Fluorescence Studies on some Hydroxypyridines including Compounds of the Vitamin B₆ Group

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1. The variations with pH (from 3*N*-sulphuric acid to 10*N*-sodium hydroxide) of the excitation and fluorescence wavelengths and fluorescence intensity of 2-, 3- and 4-hydroxypyridine and their *O*- and *N*-methyl derivatives were investigated. 2. 4-Hydroxy- and 4-methoxy-pyridine were non-fluorescent at all pH values. 3. The cations and dipolar ions of the 3-hydroxypyridine derivatives and the anion of 3-hydroxypyridine were fluorescent, but the neutral forms were not. 4. All the forms of the 2-hydroxypyridine derivatives were fluorescent. 5. Pyridoxol, pyridoxal and its 5-phosphate, pyridoxamine and pyridoxic acid and its lactone were studied similarly. All these compounds, except pyridoxal 5-phosphate, were more fluorescent than 3-hydroxypyridine. 6. The most fluorescent forms of these compounds are the anions, except for pyridoxol, where the dipolar ion was the most fluorescent form. The least fluorescent forms are the neutral molecules. The dipolar ions were appreciably fluorescent in all cases. 7. The most fluorescent form examined was the dianion of pyridoxic acid lactone. 8. The cations were all fluorescent except the cations of 2- and 3-methoxypyridine. All the cations showed excited-state ionization. The excited pK_a values of these cations were determined and the results are discussed with reference to Weller's (1952) equation relating ground- and excited-state dissociation constants. 9. The pK_a values for all ionizations undergone by the compounds examined were determined from fluorescence data. 10. Stokes shifts for the various ionic and neutral species of the compounds examined were calculated and are discussed.

Although pyridine itself is non-fluorescent (Kerr, Hayes & Ott, 1957; Bridges, 1963), some of its biologically important derivatives such as pyridoxol, pyridoxal and pyridoxamine are fluorescent in aqueous solution at pH 7 (Duggan, Bowman, Brodie & Udenfriend, 1957). The fluorimetric determination of compounds of the vitamin B₆ group, however, is usually based on their conversion into the fluorescent pyridoxic acid lactone (e.g. Huff & Pealzweig, 1944; Reddy, Reynolds & Price, 1958; Fujita, Matsuura & Fujino, 1955; Fujita, Fujita & Fujino, 1955*a,b*), or into fluorescent cyanohydrins (Bonavita & Scardi, 1959; Polansky, Camarra & Toepfer, 1964). Compounds of the vitamin B₆ group possess native fluorescence, and in the present paper a study has been made of this fluorescence over a wide pH range. To interpret the fluorescence of these compounds, the fluorescence characteristics of 2-, 3- and 4-hydroxypyridine and their *O*- and *N*-methyl derivatives have also been examined. Some of the results described below have been briefly reported (Bridges, Creaven, Davies & Williams, 1964).

EXPERIMENTAL

Materials. 2-Hydroxypyridine, m.p. 107° (from benzene), 3-hydroxypyridine, m.p. 126° (by sublimation), 4-hydroxypyridine, m.p. 145° (as supplied), and 2-methoxypyridine, b.p. 142° (redistilled), were purchased (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) and purified as indicated. 3-Methoxypyridine, b.p. 178–179° after redistillation, was prepared from 3-hydroxypyridine with ethereal diazomethane. 4-Methoxypyridine, b.p. 190° after redistillation, was prepared by heating 4-chloropyridine (m.p. 220°; Koch-Light Laboratories Ltd.) with sodium methoxide in a sealed tube at 100° for 1 hr. 2-Hydroxy-*N*-methylpyridine hydrochloride, m.p. 166°, and the free base, m.p. 33°, and 3-hydroxy-*N*-methylpyridine hydrochloride, m.p. 158° after recrystallization from ethanol, were prepared from the hydroxypyridines with methyl iodide (Albert & Phillips, 1956). Pyridoxol (pyridoxine) hydrochloride, m.p. 205–206°, pyridoxal hydrochloride, m.p. 163° (decomp.), pyridoxamine hydrochloride, m.p. 226° (decomp.), pyridoxal 5-phosphate, and pyridoxic acid, m.p. 225° (decomp.), were purchased (Koch-Light Laboratories Ltd.) and purified where necessary. Pyridoxol was prepared from its hydrochloride (Stiller, Keresztesy & Stephens, 1939), and pyridoxal and pyridoxamine from their respective hydrochlorides (Metzler & Snell, 1955). Pyridoxic acid lactone

