respectively, are of comparable energy (eq 4 and ref 36). In fact, the "doubly excited" state was found[50] to be the lowest excited singlet state in some systems with a small HOMO–LUMO energy gap $\Delta\epsilon$, and a large number of photoreactions initiated in the S_1 state are assumed to proceed via a "pericyclic minimum" of S_D character.[51]

Acknowledgments. This work is part of Project 2.012-0.78 of the Swiss National Science Foundation. Financial support from Ciba-Geigy SA, Hoffmann-La Roche SA, Sandoz SA, and the "Ciba-Stiftung" is gratefully acknowledged.

(50) B. S. Hudson and B. E. Kohler, *Chem. Phys. Lett.*, **14**, 299–305 (1972); R. P. Steiner, R. D. Miller, H. J. Dewey, and J. Michl, *J. Am. Chem. Soc.*, **101**, 1820–1826 (1979); see also ref 36.

(51) See ref 10 and, e.g., J. Michl, *Pure Appl. Chem.*, **41**, 507–534 (1975).

Equilibria and Absorption Spectra of Schiff Bases[1]

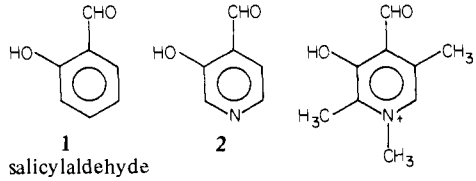
Carol M. Metzler, Allen Cahill, and David E. Metzler*

Contribution from the Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011. *Received March 21, 1980*

Abstract: Equilibria in the formation of Schiff bases between eight aromatic aldehydes including salicylaldehyde and pyridoxal 5′-phosphate with a variety of different amines, diamines, and amino acids have been investigated. The formation constants, the acid dissociation constants of the Schiff bases, and the absorption spectra of the various ionic species of the Schiff bases have been evaluated. The spectra have been resolved into components with log normal curves to provide a precise description of the band shapes and to permit estimation of tautomerization constants. Constants for ring closure in the Schiff bases of diamines have also been estimated. In addition to the major tautomer, which has an ortho quinonoid structure, smaller amounts of both a phenolic tautomer and a tautomer with a dipolar ionic pyridine ring are present in small amounts. Results of this study are correlated with those of previous investigations on related systems and with spectra of two enzymes.

Familiarity with the properties of Schiff bases (imines) of pyridoxal 5′-phosphate (**6**) with amines and amino acids is basic to an understanding of the function of this coenzyme in biological catalysis. It is also of importance because of the growing interest in using **6** and related materials as reagents for the modification of enzymes and other proteins. Numerous papers dealing with these Schiff bases have appeared. There is also a voluminous literature on the closely related Schiff bases of salicylaldehyde (**1**). However, because of the complexity of the ionic equilibrium needed to describe Schiff base forming systems, relatively few quantitative studies have appeared.

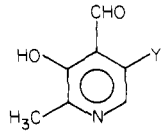
In this paper we provide a systematic description of 20 systems based on aldehydes **1**–**8**. Formation constants, acid dissociation constants, and absorption spectra of the individual ionic forms of the Schiff bases have been evaluated from absorption spectra using previously described computer-assisted methods.[2-6]



1 salicylaldehyde

2

4



Y

3	CH_3	5-deoxypyridoxal
5	CH_2OH	pyridoxal
6	CH_2OPO^{2-}	pyridoxal 5'-phosphate
7	CH_2OSO^{3-}	pyridoxal 5'-sulfate
8	$CH_2PO_3^{2-}$	

Experimental Section

Chemicals. Commercial amino acids, amines, and inorganic chemicals were used. Salicylaldehyde (**1**) (Aldrich Chemical Co., 98% pure) was distilled under vacuum at 25 °C to give a colorless product. 3-Hydroxy-4-pyridinaldehyde (**2**) was a gift from Dr. Marion O'Leary. The following were synthesized as described previously: 5-deoxypyridoxal (**3**),[7] its *N*-methochloride (**4**), and pyridoxal 5′-sulfate (**7**).[8] The phosphonate **8** was synthesized in this laboratory by C. N. Han.[9] Pyridoxal 5′-phosphate (**6**) was purchased from Sigma.

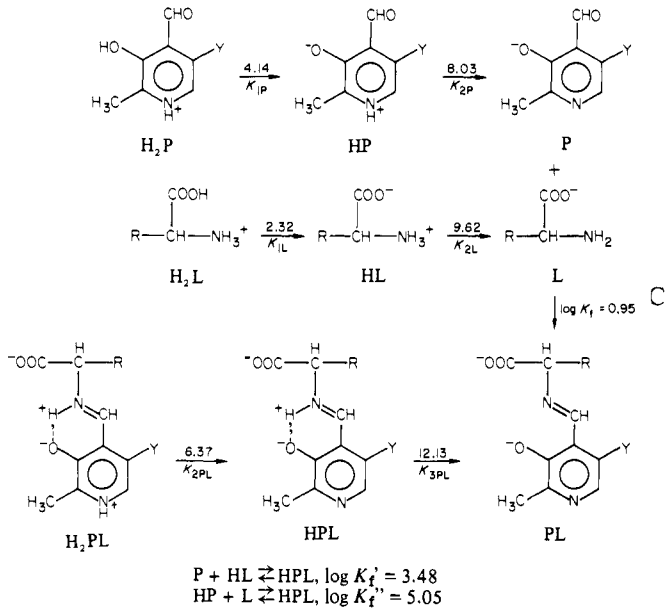
Experimental Procedure. Stock solutions of the aldehyde were prepared in water, usually at a concentration of 5×10^{-4} M. Aliquots were pipetted into volumetric flasks and appropriate amounts of the amine or amino acid component, together with buffer salts, were added. The final ionic strength was maintained at 0.2 when possible. However, solutions containing a very high concentration of the amine component (up to 1.0 M and up to 13.5 M for NH_3) were sometimes needed to permit evalu-

(1) A preliminary report appeared: *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1977**, *36*, 855. A few of the data cited here also appear in ref 6.

(2) Nagano, K.; Metzler, D. E. *J. Am. Chem. Soc.* **1967**, *89*, 2891.
(3) Johnson, R. J.; Metzler, D. E. *Methods Enzymol.* **1970**, *18A*, 433.
(4) Harris, C. M.; Johnson, R. J.; Metzler, D. E. *Biochim. Biophys. Acta* **1976**, *421*, 181.
(5) Metzler, D. E.; Harris, C. M.; Johnson, R. J.; Siano, D. B.; Thomson, J. A. *Biochemistry* **1973**, *12*, 5377.
(6) Metzler, D. E.; Harris, C. M.; Reeves, R. L.; Lawton, W. H.; Maggio, M. S. *Anal. Chem.* **1977**, *49*, 864A.
(7) Iwata, C. W. *Biochem. Prep.* **1968**, *12*, 117.
(8) Yang, In-Yu; Khomutov, R. M.; Metzler, D. E. *Biochemistry* **1974**, *13*, 3877.
(9) Han, C. N. Ph.D. Dissertation, Iowa State University, 1977.

