[CONTRIBUTION FROM THE BIOCHEMCIAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS, THE CLAYTON FOUNDATION FOR RESEARCH, AND THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

Spectra and Ionization Constants of the Vitamin B_6 Group and Related 3-Hydroxypyridine Derivatives[1]

BY DAVID E. METZLER AND ESMOND E. SNELL

RECEIVED SEPTEMBER 20, 1954

The ultraviolet absorption spectra of pyridoxal, pyridoxamine, pyridoxine and several related 3-hydroxypyridines are compared and correlated with those of a number of other pyridine and benzene derivatives. The two absorption bands in the accessible spectrum of each of the 3-hydroxypyridines appear to correspond to those of the spectra of pyridine and of benzene. The equilibrium between dipolar ionic and uncharged neutral forms of the 3-hydroxypyridines is investigated spectrophotometrically in dioxane–water and alcohol–water mixtures. In neutral aqueous solutions the dipolar ionic forms predominate but significant amounts of the uncharged forms are also present. Spectrophotometric data are used to determine semi-quantitatively the equilibria among the hemiacetal, free aldehyde and hydrated aldehyde forms of pyridoxal and to calculate the ionization constants for the phenolic group and for the ring nitrogen of 3-hydroxypyridine, pyridoxamine, pyridoxal, O-methylpyridoxal, 5-desoxypyridoxal and pyridoxamine phosphate. The effects of structure on these constants are discussed briefly.

Although the ultraviolet spectra of the vitamin B_6 group and related compounds have been discussed[2–5] and some ionization constants have been reported,[5,6] these simple properties have not yet been studied comprehensively. A detailed knowledge of them should be helpful in understanding the biological function of vitamin B_6 and the nature of the interaction of pyridoxal with amino acids and metal salts.[7] The present study represents an attempt to correlate and describe more completely these properties and from them to establish the amounts of the various ionic and non-ionic molecular species of these compounds in aqueous solution.

Spectra of 3-Hydroxypyridines.—Pyridoxine (I) is typical of the compounds under consideration. Its spectrum (Fig. 1)[2,3,5] varies with *p*H, corresponding in acidic solutions to the cation, Ia, at neutrality to neutral forms Ib and Ic,[8] chiefly the dipolar ion, Ic, and in alkaline solution to the anion, Id.

In each case the accessible spectrum consists of two absorption bands differing in the wave length of their maxima by a similar amount (Table I, Fig. 1). The changes with *p*H involve a displacement of this two band system toward either longer or shorter wave lengths. In addition, the relative intensities of the two absorption bands change. In a series of substituted 3-hydroxypyridines, II–IX (Table I), the separations of the two bands were very similar to those in pyridoxine, varying between

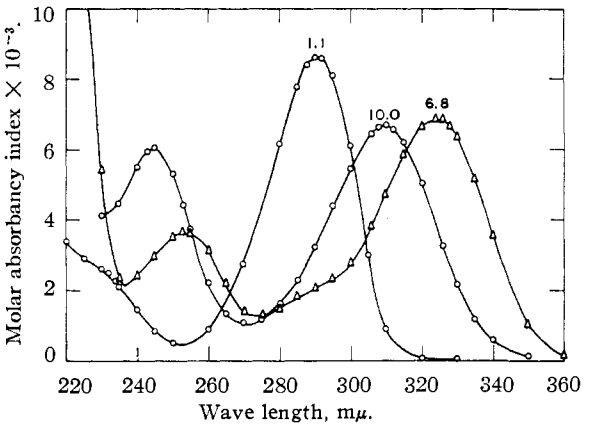
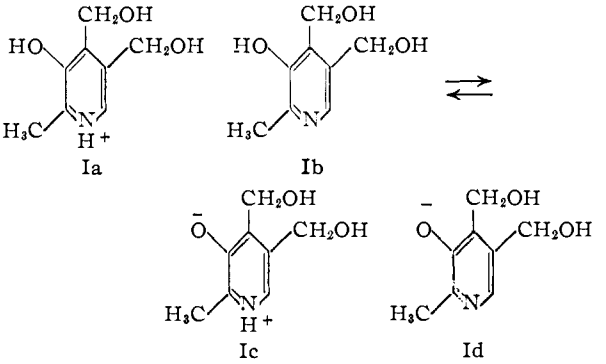


Fig. 1.—The absorption spectrum of pyridoxine at various *p*H values. The figures beside the curves indicate the *p*H of the sample.



57 and 77 mμ. It seems plausible to assume that these two bands correspond to those of the pyridine spectrum at 256 mμ[9] and at about 194 mμ.[10] These bands in turn may correspond to the two bands of the benzene spectrum at 254 and 203.5 mμ, respectively.[11]

Doub and Vandenbelt[11] have correlated the spectra of many benzene derivatives on the basis of wave length shifts of the absorption peaks caused by substituents. The effects of substitution in the

(1) Supported in part by a grant from Sharp and Dohme, Inc. Journal Paper No. J-2575 of the Iowa Agricultural Experiment Station, Ames, Iowa, Project No. 1259. Presented in part before the Federation of American Societies for Experimental Biology, 72nd Meeting, April, 1954. An abstract appears in *Federation Proc.*, **13**, 264 (1954).

(2) S. A. Harris, T. J. Webb and K. Folkers, THIS JOURNAL, **62**, 3198 (1940).

(3) (a) D. Melnick, M. Hochberg, H. W. Himes and B. L. Oser, *J. Biol. Chem.*, **160**, 1 (1945); (b) E. A. Peterson and H. A. Sober, THIS JOURNAL, **76**, 169 (1954). Data from this paper, which appeared after our table was compiled, are in good agreement with our data in Table I.