hydrochloride, m.p. 273–274°, was prepared from pyridoxic acid by a Fischer–Speier esterification as described by Heyl (1948). A chromatographic examination of all the above compounds showed that they were free from impurities that might interfere with their fluorescence.

Preparation of solutions. Stock solutions were prepared by dissolving 0.1 g. of the appropriate compound in 100 ml. of glass-distilled water. With compounds sparingly soluble in water, e.g. 2- and 3-methoxypyridine, 20 ml. of ethanol (Burroughs RR grade) was added to assist solution. Stock solutions were diluted with glass-distilled water to the desired concentration. To obtain solutions of various pH values, the aqueous solutions were titrated with 0.001N- to 10N-HCl or -NaOH. pH values between 2 and 12 were measured in a Pye Master pH-meter. Solutions of pH values 0–2 and above 12 were prepared by adding HCl or NaOH of the appropriate strength and calculating the pH value on the assumption of complete ionization. The fluorescence of uncharged forms were determined with solutions of the free bases in ethanol. Fluorescence spectra were determined in the Aminco–Bowman spectrophotofluorimeter (American Instrument Co., Silver Springs, Md., U.S.A.) by using a 150 w Osram xenon lamp (XBO-165), and ultraviolet spectra in the Unicam SP.500 spectrophotometer.

All excitation and fluorescence wavelengths quoted in this paper are instrumental values.

Use of the acidity function. All the compounds tested for fluorescence in this paper have been examined in solutions of very high acidity, i.e. 1–36N. For these solutions it has been necessary to express high acidity in terms of the acidity function, H_0 , of Hammett (1940) rather than use negative pH, which is merely a theoretical extension of the normal pH scale. H_0 is a practical measurement of the ability of the medium to protonate an uncharged base. Normality or concentration of the acid and H_0 are related and the values of H_0 used for HCl and H_2SO_4 are those of Paul & Long (1957). At pH 0 and above, H_0 and pH are virtually the same (see Albert & Serjeant, 1962).

Stability of compounds in ultraviolet light. 3-Hydroxypyridine was stable in aqueous solutions (5 μg./ml.; pH 5–8) when exposed for up to 5 hr. to light of 330 mμ from the xenon lamp of the fluorimeter. However, pyridoxol, pyridoxal and pyridoxamine photodecomposed under these conditions, their fluorescence intensities falling to half the initial values at 40, 80 and 22 min. respectively. Fluorescence-intensity readings were made within 1 min. of exposure to the exciting light.

Effect of concentration on fluorescence intensity. All the compounds studied showed some concentration quenching above 5 μg./ml. The solutions were therefore examined at concentrations of about 5 μg./ml. or less, where fluorescence intensity was proportional to concentration.

Correction of intensity values. In Tables 1–4 there are quoted observed and corrected values for fluorescence intensity. In the calculation of these values, the intensity of fluorescence of the 3-hydroxypyridine anion at pH 11 has been given the arbitrary value of 100 and all other intensities were based on this, making allowances for molecular weight. Observed values were calculated from the photomultiplier readings of the fluorimeter, whereas for the corrected values allowance was made for the variation in energy output of the xenon lamp, which was calibrated by the chemical actinometer method of Hatchard & Parker (1956).

Estimation of pK values. pK_a values were determined where possible from the fluorescence-intensity, excitation-wavelength and/or fluorescence-wavelength curves, which were drawn out in detail. The pH at which a compound fluoresced with 50% of the maximum intensity was taken as the pK_a . This was done when the intensity curve obtained was suitable for the purpose. With excitation- and fluorescence-wavelength curves, the pK_a was taken as the pH at the half-way point in a wavelength change when this change was of suitable magnitude.