0002-7863/80/1502-6075$01.00/0

Scheme I



$P + HL \rightleftarrows HPL$, $\log K_f' = 3.48$
$HP + L \rightleftarrows HPL$, $\log K_f'' = 5.05$

ation of the spectra of the most protonated and completely dissociated forms of the Schiff base. While these solutions exceeded the ionic-strength limits set, their inclusion in the data set usually caused little distortion in the formation constant or in any of the pK_a values other than the one in the pH 12 range.

After mixing, the solutions were allowed to equilibrate for a period of time ranging from 10 min, for most amino acids, to 24 h for the slowly equilibrating system salicylaldehyde + 2-aminopropane. Complete spectra of the solutions were then recorded on a Cary Model 1501 spectrophotometer equipped with a Cary-Datex digital output system. The reference cell was filled with a solution containing all components except the aldehyde. Absorbances were recorded to the nearest 0.001 unit at intervals of 200 cm^{-1}. In some cases, closer intervals, e.g., 50 cm^{-1}, were employed.

The pH of each solution was measured with a Radiometer Model PHM64 pH meter.

Computations were done at the Iowa State University Computation Center with programs written in Fortran IV. For the evaluation of equilibrium constants and spectra of individual ionic forms of the Schiff bases, it was necessary first to determine the pK values and spectra of single ionic forms for each aldehyde. This was done using a previously published procedure.[2,3] For each Schiff base system, the input data included not only the series of recorded spectra for mixtures of aldehyde and amine components but the previously established spectra of the individual ionic forms of the aldehyde and the previously determined pK values for both the aldehydes and the amine components. The pK values used in this work are listed in Table I.

From spectra of mixtures of the amine and aldehyde components in various ratios and at a variety of pH values, we evaluated simultaneously the formation constants and pK values of the Schiff bases together with the spectra of the individual ionic forms H_2PL, HPL, PL, etc., of the Schiff base using a computer-assisted method.[2,3,6] We used an updated program (available upon request) that employs the SSP subroutine ZXMIN. It provides a rapid nonlinear least-squares adjustment of the unknown equilibrium constants—usually three at a time. Thus, for the equations of Scheme I, $\log K_f$, pK_{2PL}, and pK_{3PL} could be evaluated simultaneously. When more than three constants had to be determined (e.g., for systems containing compound **6**), the data set was divided into low-pH and high-pH ranges. Using a low-pH or high-pH data set, an initial evaluation was made, and the determined pK value of the Schiff base at the extreme low pH or extreme high pH was then fixed while the entire data set was used to reevaluate the remaining constants.

For a Schiff base such as that of 5-deoxypyridoxal (**3**) with a neutral amino acid, the formation constant as well as both pK values in the Schiff base is determined quite precisely if the range of pH values and amino acid concentration used is adequate. Likewise, the spectra of all ionic forms are well defined. With some of the other compounds, one of the pK values, e.g., that of a phosphate group or an amino acid side chain, is less precisely established. Dissociation of such groups often has very little effect on spectra. Therefore, the pK values of such groups are, in essence, evaluated from the effect of pH on the degree of Schiff base formation.

Table I. Values of pK Used for Aldehyde and Amine Components in Calculations

	aldehydes	pK_a at 25 °C			source
1	salicylaldehyde		8.23		4, 5
2	3-hydroxy-4-pyridinaldehyde	4.15	6.81		4
3	5-deoxypyridoxal	4.14	8.03		4
	50% methanol	4.36	8.49		
	75% methanol	4.44	8.55		
	5 °C	4.41	8.57		
	50 °C	3.92	7.71		
4	N-methylated 3	4.34			4
5	pyridoxal	4.23	8.59		4
6	pyridoxal 5'-phosphate	3.62	6.10	8.33	4
7	pyridoxal 5'-sulfate	3.53	7.39		8
8	5'-phosphono-5-deoxypyridoxal	4.07	6.76	9.20	9

amines	pK_a at 25 °C		source
2-aminopropane		10.71	
ethylenediamine	7.35	10.01	15
ammonia		9.40	
n-butylamine		10.77	
75% methanol		10.71	
glycine	2.36	9.78	
alanine	2.35	9.83	
valine			
5 °C		10.30	
25 °C	2.32	9.62	
50 °C		9.02	
50% methanol		9.53	
75% methanol		9.35	
glutamic acid	4.25	9.67	
serine	2.21	9.15	
arginine[a]	1.82	8.99	
1,3-diaminopropane	9.03	10.94	

[a] The third pK was ignored; i.e., it was assumed the same in the free amino acid and the Schiff base.

Spectra were fitted with log normal curves using previously described methods.[4-6] From this fitting procedure the areas (integrated intensities) of the absorption bands of tautomers and cyclic forms could be estimated. Using assumed values for the molar areas, we then estimated the fraction of Schiff base present as each of the tautomers of cyclic forms. Details are given in the data supplement.

Results

We have studied 20 Schiff base forming systems involving aldehydes **1**–**8** with a variety of amines, diamines, and amino acids. The Schiff bases are numbered as in Table II. Results have been interpreted according to Scheme I, which shows the labels we have used for the various ionic forms of free aldehyde, amine (or amino acid), and Schiff base. The equilibrium constants shown in this scheme are for the system 5-deoxypyridoxal (**3**) + valine. All constants are "practical", i.e., they are defined in terms of molar concentrations with $[H^+]$ taken as 10^{-pH} and pH as the reading obtained with the meter. The negative logarithms of apparent acid dissociation constants are designated pK. The formation constant K_f is defined for reaction of the free amine with the anion of the aldehyde to give the completely unprotonated form of the Schiff base **17**. However, the computer-assisted evaluation of constants was usually done for the reaction of the anion of the aldehyde and the monoprotonated form of the amine:

$$P + HL \overset{k_f'}{\rightleftharpoons} HPL \qquad (1)$$

Using the symbols of Scheme I, the logarithm of the equilibrium constant K_f' for this reaction is

$$\log K_f' = \log K_f - pK_{2L} + pK_{3PL} \qquad (2)$$

The relationship of K_f' to K_f is shown graphically in Figure 3. When the side chain Y contains a phosphate group, an additional pK value must be defined for both aldehyde and Schiff base.