(4) D. Heyl, E. Luz, S. A. Harris and K. Folkers, THIS JOURNAL, **73**, 3430 (1951).

(5) A. K. Lunn and R. A. Morton, *The Analyst*, **77**, 718 (1952).

(6) V. R. Williams and J. B. Neilands, *Federation Proc.*, **13**, 321 (1954); *Arch. Biochem. Biophys.*, **53**, 56 (1954).

(7) D. E. Metzler, M. Ikawa and E. E. Snell, THIS JOURNAL, **76**, 648 (1954).

(8) For most of the compounds studied here, the two ionization constants are sufficiently far apart (Table III) that the spectrum at a *p*H value midway between the two *pK* values is very nearly that of the neutral forms alone.

(9) P. Krumholz, THIS JOURNAL, **73**, 3487 (1951).

(10) C. W. F. Spiers and J. P. Wibaut, *Rec. trav. chim.*, **56**, 573 (1937).

(11) L. Doub and J. M. Vandenbelt, THIS JOURNAL, **69**, 2714 (1947); **71**, 2414 (1949).

TABLE I

SPECTRA OF SOME 3-HYDROXYPYRIDINES

$\Delta\lambda$ = difference in mμ between the two absorption peaks of the spectrum. ΔOH = wave length shift in absorption peak of longer wave length upon ionization of the phenolic group. Δpy = wave length shift upon loss of the pyridinium proton.

	Compound	Conditions	λmax, mμ	$a_M \times 10^{-3}$	λmax, mμ	$a_M \times 10^{-3}$	$\Delta\lambda$, mμ	ΔOH, mμ	Δpy, mμ	Source of data
I	Pyridoxine									
	Ia, cation	0.1 *N* HCl	232[a]	2.1	291	8.6	59	+33	− 5	*b, c, d*
	Ib, neutral	Alcohol			286	5.7		+24		*b, e*
	Ic, dipolar ion	*p*H 6.8	254	3.9	324	7.2	70		−14	*b, c, d*
	Id, anion	0.1 *N* NaOH	245	6.3	310	6.8	65			*b, c, d*
II	N-methylpyridoxine									
	cation	0.1 *N* HCl			295	8.3		+35		*b*
	dipolar ion	*p*H 6.5	256	4.3	330	6.1	74			*b*
III	3-Hydroxypyridine									
	cation	0.1 *N* HCl	222	3.3	283	5.9	61	+30	− 6	*e, f*
	neutral	*p*H 6.8			277	2.2		+21		*e*
	dipolar ion	*p*H 6.8	246	4.7	313	3.0	67		−15	*e*
	anion	0.1 *N* NaOH	235	10.2	298	4.5	63			*e, f*
IV	N-Methyl-3-hydroxypyridine									
	cation	0.1 *N* HCl			288	5.8		+34		*b*
	dipolar ion	0.1 *N* NaOH	245	9	322	5.1	77			*b*
V	2-Methyl-5-hydroxypyridine									
	cation	0.1 *N* HCl			293	6.0				*f*
	anion	0.1 *N* NaOH	239	8.1	308	3.4	69			*f*
VI	2-Methyl-3-hydroxy-5-ethylpyridine									
	cation	0.1 *N* HCl			292	6.8				*f*
	anion	0.1 *N* NaOH	240	5.5	305	5.9	65			*f*
VII	4-Desoxypyridoxine									
	cation	0.1 *N* HCl	224	2.6	282	8.3	58	+31		*c*
	dipolar ion	Neutral	255	3.9	313	8.1	58		−12	*c*
	anion	0.1 *N* NaOH	244	6.9	301	7.1	57			*c*
VIII	Pyridoxamine	0.1 *N* HCl	226	2.0	292	8.2	66	+34	− 5	*c, d, e*
	monohydrochloride	98% dioxane			287	3.4		+23		*e*
		*p*H 6.7	252	4.5	326	7.9	74		−16	*d, e*
		0.1 *N* NaOH	245	6.2	310	7.2	65			*c, d, e*
IX	Pyridoxal									
	cation	0.1 *N* HCl			288	9.0		+29	− 8	*d, e*
	neutral	60% dioxane			280	4.1		+22		*e*
	dipolar ion	*p*H 6.9	252	5.8	317	8.9	65		−15	*d, e*
	anion	*p*H 10-11	240	8.4	302	5.7	62			*d, e*
X	O-Methylpyridoxine									
	cation	*p*H 2			280				0	*b*
	uncharged	*p*H 10			280					*b*
XI	O-Methylpyridoxal									
	cation	*p*H 2			289	6.4			−13	*e*
	uncharged	*p*H 8			276	3.9				*e*
XII	Pyridine									
	cation	0.05 *N* HCl			256	5.7				*g*
	uncharged	0.02 *N* NH_3	194		256	2.8	62			*g*

[a] Lunn and Morton[5] show this band as a clearly resolved peak whereas our data (Fig. 1), indicate only a shoulder. It seems probable that the peak may be obscured by a small amount of impurity absorbing at low wave lengths. On all other details our data agree with those of Lunn and Morton. [b] Reference in footnote 2. [c] Reference in footnote 5. [d] Reference in footnote 3. [e] This research. [f] Reference in footnote 24. [g] References in footnotes 9 and 10.