RESULTS AND DISCUSSION

Wavelengths given in parentheses under formulae in the succeeding pages are those of the maximum fluorescence of the compound.

3-Hydroxypyridine and its methyl ethers. The fluorescence of 3-methoxypyridine is relatively simple. Only two molecular species are involved, namely the cation (I), which fluoresces at 324 mμ (λ_{exc} 295 mμ), and the neutral molecule (II), which is non-fluorescent. The cation (I) has a maximum fluorescence intensity at about pH 1.9–3.3, and as the pH rises above this the intensity falls to zero at pH 7 (see Fig. 1), the fall following the ionization curve of the cation (I). The pK_a calculated from the intensity curve is 4.9, which is in agreement with the value 4.88 (Albert & Phillips, 1956) obtained by other methods (see Table 1). Below pH 2, the fluorescence intensity diminishes and disappears in 10N-hydrochloric acid (H_0 –3.7) and this may be due to quenching by Cl^- or H^+ ions. Whereas the cation (I) ionizes by loss of a proton from the ring nitrogen atom, 3-hydroxy-*N*-methylpyridine (III) ionizes by loss of a proton from the hydroxyl group, forming the dipolar ion (IV). Both species (III and IV) are fluorescent but the fluorescence of the cation (III) is observable only below

Fig. 1. Variation of (a) fluorescence intensity and of (b) fluorescence (Fl.) and excitation (Exc.) wavelengths with pH and H_0 of 3-hydroxypyridine (●—●), 3-methoxypyridine (○—○) and 3-hydroxy-*N*-methylpyridine (×—×) (concentration of each $5\mu\text{g./ml.}$). H_0 is the acidity function of Hammett (see the text). Intensity values are arbitrary, that of the 3-hydroxypyridine anion at pH 11 being taken as 100.

H_0-3 (8*N*-hydrochloric acid) at $335m\mu$ ($\lambda_{\text{exc.}}$ $298m\mu$). This cation shows excited-state ionization, for when the acidity function is raised from $H_0-4.15$ to -1.05 (11.6*N*- to 3*N*-hydrochloric acid) the fluorescence wavelength gradually increases to that of the dipolar ion (IV) ($395m\mu$), but the excitation wavelength ($298m\mu$) remains the same until pH about 4.5 (see Fig. 1). In this pH region, the excitation gradually changes with pH to $328m\mu$, but the fluorescence remains at $395m\mu$, and the change in excitation follows the normal ionization of the cation (III) (see Table 1 and Fig. 1). The fluorescence wavelength of 3-hydroxy-*N*-methylpyridine is thus constant from $H_0-1.05$ to pH 14, but the intensity curve shows changes in the regions $H_0-3.7$ to -0.2 and pH 4–6, the first corresponding to the excited-state ionization and the second to the normal ionization of the cation (III). The dipolar ion (IV) is more fluorescent than the cation (III). At pH 14, the fluorescence intensity of 3-hydroxy-*N*-methylpyridine increases, possibly owing to slight demethylation to the highly fluorescent 3-hydroxypyridine anion (VIII), as suggested by the wavelengths of excitation and fluorescence (Table 1).