Table II. Formation Constants and pK_a Values for Schiff Bases at 25 °C

	Schiff base	log K_f'	pK_a values		ref
9	(**1** + 2-aminopropane)	1.88		11.76	
			4.70	11.90	13
10	(**1** + ethylenediamine)	2.68	8.70	11.3	15
		2.37, 4.87	8.83	11.04	
	1 + 1,3-diaminopropane	2.20			15
11	(**1** + valine)	2.68		12.16	
12	(**2** + valine)	1.67	5.45	9.91	
	at 30 °C	1.75	5.06	9.48	16a
	(**2** + alanine, 30 °C)	1.41		9.17	16b
13	(**3** + NH_3)	0.59	6.78	~10.9	
14	(**3** + *n*-butylamine)	3.29	6.21	11.89	
15	(**3** + glycine)	2.64	6.59	11.30	
16	(**3** + alanine)	2.51	6.43	11.55	
17	(**3** + valine)	3.48	6.37	12.13	
	at 5 °C	3.88	6.59	12.8	
	at 50 °C	3.14	6.23	11.70	
	in 50% methanol	3.72	6.15	12.44	
	in 75% methanol	4.02	5.78	12.98	
18	(**3** + glutamate)	2.85	6.49	11.67	
19	(**3** + serine)	3.01	6.30	11.33	
20	(**3** + arginine)	3.56	6.12	11.32	
21	(**3** + ethylenediamine)	3.64	6.05	8.11, 11.20	
22	(**3** + 1,3-diaminopropane)	3.52	6.24	9.10 10.73	
23	(**4** + valine)	2.18[a]		9.50	
24	(**5** + valine)	2.37	5.88	10.49	
25	(**6** + valine)	3.44, 5.61	6.76	12.16	
26	(**6** + glutamate)	2.62	5.85	6.45, 11.87	
27	(**7** + valine)	3.16	5.70		
28	(**8** + valine)	3.95, 5.92	7.61	~12.9	

[a] Log K_f''.

Likewise amines may have more or fewer dissociable groups than are indicated in Scheme I. In a few cases, e.g., for comparison with formation constants of N-methylated aldehydes, it is desirable to evaluate the constant for reaction of neutral aldehyde with free amine:

$$HP + L \overset{K_f''}{\rightleftharpoons} HPL \qquad (3)$$

$$\text{where } \log K_f'' = \log K_f - pK_{2P} + pK_{3PL}$$

A summary of the equilibrium constants obtained is shown in Table II. Typical of the Schiff base spectra are those of 5-deoxypyridoxal (**3**) + valine shown in Figure 1. The three ionic forms correspond to those in Scheme I. However, it should be borne in mind that tautomeric forms may exist for the species H_2PL and HPL and that other pH-independent equilibria such as hydration to form carbinolamines may occur. Thus, spectra may contain contributions from such components when they are present. For Schiff bases of diamines, cyclic adducts may make major contributions to some ionic forms.

For all systems we prepared comparison plots of the input spectra with those calculated from the constants and the spectra found for the individual ionic forms of the Schiff base. Several of these plots are shown in Figure 2 and are representative of those obtained for most systems. In some instances, e.g., in the slowly equilibrating salicylaldehyde + 2-aminopropane system and in the system **3** + NH_3, which required measurements at very high ionic strengths, agreement was somewhat less good.

Another kind of plot[10] (Figure 3) obtained for each system is that of the pH-dependent apparent formation constant K_{pH}:

$$K_{pH} = [\text{Schiff base}]_t/[\text{aldehyde}]_t[\text{amine}]_t \qquad (4)$$

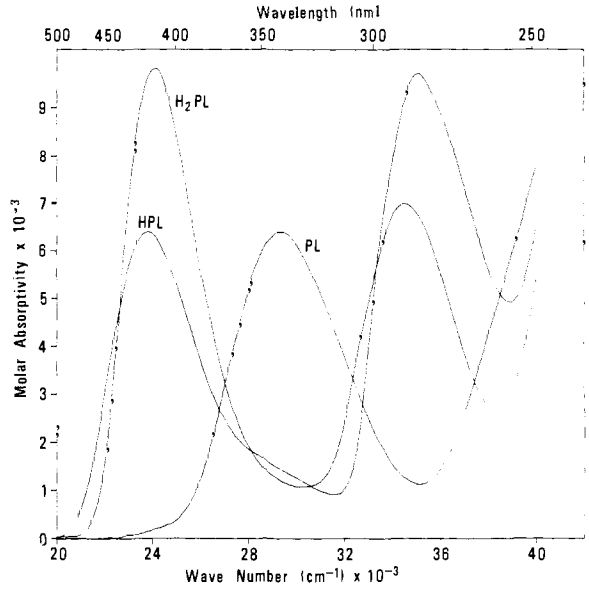


Figure 1. Calculated absorption spectra for three ionic forms of the Schiff base (**17**) of DL-valine with 5-deoxypyridoxal (**3**). The labels PL, HPL, and H_2PL refer to the unprotonated, monoprotonated, and diprotonated forms. These spectra together with the formation constant and pK_a values given in Table I (and Scheme I) were evaluated from spectra of 18 solutions in the pH range 5.1–13.0 and with valine concentrations ranging from 0.005 to 0.47 M.

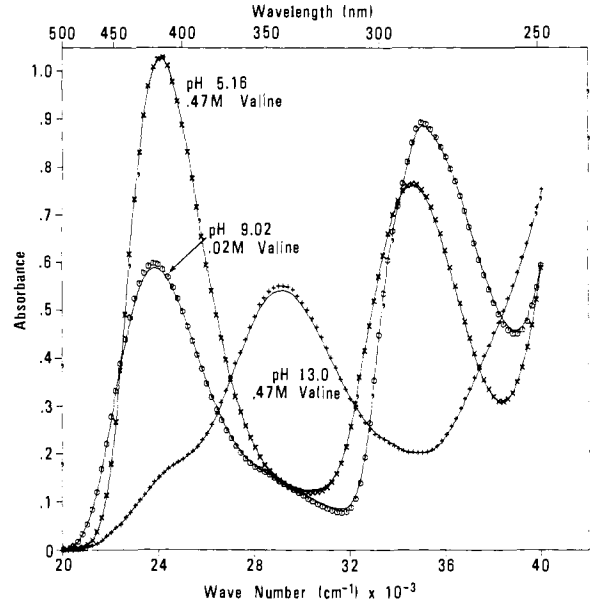


Figure 2. Comparison plots in which some of the spectra used in calculating the curves of Figure 1 are compared with theoretical curves constructed using the molar absorptivities from Figure 1 and the equilibrium constants from Scheme I. The symbols are experimental data points and the smooth curves the calculated spectra.

For the system of Scheme I

$$K_{pH} = K_f[(1 + [H^+]/K_{3PL} + [H^+]^2/K_{2PL}K_{3PL})]/[(1 + [H^+]/K_{2P} + [H^+]^2/K_{1P}K_{2P})(1 + [H^+]/K_{2L} + [H^+]^2/K_{1L}K_{2L})] \qquad (5)$$

The concentrations in eq 4 are the totals of all ionic forms. Beside the curve for 5-deoxypyridoxal (**3**) and valine in Figure 3A are the pK values for aldehyde, amino acid, and Schiff base. Their

(10) Metzler, D. E. *J. Am. Chem. Soc.* **1957**, *79*, 485.