3-position of pyridine, and of similar substitutions in benzene, are compared in Table II. Electron-donating substituents cause similar wave length shifts in the two cases, lending support to the idea that the two bands of the 3-hydroxypyridines are fundamentally related to those of benzene.[12]

Spectrophotometric comparisons of pyridoxine and its N-methyl derivative have shown[2] that the first ionization constant of pyridoxine (pK = 5.00[5]) is chiefly that of the phenolic hydroxyl group to give the dipolar ionic form Ic. This ionization

(12) The spectral shifts caused by electron-donating substituents are greater for pyridine than for benzene. However, for the electron-accepting substituents, carboxyl and carboxylate, the shifts are smaller with pyridine than with benzene. The shifts for various *meta*-substituents on the benzoate ion (which, like the pyridine ring, contains an electron-accepting group) are more nearly the same as those with pyridine derivatives in all cases.

TABLE II

EFFECT OF SUBSTITUENTS ON THE ABSORPTION SPECTRA OF PYRIDINE AND BENZENE

Wave lengths of the absorption maximum of the higher wave length band. Roman numerals refer to the compounds used (see Table I).

Parent compound	Substituent	Solvent	λmax, mμ	$a_M \times 10^{-3}$	Wave length shift (mμ)	Source of data
Pyridine	None (XII)	Water	256	2.8	0	*a*
	None	Alcohol	257	2.6	0	*b*
	3-CH_3	Alcohol	*ca.* 263		6	*c*
	3-Cl	Heptane	*ca.* 264	2.4	7	*d*
	3-OH(III)	Water	277		21	*e*
	3-OH(III)	Alcohol	278	4.2	21	*e, f*
	3-NH_2	Ether	298	3.3	41	*g*
	3-O^-	Water	298	4.5	42	*e, h*
	3-O^-	Alcohol	301	4.1	44	*e, f*
	3-COO^-	Water	261.5	5.5	3.5	*i*
Benzene	None	Water	254	0.20	0	*j*
	$-CH_3$	Water	261	0.23	7	*j*
	—Cl	Water	263.5	0.19	9.5	*j*
	—OH	Water	270	1.45	16	*j*
	$—NH_2$	Water	280	1.43	26	*j*
	$—O^-$	Water	287	2.60	33	
	$—COO^-$	Water	268	0.56	14	

[a] References in footnotes 9 and 10. [b] W. K. Miller, S. B. Knight and A. Roe, THIS JOURNAL, **72**, 1629 (1950). [c] F. Baker and E. C. C. Baly, *J. Chem. Soc.*, **91**, 1122 (1907). [d] Reference in footnote 10. [e] This research. [f] H. Specker and H. Gawrosch, *Ber.*, **75**, 1338 (1942). [g] L. C. Anderson and N. V. Seeger, THIS JOURNAL, **71**, 340 (1949). See reference in footnote 10 also, and J. D. S. Goulden, *J. Chem. Soc.*, 2939 (1952), and E. A. Steck and G. W. Ewing, THIS JOURNAL **70**, 3397 (1948). [h] Reference in footnote 22. [i] E. B. Hughes, H. H. G. Jellinek and B. A. Ambrose, *J. Phys. Colloid Chem.*, **53**, 414 (1949). [j] References in footnote 11.

causes the higher wave length maximum to shift +33 mμ (toward a higher wave length). A similar shift of +32 ± 3 mμ attends the ionization of the phenolic groups of six related compounds (Table I), and is thus characteristic of the ionization of a 3-hydroxyl group on a pyridinium ion. Neutral aqueous solutions of 3-hydroxypyridine itself contain both the dipolar ion and the uncharged neutral form (see below). Maxima corresponding to each form are present in the spectrum (Table I). Ionization of the phenolic group of the uncharged neutral form of this compound is accompanied by a wave length shift of +21 mμ. Pyridoxine, pyridoxal and pyridoxamine monohydrochloride in alcohol or dioxane–water mixtures contain the uncharged forms of type Ib,[13] and ionization of the hydroxyl group again leads to wave length shifts of about 23 mμ, a value typical for the ionization of a 3-hydroxyl group on an un-ionized pyridine ring. This shift is very similar to those observed for the ionization of various phenols in the benzene series,[11,14] *e.g.*, +17 mμ for phenol itself and for 3-hydroxytoluene and +25 mμ for 3-hydroxybenzoate.

Dissociation of the pyridinium hydrogen of the dipolar ion of pyridoxine (Ic → Id) is attended by a spectral shift of −14 mμ (to a shorter wave length) with a slight decrease in absorbancy. Similar shifts ranging from −12 to −16 mμ were observed for the loss of a proton from four other dipolar ionic forms (Table I). For dissociation of the cation to the neutral un-ionized form (*e.g.*, Ia → Ib) the shift is decreased to −5 to −8 mμ and for O-methylpyridoxine the shift is almost absent as in pyridine itself. For O-methylpyridoxal, however, a −13 mμ shift is observed (Table I).

Equilibrium between Dipolar Ion and Uncharged Forms.—Pyridoxine in aqueous solution at *p*H 6.86 should be present as the neutral (dipolar ion and uncharged) forms to the extent of about 98% as calculated from the *pK* values of 5.00 and 8.97.[5] Although the dipolar ionic form, Ic, predominates in aqueous solutions, alcoholic solutions appear to contain the uncharged form Ib.[2] We have measured the spectra of pyridoxine, 3-hydroxypyridine and pyridoxamine monohydrochloride in dioxane–water mixtures. Typical results are shown in Fig. 2. The change in the pyridoxine spectrum upon additions of dioxane (Fig. 2B) consists of a displacement of the absorption to a lower wave length with a decrease in intensity of the high wave length peak consistent with the assumption that the dipolar ionic form is being converted into the neutral non-ionic form Ib.

