			3.46		8.13	10.40	
14	5-deoxy-		3.94		8.39	9.87	
15	5′-phosphate	<2.5[j] T	3.69 T	5.76[j] T	8.61 T	10.92 T	*h*
			3.36		8.46	10.67	
16	Isopyridoxamine phosphate		3.75		8.60		*k*
			3.76	5.67[j]	8.56	10.81	
17	Isopropylidenepyridoxine				5.19		
18	5-deoxy-				6.10		

[a] Values obtained in this laboratory are for ionic strength 0.2. Most values are spectrophotometric but those identified as T are titrimetric. [b] Metzler and Snell (1955). [c] Albert and Phillips (1956). [d] Wigler and Wilson (1966). [e] Mason (1959). [f] Nakamoto and Martell (1959). [g] Lunn and Morton (1952). [h] Williams and Neilands (1954). [i] Morozov *et al.* (1966). [j] Phosphate group primarily. [k] Pocker and Fischer (1969).

TABLE VIII: Effects of Solvent and Temperature Changes on Band I of Spectra.[a]

	Cations	Neutral (Uncharged)	Dipolar Ions	Anions
		$\tilde{\nu}_0$		
Less polar solvent[b]	Decrease	Decrease (usually)	Decrease	Variable
Range (kK)	−0.1 to −0.4	+0.2 to −0.2	−0.3–0.6	
Higher temperature	Increase (small)		Decrease (larger)	Decrease
		W		
Less polar solvent	Decrease 0–1%	Decrease 2–3%	Increase 6–7%	Variable
		ρ		
Less polar solvent	Increase 1–2%	Increase ~4%	Little change	Decrease

[a] For all forms W increases as temperature is increased but the molar area remains constant. [b] Usually a change from water or aqueous buffer to 90–100% methanol.

small $\Delta\mathfrak{A}$ values the agreement with the values from the solvent mixtures is acceptable. The value of K_z in phosphate buffer (ionic strength 0.2) at 25° is 3.5 if $-\Delta\mathfrak{A}_n/\Delta\mathfrak{A}_z = 0.51$ or 3.9 if the ratio is 0.57 (from solvent data). The higher value of the apparent tautomerization constant K_z in phosphate buffer than in water is an expected consequence of the lowering of the activity coefficient of HP_z in the higher ionic strength medium.

A previous estimate for pyridoxine of $K_z = 7.4$ (Metzler and Snell, 1955) is high, because without a reliable method of curve resolution the amount of neutral, uncharged form present was underestimated.

Further confirmation of the method was obtained by examining spectra of 3-hydroxypyridine in methanol–water mixtures. An average value of $-\Delta\mathfrak{A}_n/\Delta\mathfrak{A}_z = 0.65 \pm 0.02$ was obtained for a series of solvent mixtures containing 20–100% methanol (Table X). The Δ values were taken between the spectra in each solvent and that in water. For lower methanol concentrations (5 and 10%) $-\Delta\mathfrak{A}_n/\Delta\mathfrak{A}_z$ was smaller, 0.52 and 0.58, respectively. However, the measured $\Delta\mathfrak{A}$ values

TABLE IX: Resolution of the Spectrum of the Neutral Form (HP) of Pyridoxine as a Function of Temperature.[a]

	Band I (Dipolar Ion)			Band II (Neutral, Uncharged)[c]		
Temp[b] (°C)	$\tilde{\nu}_0$ (kK)	W (kK)	$\mathcal{A}$ (km mol^{-1})	$\tilde{\nu}_0$ (kK)	W (kK)	$(\mathcal{A})$ (km mol^{-1})
5.5	30.90	3.42	291.6	35.02	3.32	32.6
25	30.83	3.45	276.4	35.25	3.34	40.5
50	30.74	3.56	240.1	35.30	3.39	58.6
	For 5.5–25°, $\Delta\mathcal{A}_n/\Delta\mathcal{A}_z = 0.52$					
	For 25–50°, $\Delta\mathcal{A}_n/\Delta\mathcal{A}_z = 0.505$					

[a] Estimated values of K_z: 4.6 at 5.5°; 3.5 at 25°; 2.1 at 50° in phosphate buffer; ionic strength 0.2. [b] Temperature was constant to within ±0.5° at 5.5° and ±0.1° at 25 and 50°. Spectra at 25 and 50° are exact spectra of the neutral form (sum of $HP_n + HP_z$) corrected for small amounts of cation and anion present. The spectrum at 5.5° was measured at pH 6.9 where 98.5% of the compound is in the neutral form. [c] The third and fourth bands were also resolved but data are not shown. The skewness of the first band was found to be 1.35–1.36 at all temperatures. That of the second (weak) band was fixed at 1.50 and, for the second band, W was fixed at the values shown for the computer resolution.

were small and we believe the ratio 0.65 obtained using the larger $\Delta\mathcal{A}$ values is more reliable.