Table III. Comparison of Low-Energy Absorption Bands of the Low-pH Forms of Two Enzymes and a Simple Schiff Base

	position, $cm^{-1} \times 10^{-3}$	nm	height, ϵ, $M^{-1}\ cm^{-1} \times 10^{-3}$	width, $cm^{-1} \times 10^{-3}$	skewness	area, km/mol
Schiff base 9, H_2PL form	24.11	414.8	9.79	3.66	1.52	395
in 75% methanol	23.76	420.9	10.49	3.55	1.48	408
aspartate aminotransferase	23.13	432.3	7.59	3.80	1.58	320
glutamate decarboxylase	23.77	420.7	10.08	3.46	1.56	386

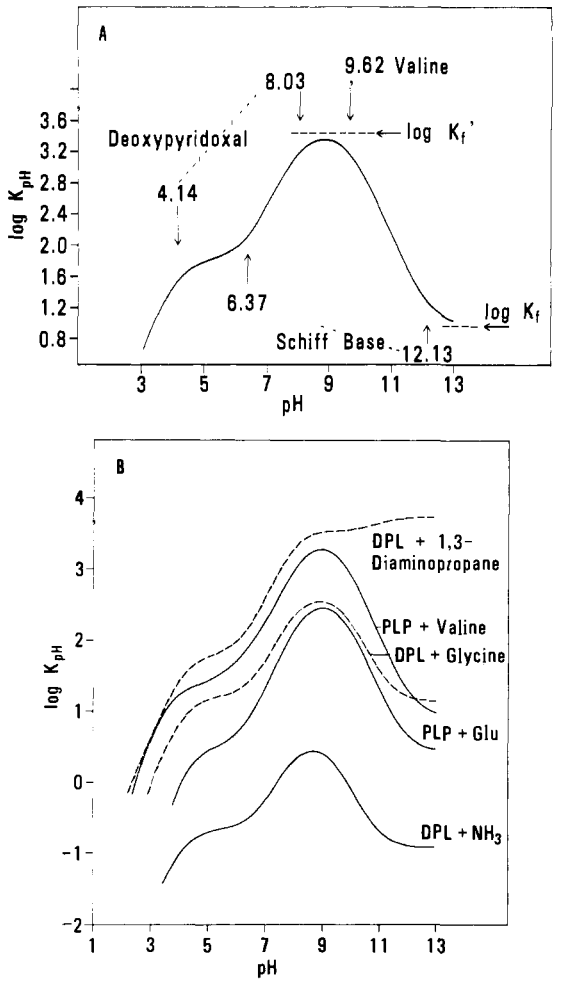


Figure 3. (A) Plot of the apparent pH-dependent formation constant K_{pH} vs. pH for the system described in Scheme I. (B) Similar plots for a few of the other systems described in this paper. Abbreviations: PLP, pyridoxal 5′-phosphate; DPL, 5-deoxypyridoxal; Glu, glutamic acid. Similar plots for all of the systems studied are given in the data supplement.

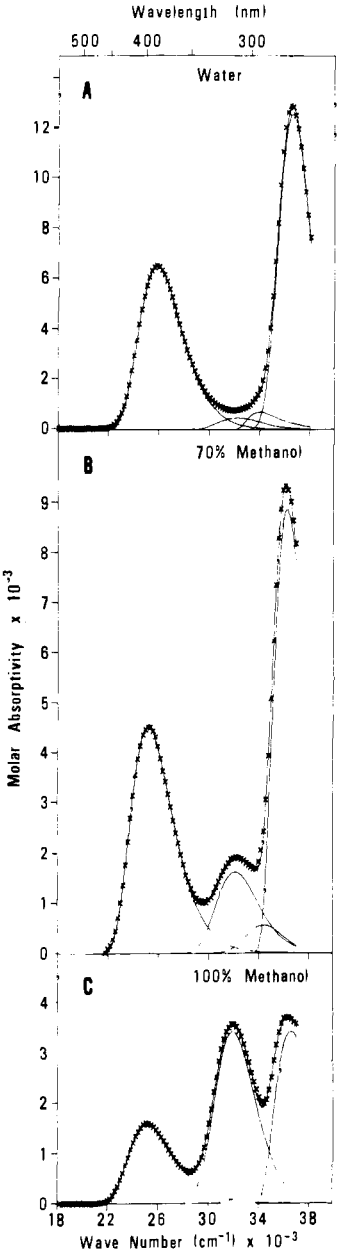


Figure 4. The spectrum of the monoprotonated form of the Schiff base (**9**) of salicylaldehyde (**1**) with 2-aminopropane: (A) in water; (B) in 70% methanol; (C) in methanol. These spectra were resolved using lognormal distribution curves and the method of Metzler et al.[5] was used to estimate molar areas and tautomerization constants. For any solution, the tautomerization constant R of eq 6 is related to the observed areas (a) and the changes in area with solvent (Δa) as follows: $R = [-\Delta a(\mathbf{9a})/\Delta a(\mathbf{9b})][a(\mathbf{9b})/a(\mathbf{9a})]$.

locations relative to one another determine the shape of the curve. Similar curves have been reported for the systems pyridoxal + alanine,[2] 5-deoxypyridoxal + alanine,[6] pyridoxal + valine,[10] and 5-deoxypyridoxal + leucine.[11]

All of the spectra calculated for Schiff bases have been analyzed by resolution with log normal distribution curves.[4-6] As is seen in Figures 4 and 5, the major bands are fitted very well by log normal curves. This was true for all of the Schiff bases. Typical log normal parameters (peak position, height, width, and skewness) are given in Table III for the major bands. However, to describe accurately the spectra, it was necessary in most instances to place minor bands in the valleys (Figure 5). For the most protonated forms (H_2PL in Scheme I), there are two major bands at about 415 and 285 nm and a weaker band, usually of less than 10% the area of the 415-nm band, located at 320–330 nm. For the HPL forms there must be at least two minor bands. These bands may represent weak electronic transitions but it seems more likely that they are an indication of the presence of minor tautomers.

It has previously been established, through the investigations of Heinert and Martell,[12] Herscovitch et al.,[13] and Inouye,[14] that

(11) Metzler, D. E.; Nagano, K. "Pyridoxal Catalysis: Enzymes and Model Systems", IUB Symposium Series, Vol. 35; Interscience: New York, 1968; pp 81–98.

(12) Heinert, D.; Martell, A. E. *J. Am. Chem. Soc.* **1963**, *85*, 188.
(13) Herscovitch, R.; Charette, J. J.; de Hoffman, E. *J. Am. Chem. Soc.* **1973**, *95*, 5135.
(14) Inouye, S. *Chem. Pharm. Bull.* **1967**, *15*, 1540.

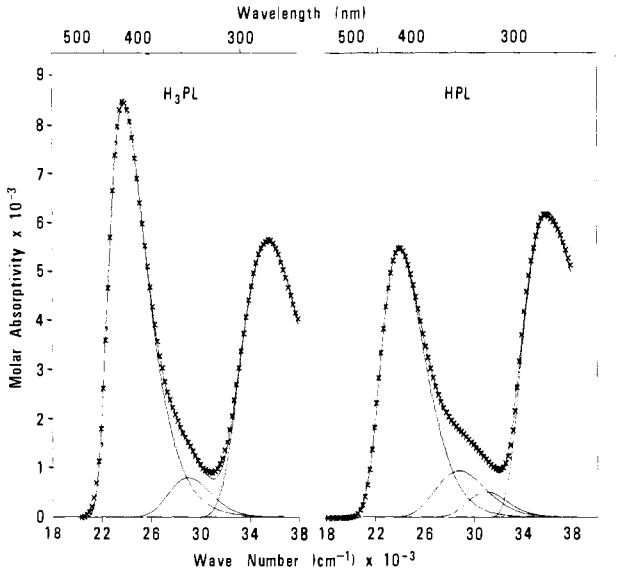


Figure 5. Spectra of the two ionic forms H_3PL and HPL of Schiff base **26** of pyridoxal phosphate with glutamate fitted with lognormal distribution curves.