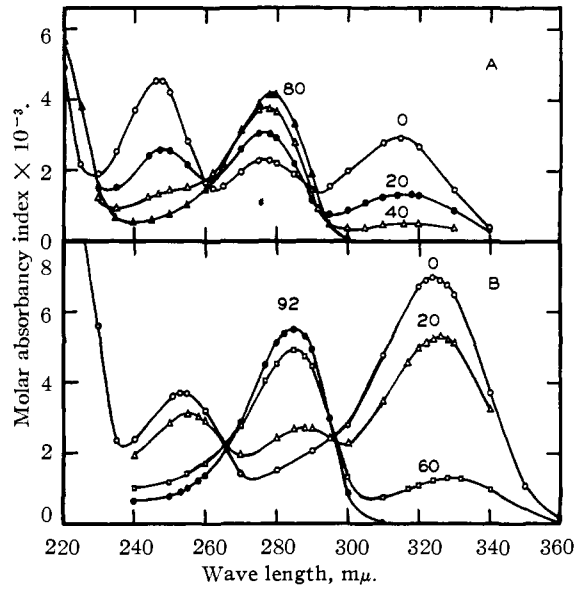


Fig. 2.—The absorption spectra of various 3-hydroxypyridines in dioxane–water mixtures: A, 3-hydroxypyridine; B, pyridoxine. The figures by the curves give the dioxane concentrations as volumes of dioxane in 100 volumes of solution. Boiled water of *p*H approximately 6.8 was used.

The presence of isosbestic points in Fig. 2B suggests that a single equilibrium is being altered by the solvent change. Similar though less clear-cut changes occur with 3-hydroxypyridine (Fig. 2A) and with pyridoxal and pyridoxamine monohydrochloride. In the case of 3-hydroxypyridine, a substantial amount of the neutral non-dipolar ionic form is present in aqueous solution and complete conversion of the dipolar ion to the uncharged form occurs at a lower dioxane concentration than with pyridoxine. Pyridoxal is apparently converted to the uncharged form nearly as readily as pyridoxine. In addition, at high dioxane concentrations other spectral changes occur, perhaps involving a shift in the equilibria among free aldehyde, hydrate and

(13) In pyridoxamine, this form bears a single positive charge associated with the primary amino group.

(14) N. D. Coggeshall and A. E. Glessner, Jr., THIS JOURNAL, **71**, 3150 (1949).

hemiacetal forms (discussed below) as well as a marked decrease in the intensity of the absorption. Pyridoxamine monohydrochloride undergoes a similar change which is complete only at high dioxane concentrations. A very pronounced decrease in intensity also occurs as dioxane is added.

From data of the type given in Fig. 2 we have made rough estimates of the ratio, K_Z, of dipolar ion to neutral uncharged forms in the solutions of each of the compounds at the various dioxane concentrations used (Experimental section). A plot of log K_Z *versus* the solvent composition is given in Fig. 3. Simple electrostatic theory[15] predicts that the curves in Fig. 3 should not be linear but that a plot of log K_Z *versus* the inverse of the solvent dielectric constant should be; evidently this theory is inadequate for these compounds. A few data for alcohol–water mixtures were obtained. Except at low solvent concentrations it was found that for solutions of a given dielectric constant, log K_Z was distinctly more negative in the alcoholic than in the dioxane containing mixtures.

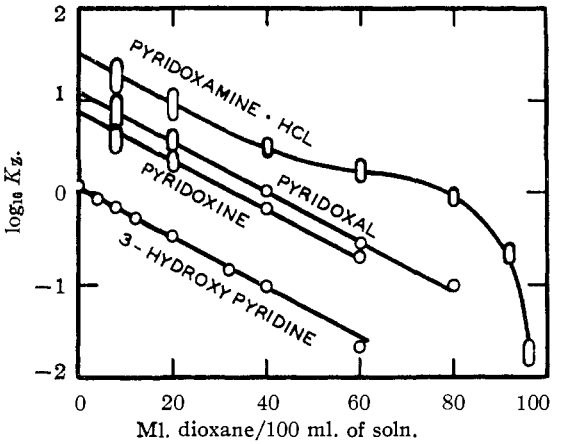


Fig. 3.—Variation in the ratio of dipolar ion to uncharged form, K_Z, with solvent composition for 3-hydroxypyridines in dioxane–water mixtures.

By extrapolation (Experimental section), the percentages of the non-dipolar ion form present in aqueous solution were: 3-hydroxypyridine, 46%; pyridoxine, 12%; pyridoxal, 8% and pyridoxamine monohydrochloride, 3%. The corresponding values of K_Z are 1.2, 8, 12 and 40, respectively.

5-Desoxypyridoxal.—This compound, XIII, is of special interest since its structure, like that of the coenzyme, pyridoxal-5-phosphate, precludes occurrence of hemiacetal forms. Its properties may thus be expected to resemble closely those of the coenzyme. Its spectrum is almost identical with that of pyridoxal-5-phosphate.[16,17] Hence the results of the interpretation of the spectrum of 5-desoxypyridoxal can also be applied to the coenzyme.

The spectrum of 5-desoxypyridoxal at *p*H 1 (Fig. 4A) contains a sharp peak at 294.5 mμ corresponding to the 291 mμ peak of pyridoxine and another peak at 342 mμ. The presence of these two absorption bands suggests that the compound exists at *p*H 1 as a mixture of two species, probably the free aldehyde form XIIIa and the hydrated form XIIIb. The 294.5 mμ peak