Measurements of the spectrum of 3-hydroxypyridine in phosphate buffer at three temperatures gave $-\Delta\mathcal{A}_n/\Delta\mathcal{A}_z = 0.53$ (6–25°) and 0.62 (6–50°). We regard the latter value, obtained with larger $\Delta\mathcal{A}$ values, as more reliable.

The overall consistency of the results with 3-hydroxypyridine can be judged from Table X. Measured areas of bands of the dipolar ion and uncharged forms in both the water–methanol and buffered solutions at three temperatures are shown. Assuming that $-\Delta\mathcal{A}_n/\Delta\mathcal{A}_z = 0.65$ for both sets of data the sum $\mathcal{A}_n + 0.65\mathcal{A}_z$ should be constant and equal to $\mathcal{A}_n°$. The value of K_z is also shown for various solvents and for three temperatures in buffered solutions. In pure water $K_z = 0.88 \pm 0.16$ if we assume ±5% errors in both $\mathcal{A}_n$ and $\mathcal{A}_z$. As anticipated K_z increases in buffered solutions (to 1.05).

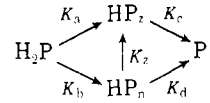
Previous estimates (Metzler and Snell, 1955; Mason, 1957, 1958) of K_z for 3-hydroxypyridine are 1.17 and 1.27. These were obtained by comparisons with methylated derivatives. For example, ϵ_0 for the dipolar ion of 3-hydroxypyridine methochloride is 5.44×10^3 l. mol^{-1} cm^{-1} (5.81×10^3 l. mol^{-1} cm^{-1} according to Mason, 1957, 1958). Assuming the same ϵ_0 for the dipolar ion of 3-hydroxypyridine itself and using the observed value of ϵ for the dipolar ion peak in phosphate buffer we obtain $f_z = 2.98/5.44 = 0.55$ and $K_z = 1.2$. The agreement with the value of 1.05 obtained by temperature variation (Table X) is satisfactory. The assumption of equal ϵ values for the methylated and protonated forms is apparently a reasonable one in the present case (but it may not be in other cases). The new method has the advantage that it is not necessary to have methylated derivatives available to obtain tautomerization constants.

Nakamoto and Martell (1959) calculated K_z for 2-hydroxymethyl-3-hydroxypyridine (7) as 1.8, in good agreement with our value of 1.9. These investigators assumed that the ratio $\epsilon_0(H_2P)/\epsilon_0(HP_n)$ was the same for compound 7 as for 3-methoxypyridine for which the ratio could be measured.

From the values of K_z in phosphate buffer (ionic strength 0.20) at 25° the apparent values of $\Delta G°$ for conversion of the neutral, uncharged form, HP_n, to the dipolar ion, HP_z, are estimated as −0.74 and −0.03 kcal mol^{-1}, respectively, for pyridoxine and for 3-hydroxypyridine. From the variation of K_z with temperature $\Delta H°$ for the same process is approximately −3.1 kcal mol^{-1} for both compounds.

We have not conducted solvent or temperature change experiments for compounds other than pyridoxine and 3-hydroxypyridine. However, we note that the ratio of molar areas $\mathcal{A}_n°/\mathcal{A}_z°$ is ~0.6 for both of these compounds. If it is assumed that the same ratio holds for other 3-hydroxypyridine derivatives, K_z can be estimated for them from spectral resolution data. The measured band areas for band I are given in Table V together with the estimates of K_z obtained in this way.

From the stepwise pK_a values of Table VII and the values of K_z from Table V the microscopic dissociation constants of the —OH and =NH$^+$ groups can be evaluated (as pK values) as follows: $pK_a = pK_1 + \log(1 + 1/K_z)$; $pK_b = pK_1 + \log$



$(1 + K_z)$; $pK_c = pK_1 + pK_2 - pK_a$; $pK_d = pK_a - pK_b + pK_c$. Any three of the microscopic pK values can be taken as

TABLE X: Areas (in km mol^{-1}) of First (Low-Energy) Bands of Dipolar Ion (HP_z) and Neutral (HP_n) Forms of 3-Hydroxypyridine.