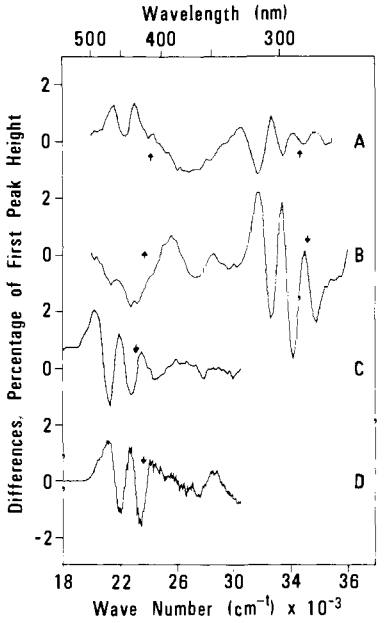
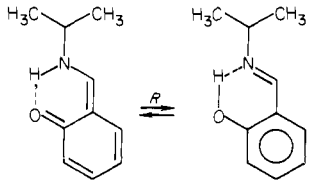


Figure 6. Portions of the "fine structure plots" given by graphing the differences between experimental points and corresponding points on the fitted curve (the sum of the lognormal bands). The small arrows mark the positions of the principal band maxima, i.e., $\sim\nu_0$ of the log normal curve. (A) Schiff base **17** of 5-deoxypyridoxal + valine, H_2PL form. (B) Schiff base **17**, HPL form. (C) Aspartate aminotransferase of pig heart, cytosolic isoenzyme, low-pH form. (D) Glutamate decarboxylase of *E. coli*, low-pH form.

in the Schiff base of **1** with 2-aminopropane the quinonoid tautomer (**9a** in eq 6) predominates in aqueous solution. In less polar solvents, the equilibrium is shifted in favor of the phenolic tautomer **9b**.



(6)

9a 9b

By studying this system in water and in water–methanol mixtures, we have attempted to use the method proposed by

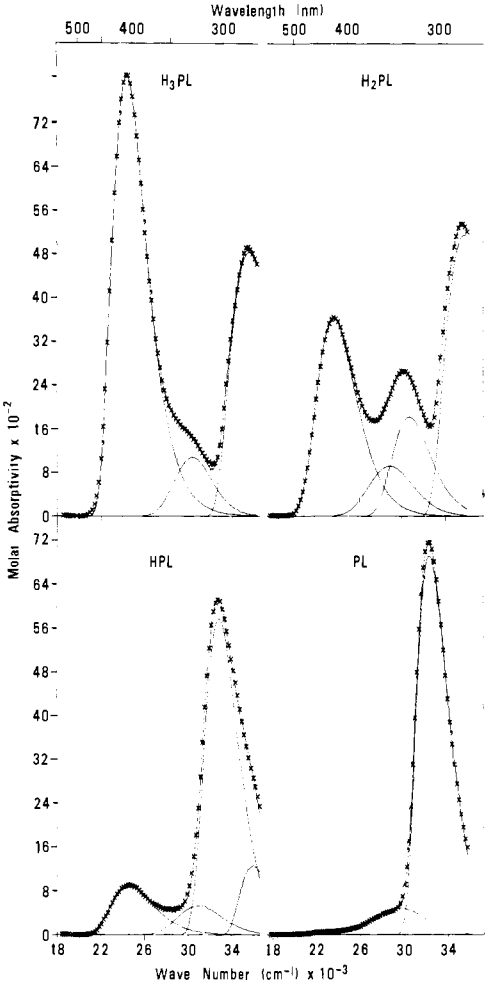


Figure 7. Spectra of the four ionic forms of Schiff base **21** of 5-deoxypyridoxal plus 1,3-diaminopropane analyzed with log normal curves.

Metzler et al.[5] to obtain a direct estimate of the tautomerization constant R in eq 6. The minor, almost buried band at 306 nm (32.7×10^3 cm^{-1}) in aqueous solution (Figure 4A) rises as the band of **9a** at 388 nm (25.8×10^3 cm^{-1}) falls in going to partially methanolic solutions (Figure 4B). From resolution with log normal curves the ratio of changes in area for the two bands Δa(**9b**)/Δa(**9a**) was found to be about 0.58. From the analysis shown in Figure 4A, this led to a value for R of about 0.088.

The spectra of Schiff bases derived from aldehydes **2**, **3**, and **5–8** have three or more ionic species as is shown in Figure 1. The spectra for forms H_3PL and HPL of Schiff base **25** formed from pyridoxal phosphate and glutamic acid and analyzed with log normal curves are shown in Figure 5. The minor tautomers represented by the weaker bands are considered in the Discussion section.

During the fitting of spectra with log normal curves, we obtained plots of the differences between the experimental points and the corresponding points on the smooth fitted curve, the sum of several log normal components. These difference plots or "fine structure plots" often reveal vibrational fine structure in the absorption bands.[4-6] Typical fine structure plots for several Schiff bases are shown in Figure 6.

The spectra of Schiff bases **10** and **21** derived from ethylenediamine and **22** derived from 1,3-diaminopropane (Figure 7) are of special interest. Formation of a cyclic adduct is possible, at least in the latter case. In addition, stabilization of a minor tautomer is apparently observed in some ionic forms (see Discussion).

Discussion

Equilibrium Constants. It is useful to relate the properties of the simplest Schiff base (**16**) studied to that of the components.

Salicylaldehyde has a pK of 8.35 and 2-aminopropane of 10.7. The Schiff base **9** (Scheme I) has a pK (11.8) higher than either, reflecting the chelate effect on the hydrogen bonding indicated in structure **9**. A high pK for the corresponding proton is seen in most of the other Schiff bases as well. For example, Schiff bases of valine with **2**, **3**, **5**, and **6** have pK values of 9.9, 12.1, 10.5, and 12.2, respectively. The lower pK for **2** correlates with the lower pK of the aldehyde; however, it seems surprising that the Schiff base of pyridoxal (**5**) with valine also has a low pK (10.5). This must result from some stabilizing interaction in the anion, perhaps involving hydrogen bonding between the imine group and the 5′-hydroxyl group.