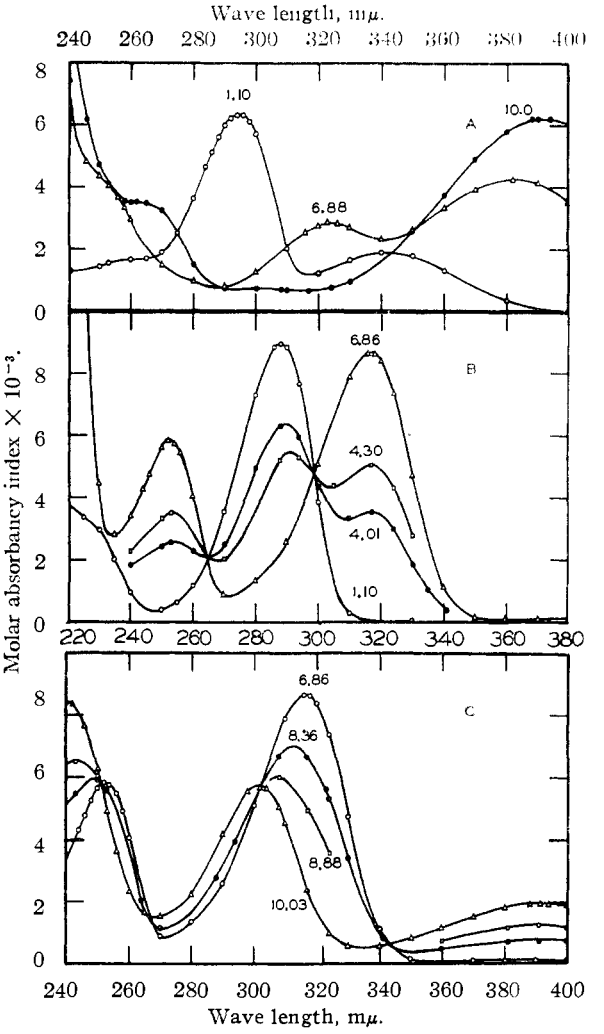
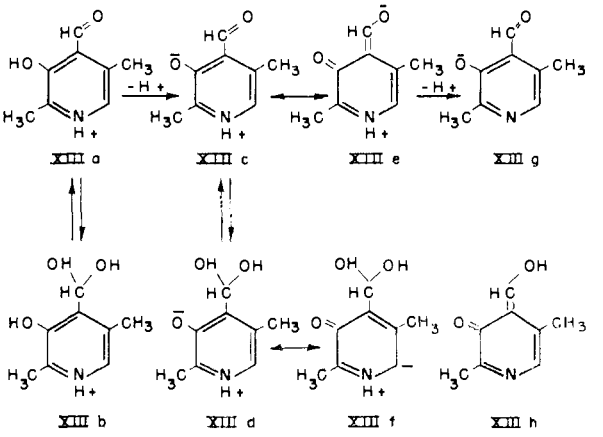


Fig. 4.—The spectra of 5-desoxypyridoxal and pyridoxal at various *p*H values: A, 5-desoxypyridoxal; B, pyridoxal in acidic solutions; C, pyridoxal in basic solutions. The figures beside the curves denote the *p*H of the solutions.



(15) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, pp. 96–111.

(16) D. Heyl, S. A. Harris and K. Folkers, THIS JOURNAL, **75**, 653 (1953).

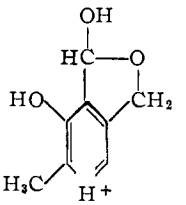
(17) M. Viscontini, C. Ebnother and P. Karrer, *Helv. Chim. Acta*, **34**, 1834 (1951).

must belong to the latter whose structure is very similar to that of pyridoxine, and the 342 mμ peak to the free aldehyde form. The addition of the carbonyl group to the chromophoric system would be expected to cause a shift of the absorption bands to higher wave lengths.[18]

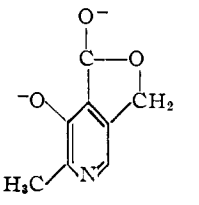
The spectrum of the isolectric form of desoxypyridoxal (*p*H 6.88) contains a peak at 324 mμ, apparently representing the hydrated dipolar ionic form XIIId (shifted 29.5 mμ from its position in acid solution, corresponding to the loss of the phenolic proton, *cf.* Table I), and a stronger peak at 381 mμ (shifted 39 mμ from its position in acid solution) representing the free aldehyde form XIIIc and XIIIe. The greater stability of the free aldehyde form relative to the hydrate in neutral solution than in acid solution (as indicated by the relative heights of the peaks) is attributed to the increased resonance involving structures of type XIIIe in the dipolar ionic form. The uncharged structure XIIIh which has been suggested probably does not occur to any significant extent.

In alkaline solution the peak corresponding to the hydrated form undergoes the expected shift to lower wave lengths (about −18 mμ) but becomes very weak, whereas the peak corresponding to the free aldehyde form XIIIg increases in intensity and is shifted 10 mμ toward a longer wave length. This shift as well as the increased stability of the free aldehyde form over the hydrated form can be explained by the increased resonance involving a structure analogous to XIIIe caused by the loss of the pyridinium proton. Loss of the proton decreases the contribution of structures of type XIIIf which are important in the dipolar ion and would tend to stabilize the hydrated form.[19]

Pyridoxal.—In addition to the two types of structure present in desoxypyridoxal, pyridoxal itself can exist at all *p*H values as a hemiacetal, *e.g.*, IXa.[4] The spectrum of this form is probably very similar to that of the hydrated aldehyde form.



IXa



IXb

At *p*H 1, a single peak above 250 mμ is observed at 288 mμ (Fig. 4B). The hemiacetal IXa must predominate. The neutral dipolar ionic form of pyridoxal displays in addition to the corresponding peak at 317 mμ, a very weak (molar absorbancy index = 0.11×10^3) absorption at about 390 mμ, evidently (by comparison with the spectrum of desoxypyridoxal) representing the free aldehyde form. In alkaline solutions (*p*H 10–11) (Fig. 4C) as with desoxypyridoxal, the amount of the free aldehyde form increases.[20]

In very alkaline solutions, the yellow color of pyridoxal solutions bleaches and the 302 mμ (hemiacetal) peak increases in intensity and is shifted slightly toward lower wave lengths. The *pK* for the ionization is nearly 13, hence the *p*H of complete ionization was not attained. The colorless form above *p*H 13 is probably the ion IXb.