Solvent	$\mathcal{A}_z$	$\mathcal{A}_n$	$\mathcal{A}_n + 0.65\mathcal{A}_z$	K_z
H_2O, 25°	102.4	75.6	142.2	0.88
5% methanol	96.2	78.8	141.3	0.79
10% methanol	89.3	83.2	141.2	0.70
20% methanol	74.8	93.1	141.7	0.52
30% methanol	53.2	107.8	142.4	0.32
40% methanol	42.5	114.8	142.4	0.24
60% methanol	18.4	131.8	143.8	0.091
80% methanol	6.4	140.2	144.4	0.030
100% methanol	0	144.2	144.2	
			Av 142.6 ± 1.0	
H_2O, 6° [a]	133.8	58.8	145.8	1.48
H_2O, 25° [a]	112.9	69.9	143.3	1.05
H_2O, 50° [a]	91.0	85.4	144.5	0.69
			Av 144.5 ± 0.8	

[a] These three are in phosphate buffer accounting for the fact that K_z is higher than in pure water (at top of table).

independent, but the fourth is a function of the other three. Three microscopic constants contain the same information as K_1, K_2, and K_z. However, it may be preferable to consider the microscopic constants in attempting to understand the electronic and hydrogen bonding effects of ring substituents.

Evaluation of the microscopic dissociation constants of pyridoxamine and its derivatives is more complex. There are three HP forms, protonated on the ring nitrogen, oxygen, and primary amino group, respectively. Log normal fitting of the HP forms with a single curve for band I gives abnormally large bandwidths: pyridoxamine, 3.77; 5-deoxypyridoxamine, 4.22; pyridoxamine phosphate, 3.62; isopyridoxamine phosphate, 3.86 kK. From Table I we see that the widths of forms P and H_2P for the same compounds range between 3.30 and 3.40 kK. Thus, the broadening of the HP bands strongly indicates the presence of a mixture of the tautomers. The abnormal width of the corresponding ionic form of *N*-pyridoxylglutamic acid has already been noted by Khomutov *et al.* (1971). These authors suggest the same explanation for the broadening.

Is it possible to resolve the spectra of the HP forms of the pyridoxamine derivatives into three components? A tentative answer can be given in the affirmative. For a first test we chose 5-deoxypyridoxamine for which the broadening of the HP band was greatest. Then we fixed the widths and skewnesses of band I for all three components at reasonable values based on those in Table II. For the first three bands these were: 3.34, 1.36; 3.34, 1.43; 3.33, 1.52. Figure 7 shows the results of such a resolution. An excellent fit is obtained for the first three bands. However, the relative heights of the fourth and fifth bands (representing bands II) seem unrealistic.

Questions that must be raised about Figure 7 concern the uniqueness of fit and the reliability of the areas measured. A series of resolutions was conducted in which the widths of the first two bands were varied from 3.30 to 3.37. Sometimes all the parameters were fixed and at other times they were allowed to vary freely. A number of excellent fits (comparable to the one in Figure 7) were obtained. Some of these were rejected because one band was unrealistically narrow or the skewness was far outside the limits indicated by the data of Table I. For seven very good fits[8] the following maximum ranges of variation in $\tilde{\nu}_0$ were observed for the first three bands: 31.20 ± 0.03, 32.55 ± 0.05, 35.30 ± 0.03. The areas of the same three bands varied over the ranges 12.4–14.4, 11.5–9.5, and 5.2–3.4. If the input parameters were varied far enough from those used for Figure 7, area values outside the ranges indicated were sometimes obtained. However, if the fitting procedure did not lead to "convergence" back into the indicated ranges the standard deviation of the absorbance points in the fit obtained was always outside the acceptable range. Thus, we feel that the results of Figure 7 (together with other unpublished resolutions) indicate the utility of log normal fitting in the analysis of spectra containing heavily overlapping absorption bands.

From the areas obtained in Figure 7, and making reasonable assumptions about molar areas,[9] one can easily estimate the two tautomerization constants needed to fully describe the system and to compute all microscopic dissociation constants for these compounds.