The pK values also vary with those of the parent amine. For example, for 5-deoxypyridoxal plus ammonia, the pK of the Schiff base is 10.9, while with *n*-butylamine, whose pK is about 1 unit higher than that of ammonia, the pK of the Schiff base is 11.9. For the Schiff base of **3** + glycine, the pK is 11.3, only slightly greater than that with ammonia. As the side chain becomes more bulky, the pK increases, to 11.6 with alanine and 12.1 with valine. The pK is further increased by addition of methanol to the solvent. Thus, in 75% methanol, the pK is 13.0. This is presumably the result of increased electrostatic interaction between the phenolate oxygen and the proton hydrogen bonded to it with a decrease in polarity of the solvent. The high pK values for Schiff bases of valine and butylamine may be a result of a similar effect induced by the large alkyl groups.

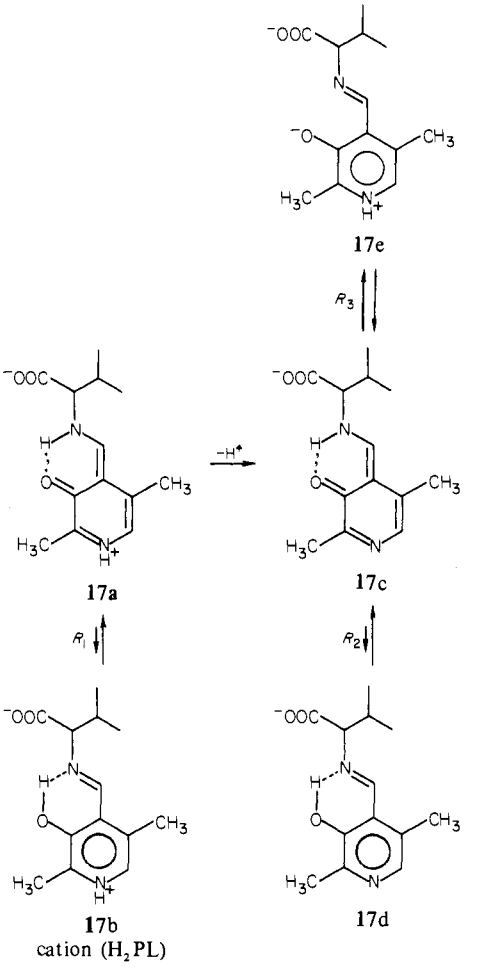
On the other hand, the pK for the ring nitrogen of Schiff bases of 3-hydroxypyridine-4-aldehydes is affected little by structure or by solvent. For most of the Schiff bases, this pK is 6.4 ± 0.2.

The formation constants of the Schiff bases studied vary considerably from one compound to another and also as a function of pH. Several representative curves of K_{pH} vs. pH are given in Figure 3B. Values of log K_f' are summarized in Table II. The values of K_{pH} are highest in the region 8–9 and drop at higher pH as the pK values of the amine and aldehyde components are exceeded. At still higher pH, K_{pH} approaches a constant value equal to K_f. The values of K_f are considerably less variable among the various Schiff bases than is K_f'. Log K_f usually falls in the range 0.6–1.4. However, there are exceptions; e.g., for **3** + ammonia log K_f is −0.9 but for **3** + butylamine it is 2.2.

The stabilization of the high-pH form of the Schiff base of pyridoxal + valine suggested by the low pK of 10.5 also accounts for the relatively high value of K_f for this system. Schiff bases for which cyclic adducts can form have relatively high values of K_f.

Previous studies have covered several aspects of the chemistry of some of the Schiff bases examined in this study. The systems salicylaldehyde + ethylenediamine and salicylaldehyde + 1,3-diaminopropane have been investigated in detail by McQuate and Leussing.[15] Schiff bases of **2** + glycine, alanine, valine, and glutamic acid were studied by French et al.[16a] and by Auld and Bruice.[16b] The Schiff base of **3** with leucine has also been studied.[2] In this instance, some evidence for the presence of 2:1 species having two molecules of amino acid to one of aldehyde was obtained. However, no indication of such complexes was obtained for the Schiff bases examined in this study. A number of Schiff bases of pyridoxal (**5**)[10,17] and a larger number with pyridoxal phosphate (**6**)[17-22] have been investigated. Recent studies using ^1H and ^{13}C NMR should also be mentioned.[23-26]

Scheme II



Some formation constants and pK values from these published studies are included in Table II. In general, these are consistent with the results of our study.

Tautomerism. A characteristic of Schiff bases of pyridoxal phosphate that may be of biological significance is the ability to exist in a variety of tautomeric forms. The solvent-dependent equilibrium between the two tautomers of Schiff bases of salicylaldehyde (eq 6, Figure 4) has been studied previously by Heinert and Martell[12] and by Charette and co-workers.[13,27,28] Tautomer **9a** predominates in aqueous solution while **9b** is favored in methanol and other nonaqueous solvents. Our estimate of the tautomeric ratio R (eq 6) for the Schiff base of salicylaldehyde and 2-aminopropane of 0.088 agrees well with that of 0.062 obtained by Herscovitch et al.[13] from a series of infrared spectral measurements in the same water–ethanol solvent pair. Nevertheless, there is considerable uncertainty primarily because of the presence of what is probably a minor electronic transition giving rise to an additional absorption band at 292 nm (Figure 4A; discussion in data supplement).

The spectrum of Schiff base **11**, formed from **1** + valine, is similar to that of **9**. We estimate the tautomeric ratio R as <0.15. Cations of the Schiff bases of compounds **2**–**8** also undergo the same type of tautomerism as is indicated for Schiff base **17** in Scheme II. Analysis with log normal curves reveals that the spectra of these cations have at least one minor absorption band in the valley at about 330 nm (30.3×10^3 cm^{-1} for **17**; 29.2×10^3 cm^{-1} for **26**, Figure 5). It is probable that at least a substantial

(15) McQuate, R. S.; Leussing, D. L. *J. Am. Chem. Soc.* **1975**, *97*, 5117.
(16) (a) French, T. C.; Auld, D. S.; Bruice, T. C. *Biochemistry* **1965**, *4*, 77. (b) Auld, D.; Bruice, T. C. *J. Am. Chem. Soc.* **1967**, *89*, 2083.
(17) Arrio-Dupont, M. *Photochem. Photobiol.* **1970**, *12*, 297.
(18) Matsuo, Y. *J. Am. Chem. Soc.* **1957**, *79*, 2011, 2016.
(19) O'Leary, M. H. *Biochim. Biophys. Acta* **1971**, *242*, 484.
(20) Tobias, P. S.; Kallen, R. G. *J. Am. Chem. Soc.* **1975**, *97*, 6530.
(21) Schonbeck, N. D.; Skalski, M.; Shafer, J. A. *J. Biol. Chem.* **1975**, *250*, 5343.
(22) Olivio, F.; Rossi, C. S.; Siliprandi, N. "Chemical and Biological Aspects of Pyridoxal Catalysis", IUB Symposium Series, Vol. 30; Macmillan: New York, 1963; p 91.
(23) Karube, Y.; Matsushima, Y. *Chem. Pharm. Bull.* **1977**, *25*, 2568.
(24) Harruff, R. C.; Jenkins, W. T. *Org. Magn. Reson.* **1976**, *8*, 548.
(25) Jaworski, R. A.; O'Leary, M. H. *Methods Enzymol.* **1979**, *62*, 436.
(26) Tsai, M.-D.; Byrn, S. R.; Chang, C.; Floss, H. G.; Weintraub, H. J. R. *Biochemistry* **1978**, *17*, 3177.
(27) Bruyneel, W.; Charette, J. J.; de Hoffman, E. *J. Am. Chem. Soc.* **1966**, *88*, 3808.
(28) Herscovitch, R.; Charette, J. J.; de Hoffman, E. *J. Am. Chem. Soc.* **1974**, *96*, 4954.
(29) Metzler, D. E.; Snell, E. E. *J. Am. Chem. Soc.* **1955**, *77*, 2431.