Dissociation Constants.—The characteristic changes in absorption spectra can be used to calculate the dissociation constants of both ionizing groups of the 3-hydroxypyridines considered here. Lunn and Morton[5] have made such calculations for pyridoxine and we have employed similar methods to evaluate the constants for a number of other 3-hydroxypyridines (Experimental section). Ionization of groups not directly attached to the aromatic ring, *e.g.*, the primary amino group of pyridoxamine, have little effect upon the spectrum, and hence could not be determined spectrophotometrically. In these cases, ionization constants were determined by electrometric titration. The values obtained, expressed as apparent acid dissociation constants are given in Table III as *pK* values designated pK_1, pK_2, etc., in the order of their numerical values. Some values reported by other workers are included for comparison. Spectrophotometric values are for ionic strength 0.1; the titration values were not corrected to any definite ionic strength.

TABLE III

pK VALUES FOR VARIOUS 3-HYDROXYPYRIDINES

	Compound	pK_1	Max. dev.	pK_2	Max. dev.	pK_3
III	3-Hydroxypyridine	5.10	0.16	8.60 8.72[a]	0.08	
VIII	Pyridoxamine	3.31 3.54[c] 3.4[d]	.02	7.90[b] 8.21[c]		10.4[b] 10.63[c]
IX	Pyridoxal	4.20 4.23[c]	.02	8.66 8.70[c]	.06	13
I	Pyridoxine	5.00[c,d]		8.96[c] 8.97[d]		
XI	O-Methylpyridoxal	4.75	.04			
XIII	5-Desoxypyridoxal	4.17	.04	8.14	.08	
XIV	Pyridoxamine-5-phosphate	<2.5[c] (phosphate)		3.25 3.69[c]	.07	

[a] A. Albert and A. Hampton, *J. Chem. Soc.*, 505 (1954). [b] Obtained by titration. [c] Reference in footnote 6. [d] Reference in footnote 5.

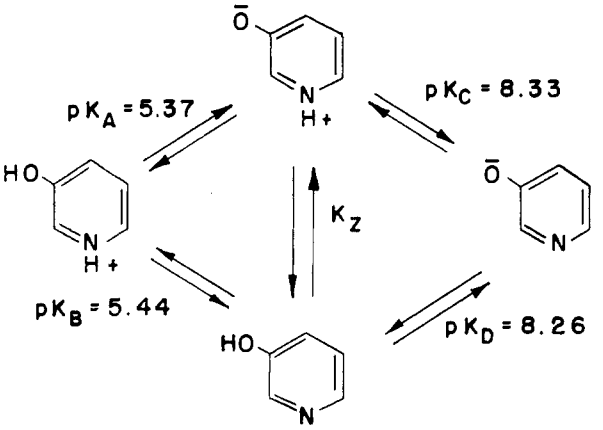
In most cases it is possible to assign the *pK* values to particular ionizable groups on the basis of the spectral changes. In a few cases this is not possible, *e.g.*, an isoelectric solution of 3-hydroxypyridine contains both dipolar ion and uncharged forms. Hence, K_1 involves both the hydroxyl and pyridinium groups. If K_{A_1} K_B, K_C and K_D

(18) It is probable that a weak absorption band at about 255 mμ (Fig. 4A) also belongs to the free aldehyde form. The 87 mμ separation from the upper band is greater than the usual 55-77 mμ but is not surprising in view of the altered nature of the chromophore.

(19) The following rough estimates of the ratio of hydrated to free aldehyde form have been made by assuming that the ratio is the same as the ratio of the absorbancies at the spectral peaks corresponding to the two forms; in acid solution, 3.4; in neutral solution, 0.66; in alkaline solution, 0.06.

(20) If it is assumed that absorption at 317 mμ in neutral solutions and at 302 mμ in alkaline solutions is caused by a mixture of both hemiacetal and hydrated forms, and that the ratio of hydrate to free aldehyde form is the same as in desoxypyridoxal, one can estimate the ratio of hemiacetal to free aldehyde forms from the heights of the absorption peaks. This ratio was estimated as 80 in neutral solution and 2.8 in alkaline solution. As one would expect, the ratio of hemiacetal to hydrated forms is of the same order of magnitude for the dipolar ion (1.2×10^2) and for the anion (5×10^3).

are the dissociation constants for single groups as shown below, and K_Z the ratio of dipolar ion to uncharged form, it can readily be shown[15] that $K_1 = K_A + K_B$, $1/K_2 = 1/K_C + 1/K_D$ and $K_Z = K_A/K_B = K_D/K_C$.



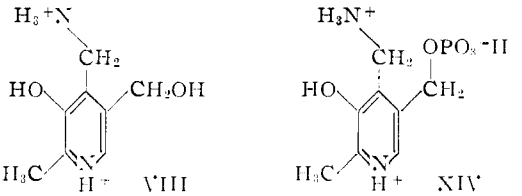
$pK_1 = 5.10$, $pK_2 = 8.60$, $\log K_Z = 0.07$

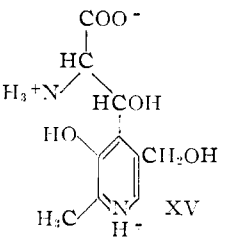
Thus if K_1, K_2 and K_Z are measured, the values of K_A, K_B, K_C and K_D can be determined. Values of these constants calculated for 3-hydroxypyridine (using the previously obtained estimate of K_Z) are given in the above diagram. With the 3-hydroxypyridines, pK_A is about 3 units less than pK_D (2.9 for 3-hydroxypyridine, 3.0 for pyridoxine, 3.3 for pyridoxal and about 3 for pyridoxamine monohydrochloride). The comparable difference for α-amino acids has been estimated as about 2.0 units.[15] The much greater interaction in the case of the 3-hydroxypyridines probably results from a lower effective charge separation in these compounds than in amino acids, the negative charge of the phenolate ion being distributed in part over the carbon atoms of the pyridine ring.