Fine Structure Plots. In Figure 8 the fine structure plots for a number of compounds have been grouped together. In all of these cases spectra were recorded at closely spaced intervals (0.05 kK). The similarities between the fine structure plots for different compounds of the same ionic type are striking. In particular cations and uncharged neutral forms of all compounds display a sharp "spike" on the low-energy side of the peak possibly marking the position of the underlying 0–0 transition. In 3-hydroxypyridine this comes just 1.20 kK below $\tilde{\nu}_0$ (*e.g.*, at 34.64 kK in Figure 3), but in pyridoxine and other substituted 3-hydroxypyridines it lies about 0.9 kK below $\tilde{\nu}_0$. The intensity of the fine structure is greatest in the neutral uncharged forms and is strongly increased in less polar solvents. Up to three vibronic sub-bands can be resolved. In methanol and dioxane the sharp band in the spectrum of 3-hydroxypyridine remains exactly 1.20 kK below $\tilde{\nu}_0$ but in hexane the distance changes abruptly to 0.6 kK. This suggests that some drastic alteration in environment (possibly a result of dimerization) occurs when hydrogen binding to the solvent is no longer possible.

An example of the use of fine structure plots in studying changes in environment of a chromophore is demonstrated in Figure 9. The lower curve is the plot for the monocation–dipolar ion form of free pyridoxamine phosphate while the upper curve is for pyridoxamine phosphate bound to apoaspartate aminotransferase. There is a very small spike about 0.6 kK below the peak position in pyridoxamine (Figure 8) and an even weaker peak in free pyridoxamine phosphate. This spike is greatly intensified upon binding of the coenzyme suggesting that the pyridoxamine phosphate is located in a nonpolar environment at the active site. This conclusion is further supported by a shift in peak position upon binding from 30.59 to 30.13 kK. Furthermore, the width is narrowed from 3.37 to 3.30 kK and ρ increases from 1.38 to 1.54. All of these changes are consistent with transfer of the chromophore to a less polar environment.

Discussion

The present work, together with preceding results, establishes the value of fitting absorption spectral bands with log normal distribution curves. In the first place, the method provides an extremely precise and reproducible way to describe spectra. The surprising constancy of the band parameters over a series of closely related compounds means that the method can be used in other ways as well. Measurement of bandwidth can be an excellent criterion of purity. Changes in widths and skewness values with changes in temperature or with solvent can be measured readily. Such data may be of value in interpreting changes in the environment of a chromophore.

In this connection it is pertinent to mention that we have confirmed the large decrease in $\tilde{\nu}_0$ in going from water to less polar solvents reported by Nakamoto and Martell (1959). This is equivalent to a "blue shift" in bringing the chromophore from a less polar to a more polar solvent. Such a blue shift has often been taken as indicative of an $n-\pi^*$ transition. However, as Nakamota and Martell point out, $\pi-\pi^*$ bands of the dipolar ions of 3-hydroxypyridines shift in the same direction. Furthermore, it is well known that $\pi-\pi^*$ bands of tyrosine and tryptophan also undergo blue shifts on transfer to polar solvents. According to Nakamota and Martell the uncharged tautomers of 3-hydroxypyridines undergo the

[8] For which the standard deviation of the experimental points from the fitted curve was no more than twice that of the curve shown in Figure 7.

[9] Equation 4 cannot be used for it applies to mixtures of two tautomers only.

"expected" red shift. From our results it appears that this conclusion was probably based on an illusion resulting from overlapping of bands. We find that band I of the neutral tautomers also undergoes a small blue shift.

A major application of methods described in this paper is the resolution of overlapping absorption bands. Such resolution provides a straightforward method of evaluating tautomeric equilibria, a matter of considerable biological significance. From measurement of these tautomeric equilibria microscopic acid dissociation constants can be evaluated. While we will consider this matter in a later publication, it is pertinent to discuss a few points at this time. Considering dissociation of protons from the cations of 3-hydroxypyridines we find that substitution of the weakly electron attracting CH_2OH groups of pyridoxine in either the 4 or 5 position by a methyl group (4- and 5-deoxypyridoxine) produces a distinct increase in the pK values. Methylation of pyridoxine in the 6 position has a similar effect. Thus, pK_a (OH group) increases from 4.94 to 5.36, 5.54, and 5.42, respectively, in the 4-deoxy, 5-deoxy, and 6-methyl derivatives. Likewise, pK_b ($=NH^+$ group) increases from 5.52 to 6.22, 6.36, and 6.13, respectively. At the same time substitution of $CH_2NH_2^+$ for CH_2OH in the 4 position leads to much stronger inductive electron withdrawal; in pyridoxamine pK_a is 3.51 and pK_b is 4.43.