part of this band (or bands) represents the tautomer, e.g., **17b**. A corresponding band is present in the Schiff base of the N-methylated aldehyde **4** with valine (**23**) at 336 nm (29.8×10^3 cm^{-1}). The tautomerization constant R was estimated as 0.093 for **17** and R_1 (Scheme II) as 0.11 for the average of Schiff bases **14–28**.

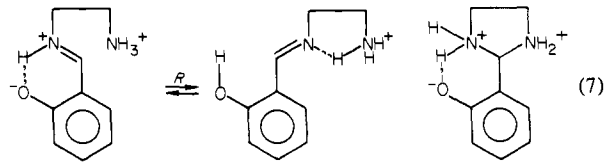
In the neutral (HP) forms of the Schiff bases both tautomers **17d** and **17e** may be present along with the major form **17c**. From the work of Heinert and Martell,[12] it is clear that **17d** is formed in nonpolar solvents but careful analysis with lognormal curves shows that it is not possible to fit the spectra in solvent mixtures with just two bands having reasonable width, skewness, and peak positions. We conclude that, in addition to **17d**, there is an equal amount of the dipolar ionic tautomer **17e**. This appears to absorb at about 347 nm (28.8×10^3 cm^{-1}), a position similar to that of the 351-nm absorption band of the *N*-methylated Schiff base **23**. For the latter the spectrum above 280 nm is represented well by a single log normal band, although there is a small misfit on the high-energy side as has been observed for other anions.[4]

Tautomer **17d** appears to absorb at about 330 nm (30.3×10^3 cm^{-1}). As shown by Heinert and Martell, the corresponding form of Schiff base **11** predominates in methanolic and dioxane–water solutions. Although it may seem surprising that **17d** absorbs at the same position as **17b**, Heinert and Martell observed only a 4-nm difference in positions between the corresponding forms of Schiff base **11** in dioxane.[12] The content of the minor tautomers is hard to evaluate. However, from analysis with log normal curves, as shown in Figure 5, we estimate for Schiff bases **14–28** that in aqueous solutions from 4 to 12% of the dipolar ionic tautomers (e.g., **17e**) and from 7 to 15% of the uncharged ring tautomer (e.g., **17d**) are present. Somewhat larger amounts (18–26%) of the uncharged ring tautomer appear to be present in **19**, **20**, and **28**. The content of **17d** is estimated as 8.6% in water and as increasing to 32% in 75% methanol while the content of the dipolar ionic tautomer **17e** decreases from 9 to 7%, i.e., roughly in proportion to the amount of the major tautomer **17c**.

The fraction of tautomer **17e** might be estimated in another way by assuming its formation constant to be the same as that of the N-methylated aldehyde **4** with valine to give Schiff base **23**. Using the values of log $K_f'' = 2.18$ for **23** and log $K_f'' = 5.05$ for **17** given by eq 3, we estimate only 0.13% tautomer **17e** present. This is lower than that estimated from the spectrum. We tend to believe the latter, but there is some uncertainty.[17]

Most pyridoxal phosphate dependent enzymes contain a Schiff base of a lysine side chain with **6**. Among the various enzymes there appear to be Schiff bases with structures corresponding to all three of the tautomers **17c–e**. That corresponding to the major tautomer **17c** may be present in tryptophanase at high pH.[30] The dipolar ionic form (corresponding to **17e**) is apparently present in aspartate aminotransferases[31,32] and in some other enzymes, while the form with an uncharged ring (analogous to **17d**) is present in glycogen phosphorylase.[33,34] Since the latter is favored by a medium of low dielectric constant, a hydrophobic environment has been suggested for the coenzyme in phosphorylases. On the other hand, the stabilization of the dipolar ionic form of the ring in aspartate aminotransferase is probably a result of interaction of groups in the binding site with the protonated ring nitrogen and with the dissociated phenolic oxygen.[35,36] Results of recent X-ray crystallographic studies show that for the aspartate aminotransferases a carboxylate group of an aspartate residue interacts tightly with the ring nitrogen of the coenzyme and a phenolic OH group of a tyrosine side chain reacts with the 3-O atom.[36] The possibility of interconversion among various tautomers during the course of an enzymatic reaction should be considered.[35]

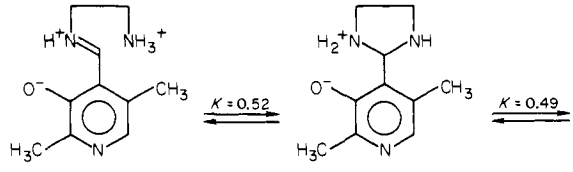
Schiff base **10** formed from **1** and ethylenediamine is of special interest. The spectrum of the monoprotonated form (HPL of Scheme I) closely resembles that of **9**, but the spectrum of the diprotonated (H_2PL) form contains bands of nearly equal intensity at 399 and 309 nm. The former clearly represents structure **10a** but the latter could be either **10b** or the cyclic adduct **10c**. Since



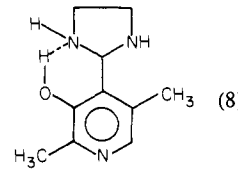
(7)

10a **10b** **10c**

there is little or no evidence of cyclization in the monoprotonated or anionic forms, we favor **10b**. By resolving with log normal curves, assuming the molar area of **10a** the same as that of **9a** (eq 6) and neglecting any contribution from **10c**, we estimated $R = [\mathbf{10b}]/[\mathbf{10a}] = 2.0$. A similar value of 1.8 was estimated by McQuate and Leussing, whose detailed analysis of this system[15] is in agreement with ours. The high value of this tautomerization constant compared to that for **9** or for the HPL form of **10** may be attributed to formation of the strongly hydrogen-bonded structure indicated for **10b**. A similar situation holds for Schiff base **21** derived from **3** + ethylenediamine. In this case the tautomerization constant R for the H_3PL form is estimated as 1.04 and for the H_2PL form as 4.0. It is possible that some cyclization has also occurred in the H_2PL form to give a form similar to **10c**. In the HPL form the cyclic species accounts for 56% of the total and in the PL form for 72%. For **21** we estimate the equilibrium constant for cyclization as 1.9 in the PL form and as 0.52 in the HPL form **21a**. However, about 13% of the HPL



21a **21b**



(8)

21c

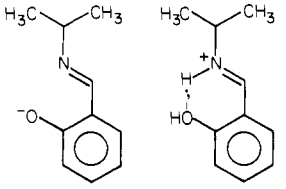
form of the Schiff base probably exists as the uncharged, cyclic structure **21c**. Thus, the effective cyclization constant for **21a** is 0.77. These values compare well with those of 1.2 and 0.84 estimated by Tobias and Kallen[20] for the PL and HPL forms of the Schiff base of **6** and ethylenediamine. However, we disagree with those authors in their assignment of the 334-nm band in the H_2PL form of these Schiff bases to a cyclic species. There is no reason to expect a large amount of a cyclic species in the HPL form. However, the previously mentioned stabilization by hydrogen bonding would favor a tautomer analogous to **10b**.