A problem similar to that considered above exists with respect to the order of dissociation of the pyridinium and the primary amino groups of pyridoxamine. That K_2 is associated largely with the pyridinium group is evident from the spectral changes. However, Lunn and Morton[5] report that the lack of accurate isosbestic points indicates the coexistence of three or more different species in alkaline solutions. Pyridoxamine-5-phosphate is another compound in which some uncertainty regarding the order of ionization exists. Thus dissociation of the second hydrogen of the phosphoric acid group may occur to some extent before the phenolic group ionizes. In both of these cases a careful comparison of titration curves and spectra could probably provide a more accurate estimate of the pK values for the individual groups.

The pK value of the hydroxyl group of phenol is 9.9, of the neutral uncharged form of 3-hydroxypyridine, 8.3, (pK_D) and of its anion, 5.4 (pK_A). The large acid-strengthening effect of the ring nitrogen and the even greater additional effect of the positive charge (previously discussed) are to be expected. The effect of the uncharged ring nitrogen is similar in magnitude to that of a nitro group.[21]

The pK_A value of 5.05 for the hydroxyl group of the pyridoxine cation, Ia, is similar to that of 3-hydroxypyridine. The pyridoxamine cation VIII is a much stronger acid of $pK_A = 3.32$ because of the additional positive charge on the primary amino group which can approach the hydroxyl group closely as indicated. Pyridoxamine phosphate XIV which in this pH range should carry an additional negative charge on the phosphate group, has a pK value for its hydroxyl group very similar to that of pyridoxamine (Table III), apparently because the negative charge is too far removed to have a noticeable effect on the ionization of the hydroxyl group.





β-Pyridoxylserine, XV,[22] has a less strongly acidic hydroxyl group ($pK_2 = 3.86$) than pyridoxamine because of the presence of the charged carboxylate group.

For 5-desoxypyridoxal, the ionization constants of the phenolic group were estimated as 4.5 for the hydrated form XIIIb, and 3.8 for the free aldehyde form, XIIIa. The hydrated form is a somewhat stronger acid than pyridoxine while the free aldehyde form is still stronger because of the stabilization of the dipolar ion form by resonance. As shown earlier, pyridoxal in the cationic and dipolar ionic forms exists predominantly as the hemiacetal. Thus pK_A (4.20) is nearly equal to the pK of the phenolic group of the hemiacetal form IXa, and does not differ greatly from that of the hydrated form of desoxypyridoxal.

The dissociation of the pyridinium group has been considered in a manner similar to that described above for the phenolic group. The results are in general not unexpected. However, the high value of pK_2 (9.98) for 4-desoxypyridoxine[5] does seem surprising.

(21) We have calculated an effective "substituent constant" σ as has been defined by Hammett (L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940, pp. 184-191) for the pyridine nitrogen. Using Hammett's ρ-value for the ionization of phenols in water at 25°, σ for the pyridine nitrogen in the *meta*-position is calculated from our data as 0.84. An independent σ-value was calculated from data of K. Kindler (*Ber.*, **69B**, 2792 (1936)) for the rate of saponification of the ethyl ester of nicotinic acid, as 0.62, σ for a *meta*-nitro group is 0.71.

(22) D. E. Metzler, J. B. Longenecker and E. E. Snell, THIS JOURNAL, **76**, 639 (1954).

Experimental

Apparatus and Materials.—Spectral measurements were made with Beckman model DU spectrophotometers with cell holders thermostated at 25°. The molar absorbancy indices given are for 1×10^{-4} *M* solutions. Beckman model G *p*H meters were employed, measurements being made at about 25°. Beckman type E high *p*H electrodes were used above *p*H 9. Electrometric titrations were carried out with the *p*H meter. Errors due to carbon dioxide absorption were not completely eliminated but were minimized by using carbonate-free sodium hydroxide and boiled water and by titrating as rapidly as possible.

Pyridoxamine phosphate and pyridoxal phosphate were prepared by the methods of Peterson, Sober and Meister[23] by Miss Joanne Olivard. 3-Hydroxypyridine was prepared by Dr. Miyoshi Ikawa. 5-Desoxypyridoxal, 3-O-methylpyridoxal, pyridoxal hydrochloride, pyridoxamine dihydrochloride and pyridoxine hydrochloride were gifts from Dr. Karl Folkers of Merck & Co., Inc. The neutral form of pyridoxine was prepared by neutralization of an aqueous solution with sodium hydroxide, evaporation to dryness, extraction of the residue with alcohol,[24] and crystallization (twice) of the product. Possible contamination with a trace of salt would have no effect on the conclusions drawn. The neutral form of pyridoxal was prepared by dissolving 2 millimoles of the hydrochloride in 0.4 ml. of water and adding 1.6 milliequivalents of sodium hydroxide (to bring to *p*H near 7) in 0.9 ml. of water and allowing the neutral product to crystallize overnight. The product was washed with cold water and dried. Pyridoxamine monohydrochloride was prepared by dissolving 1.7 millimoles of the dihydrochloride in 0.4 ml. of water and adding an equivalent amount of sodium hydroxide in 0.7 ml. of water. The crystals obtained were washed with water and dried *in vacuo*.