The strong interaction of the ring nitrogen and the phenolic group in the 3 position is well established (Metzler and Snell, 1955). Thus, pK_a for pyridoxine is 4.94 but pK_d, the pK of the same OH group with the $=NH^+$ proton dissociated, is 8.20. Thus the acidity of the phenolic group is affected by over three orders of magnitude by protonation or deprotonation of the ring nitrogen. In the case of pyridoxamine the change amounts to four orders of magnitude, a fact that follows from the high value of K_z. Ivanov and Karpeisky (1969) have pointed out the possible significance of protonation and deprotonation of the ring nitrogen as a "switching mechanism" for regulating the acidity of the OH group as well as the electron withdrawal or donation at the 4′ position. We also note that, just as substitution of $CH_2NH_3^+$ for hydroxymethyl affects the acidity of groups in the ring markedly, so changes in the state of protonation of ring groups will also have a large effect on the pK of the amino group of pyridoxamine. The same will be true for amino groups of transient intermediates formed during the action of pyridoxal phosphate dependent enzymes.

Turning to the values of $\tilde{\nu}_0$, pyridine can be treated as a substituted benzene with a strongly electron attracting group. Bathochromic shifts from the positions of the benzene bands are expected to be greatest when a strong electron donating group is also present in a meta position. The stronger the electron donating group and the stronger the electron accepting group the greater the expected bathochromic shift (Petrushka, 1961a,b; Stevenson, 1965). This leads directly to the predicted order of bathochromic shifts within the 3-hydroxypyridine series: $HP_z > P^- > H_2P^+ > HP_n$. As is observed with benzene derivatives, substitution of additional methyl groups or hydroxymethyl groups on the pyridine ring leads to bathochromic shifts of 0.5–1.0 kK. The spectrum of pyridoxine is little altered by replacement of the 5-CH_2OH group with a methyl group or by formation of a phosphate ester. On the other hand band I undergoes a surprisingly large hypsochromic shift (0.46–0.96 kK) when the 4-CH_2OH is replaced with methyl. The explanation for this shift is not obvious. Band II is relatively insensitive to the same change.

Our method of portraying small amounts of vibronic fine structure in the absorption bands should be useful in studies of solvent effects, hydrogen bonding, and binding to macromolecules. The spectral changes observed upon binding of pyridoxamine phosphate to an apoenzyme illustrate the small but clear-cut alterations that can be observed when the environment of a chromophore is changed.

It is established that transfer of pyridoxamine or of pyridoxamine phosphate from water to methanol leads to extensive conversion from the dipolar ion into the uncharged tautomer (Metzler and Snell, 1955; Matsushima and Martell, 1967). Furthermore, at the pH of our binding experiment (8.3) free pyridoxamine phosphate is partly dissociated to the anionic ring form. Nevertheless, it is clear from the data given that when it binds to the protein, the coenzyme is fixed as a single ionic form; that form is the dipolar ion, despite the fact that the properties of the absorption band suggest a less polar environment. An explanation may lie in the presence of a specific hydrogen bonding group in the protein designed to hold the coenzyme in the proper ionic form (Yang, 1973). This same hydrogen bonding may be responsible for the intensity of the fine structure.

When pyridoxamine phosphate binds to apoaspartate aminotransferase is the primary amino group of the coenzyme protonated? We cannot give a definitive answer to this question but the 0.46-kK bathochromic shift from the position of the free monocation–dipolar ion suggests that the $CH_2NH_3^+$ form is bound. The bathochromic shift is of the same magnitude as that observed for transfer of dipolar ions from water to methanol (Table X). From the resolution of the spectrum of the monoprotonated form of 5-deoxypyridoxamine (Figure 9) a hypsochromic shift of 0.5 kK is observed for deprotonation of the aminomethyl group. If the bound form were the CH_2NH_2 form a total bathochromic shift of nearly 1.0 kK would be required. We believe this unlikely.

Spectral band-shape analysis may be of general utility in investigations of the binding of small light-absorbing molecules to proteins. For chromophores absorbing at low energy where the protein band does not interfere, the application is straightforward. It may also be possible to apply the method to binding of nucleotides to proteins, even though the absorption bands overlap. Some nucleotides also have sharp spikes in their fine structure plots and changes in this fine structure upon binding may be observable.

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