For the Schiff base **22** of **3** with 1,3-diaminopropane (Figure 7) closed-ring species predominate at high pH, the cyclization constants for PL and HPL forms being about 11 and 7, respectively. However, in the HPL form, a tautomer analogous to **10b** probably is again a major contributor. A more complete analysis is available in the supplementary data. However, since the equilibria in this system are exceedingly complex, they probably cannot be worked out fully without examination of the solvent and temperature dependence of the spectra.

Absorption Spectra. The spectrum of the nonionic tautomer **9b** in nonpolar solvents can be related directly to that of salicylaldehyde (**1**) in water.[37] The latter contains bands at 325, 256,

(30) June, D. S. Ph.D. Dissertation, Michigan State University, 1979.
(31) Jenkins, W. T.; Sizer, I. *J. Am. Chem. Soc.* **1957**, *79*, 2655.
(32) Davis, L.; Metzler, D. E. In "The Enzymes", 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1972; Vol. VII, p 64.
(33) Johnson, G. F.; Tu, J.-I.; Bartlett, M. L. Shonka; Graves, D. J. *J. Biol. Chem.* **1970**, *245*, 5560.
(34) Shaltiel, S.; Cortijo, M. *Biochem. Biophys. Res. Commun.* **1970**, *41*, 594.
(35) Metzler, D. E. *Adv. Enzymol.* **1979**, *50*, 1.
(36) (a) Ford, G. C.; Eichele, G., and Jansonius, J. N., *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 2559. (b) Arnone, A., private communication.

and 213 nm (30.77, 39.12, and 46.9 × 10^3 cm^{-1}, respectively). The relative intensities of the three bands increase toward higher energies. The spectrum of **9b** in cyclohexane has three bands at 317, 256, and 224 nm (31.5, 39.1, and 44.6 × 10^3 cm^{-1})[38] and the intensities increase with increasing wavenumber. The center bands of both **1** and **9b** display distinct vibrational structure in nonpolar solvents. In a similar manner, the spectra of the anion of **1** and that of the Schiff base **9c** consist of three bands, the first two being of similar intensity. Positions are 377, 256, and 226



9c

9d

nm (26.52, 37.75, and 44.15 × 10^3 cm^{-1}) for **1**. Again, the Schiff base bands are at the somewhat higher energies of 28.9, 39.3 and approximately 45.0 × 10^3 cm^{-1}. Because of their instability in solution, we have not studied the cationic forms of the Schiff base such as **9d**. These have been investigated in solution by Bruyneel et al.,[27] by McQuate and Leussing,[15] and in methanolic solutions by Heinert and Martell.[12] The positions of the first two bands of the cation of **9d** are shifted bathochromically from those of the neutral form.

Tautomer **9a** does not correspond in structure to any form of salicylaldehyde and its spectral bands are shifted to considerably lower energies: 25.9, 36.4, and 45.0 × 10^3 cm^{-1} (386, 275, and 220 nm).

The most notable fact about the spectra of the Schiff bases derived from aldehydes **3–8** is that they differ very little one from another as is documented in the data supplement. The N-methylation in **23** causes a small bathochromic shift while the anionic form of the Schiff base **24** of pyridoxal has a maximum at 366 nm compared to 340 nm for the average of the other anions. A possible interpretation is that a hydrogen-bonding interaction involving the 5′-OH group of the anion of **24** helps hold the imine group more nearly coplanar with the ring than in the other anions.

The widths of the lowest energy absorption bands are also nearly constant, averaging (3.68 ± 0.09) × 10^3 and (4.03 ± 0.10) × 10^3 cm^{-1} for the H_2PL and HPL forms. The width of the absorption bands of the anions is somewhat more variable within the range 4.04 × 10^3 to 5.68 × 10^3 cm^{-1} (data supplement). The average skewnesses of the absorption bands as defined previously[5] are 1.50, 1.26, and 1.32 for H_2PL, HPL, and PL forms.

A comparison with spectra of enzymes is of interest. The shapes of the low-energy absorption bands of a highly purified aspartate aminotransferase of pig heart and of a glutamate decarboxylase from *E. coli* are very similar to those of the Schiff base of 5-deoxypyridoxal plus valine (Table III). However, the distinct differences in bandwidths and positions must be explained by significant differences in the environment of the coenzyme in the two proteins and in the model systems.

Although the absorption bands of vitamin B_6 derivatives appear smooth, a small amount of vibrational fine structure amounting to about 1% of the peak height is often detected (Figure 6). This is usually apparent in the fine structure plot as a sharp spike, which may mark the position of the 0–0 band, on the low-energy side of an absorption band. There may also be other lower spikes, often at a spacing of about 1600 cm^{-1}. In the H_2PL forms of Schiff base **17**, the sharp spike lies about 1100 cm^{-1} below the band maximum and is 1100 ± 200 cm^{-1} below the maximum for many of the other Schiff bases studied. The spike is much weaker in the HPL forms (Figure 6). However, it is clearly present at 1200 and 970 cm^{-1} below the maxima for the two enzymes aspartate aminotransferase and glutamate decarboxylase. This fact suggests that in these enzymes at low pH the coenzyme is present in an N(ring)-protonated form. In the higher energy band (289 nm for **17**), very strong vibrational structure is always present (Figure 6) as it is in salicylaldehyde. Two distinct peaks are always present in the fine structure plots about 200 and 1800 cm^{-1} below the band maximum. These peaks have not been observed in enzymes and the strong 280-nm absorption bands of aromatic amino acids will interfere with their observation. However, the vibrational structure of the Schiff base is intense enough that it may be possible to observe it in an enzyme such as tryptophanase where the N-(ring)-unprotonated form is thought to be present at high pH.[30]

Acknowledgments. This work was supported by a grant (AM-01549) from the U.S. Public Health Service and by the Sciences and Humanities Research Institute of Iowa State University. We are grateful to Marion O'Leary, Chi-Neng Han, Chuzo Iwata, and Kevin Kelly for providing compounds used in this work.

Supplementary Material Available: Additional spectra; tables of band parameters for spectra together with estimates of tautomerization constants (57 pages). Ordering information is given on any current masthead page.

(37) Stevenson, P. E. *J. Mol. Spectrosc.* **1965**, *15*, 220.
(38) Seliskar, C. J. *J. Mol. Spectrosc.* **1974**, *53*, 140.