Dioxane for spectrophotometric measurements was obtained by purification of commercial material by the method of Fieser[25] followed by distillation through an efficient fractionating column. The product had an absorbancy of less than 0.3 compared with pure water at 260 mμ. It was stored in the frozen state and was distilled immediately before use to remove traces of peroxide.

Calculation of K_Z.—It is not possible to calculate values for K_Z rigorously from the spectral data of Fig. 2, but rough estimates can be made, and extrapolation of a plot of log K_Z against solvent composition adds somewhat to the certainty of the results. In the case of pyridoxine, rather sharp isosbestic points are present (Fig. 2B). This suggests that the molar absorbancy indices of the individual species do not change with solvent composition and that K_Z could be calculated for any solvent composition if it were known for one particular composition. By comparison of the shape of the spectrum of pyridoxine in aqueous solution with that of pyridoxamine monohydrochloride (which contains very little of the non-dipolar ionic form), it was estimated that between 6 and 16% of the isoelectric pyridoxine was present as the uncharged form. This form is responsible for the small shoulder in the spectrum of neutral pyridoxine between 280 and 290 mμ which is still evident in the spectrum of the isoelectric compound which Lunn and Morton calculated by a method of regression.[5] On the basis of this estimate, K_Z was calculated for each dioxane–water mixture and log K_Z plotted against the percentage dioxane (Fig. 3). The nearly straight line curve was extrapolated to obtain a somewhat more accurate estimate of K_Z in water itself.

For the other three compounds, accurate isosbestic points are not present. It appears that both the molar absorbancy indices and the positions of the absorption maxima for the various species change with solvent composition. Even so, by methods similar to those employed for pyridoxine, estimates of K_Z were made and extrapolated to pure water. No reliable estimate of the possible error in these data can be offered; the size of the points in Fig. 3 represent a subjective judgment of the accuracy. We feel that errors in log K_Z in water and hence in the derived values of K_A, K_B, etc., probably do not exceed 0.1 to 0.2 unit.

Calculation of Ionization Constants.—The spectrum of each compound was measured as a function of *p*H in buffers of approximately 0.1 ionic strength. The following buffers were employed: hydrochloric acid and potassium chloride, *p*H 1 to 2.5; formate, *p*H 2.5 to 4; acetate, *p*H 4 to 6; phosphate, *p*H 5.6 to 8.3; bimaleate, *p*H 6.9 to 7.4; carbonate, *p*H 8.9 to 12.0 and phosphate or sodium hydroxide plus potassium chloride above 12. These buffers all appear to be free from serious interaction with the compounds being studied as judged by the continuity of the results in passing from one buffer series to another. Borate and glycine-containing buffers were avoided since the former complexes with all of the vitamin B_6 compounds and the latter interacts with pyridoxal.

The spectrum of the vitamin B_6 compounds changes markedly with *p*H when dissociation of the phenolic or pyridinium groups occurs. Lunn and Morton have reported[5] the existence of isosbestic points in the spectra of pyridoxine in the *p*H regions 3–7 and 7–11. Figures 4B and C show the presence of similar isosbestic points in the pyridoxal spectrum. The presence of isosbestic points is a strong indication that only one dissociation step is occurring in each of these regions and that it should be possible to calculate the ionization constants directly from the spectrophotometric data. To do this the molar absorbancy indices of the various compounds at selected wave lengths were plotted against *p*H. Such data are shown for pyridoxal in Fig. 5. Limiting values at the ends of each dissociation step were estimated visually or by successive approximations.[26] From the limiting values and each point on the curve for *p*H values within about 0.9 unit of the *pK* values an independent value of *pK* was computed. From 3 to 5 values were obtained for each titration step at each of two or more different wave lengths. These usually agreed within a few hundredths of a *p*H unit. The average values for each *pK* and the maximum deviations from the means are recorded in Table III.

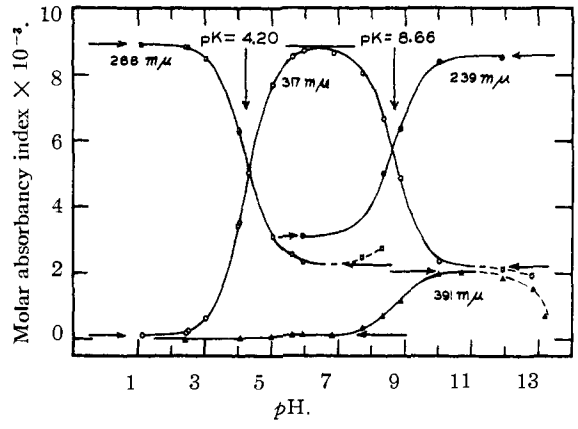


Fig. 5.—Absorbancy of a pyridoxal solution at selected wave lengths as a function of *p*H. Points are experimental; lines are theoretical curves based on the calculated *pK* values and the estimated limiting values which are indicated by the horizontal arrows.

Acknowledgment.—We are indebted to Dr. George Hammond for several helpful suggestions.

AUSTIN, TEXAS
AMES, IOWA

(23) In E. E. Snell, "Biochemical Preparations," Vol. 3, John Wiley and Sons, Inc., New York, N. Y., pp. 29, 34.

(24) E. T. Stiller, J. C. Keresztesy and J. R. Stevens, THIS JOURNAL, **61**, 1237 (1939).

(25) L. F. Fieser, "Experiments in Organic Chemistry," 2nd Edition, D. C. Heath and Company, New York, N. Y., 1941, p. 369.

(26) Compare with the more rigorous method employed by Lunn and Morton with pyridoxine.[5]