3-Hydroxypyridine can exist in solution at various pH values in four forms, namely the cation (V), dipolar ion (VI), neutral molecule (VII) and anion (VIII). The above observations on the methyl ethers suggest that the neutral molecule (VII) is unlikely to be fluorescent, and in ethanol 3-hydroxypyridine is, in fact, virtually non-fluorescent. In aqueous solution, the two forms occur in the proportion of 54% of the dipolar ion (VI) to 46% of the neutral molecule (VII) (Metzler & Snell, 1955). The 3-hydroxypyridine cation (V), like its *N*-methyl derivative (III), shows excited-state ionization. The fluorescence of the cation (V) occurs at about $340m\mu$ and is observable only at $H_0-3.7$ to -3.25 . Above the latter H_0 , the fluorescence wavelength changes to that of the dipolar ion ($392m\mu$), which persists to pH 7. The excitation wavelength ($283m\mu$), however, remains unchanged to pH 4, and then changes gradually to $325m\mu$ over the pH range at which normal ionization occurs. The pK_a calculated from the excitation curve is 4.80 (see Table 1). Both excitation and fluorescence wavelengths change again at pH 7–8, owing to ionization to the anion (VIII). The pK_a for this ionization, estimated from the fluorescence-intensity curve, is 8.5 (see Table 1). The intensity curve (see Fig. 1) shows that the cation and dipolar ion are not strongly fluorescent. The dipolar ion (VI) fluoresces maximally at about $H_0-0.2$ to pH 3.0, but this is followed by a slight fall in intensity between pH 3 and pH 5, which is probably due to the formation of varying amounts of the non-fluorescent neutral form (VII). At pH 6 the intensity begins to increase slowly, then rapidly at pH 8, until at pH 11 the intensity reaches a maximum due to the highly fluorescent anion (VIII).

The neutral molecules (II and VII), not unexpectedly (see Williams & Bridges, 1964), appear to be non-fluorescent. The neutral form of 3-hydroxyquinoline, which is a comparable form, is also non-fluorescent in water (Haylock, Mason & Smith, 1963).

2-Pyridone and its methyl ethers. The fluorescence of 2-methoxypyridine (see Fig. 2) is similar to that of its 3-isomer. The cation (IX) fluoresces at $325m\mu$ with a maximum intensity confined to a very narrow pH range (about 2.5). Above this the intensity falls with increasing pH, and follows the ionization curve closely. The pK_a , estimated from the intensity curve, is 3.2, which compares well with the value 3.28 (Albert & Phillips, 1956) obtained by other methods. Between pH 2.5 and pH 4.2, the excitation and fluorescence wavelengths change and at pH 4.2 the maximum fluorescence wavelength is $315m\mu$, which is that of the neutral molecule (X). The fluorescence of the neutral molecule (X) is, however, relatively weak but persists to pH 14. Below pH 2 the fluorescence of the

Table 1. *Fluorescence of 3-hydroxypyridine and its methyl ethers*The concentration of each compound was 5 $\mu\text{g./ml.}$

Compound	Molecular species	Wavelength ($m\mu$)			Relative fluorescence intensity†			pH range of max. fluorescence	pK _a			Literature values
		Of max. absorp- tion*	Of max. excita- tion	Of max. fluores- cence	intensity†		Int.		Exo.	Fl.		
					Observed	Corrected‡						
3-Methoxypyridine	Cation	284	295	324	30.2	39.0		pH 2.5-3.5	4.9	—	—	4.88¶
	Neutral form	276	—	None	0.0	0.0						
3-Hydroxy-N-methyl- pyridine	Cation	288	298	335	3.0	3.8		about H ₀ -4	5.1	4.4	—	4.96¶
	Cation (excited)	—	298	395	18.3	23.0		H ₀ -0.3 to pH 1.0	—	—	-3.0**	
3-Hydroxypyridine	Dipolar ion	320	328	395	38.4	28.6		pH 6-14				
	Cation	283	298	about 340	2.0	2.5		about H ₀ -4	—	4.8	—	4.86¶, 5.10††
	Cation (excited)	—	298	392	7.1	8.9				—	-3.0**	
	Dipolar ion	315	325	392	6.1	4.7		pH 1-2	8.5	7.9	7.85	8.60††, 8.72††
	Neutral form	278†	—	None	0.0	0.0						
	Anion	298	309	365	100.0	100.0		pH 10-14				

* Mason (1959).

† In ethanol (Moehrer, 1950).

‡ Intensity of 3-hydroxypyridine anion at pH 11 = 100.

§ See the text.

|| pK_a values estimated from intensity curve (Int.), or excitation-wavelength change (Exc.), or fluorescence-wavelength change (Fl.) where convenient (see Fig. 1).

¶ Albert & Phillips (1956).

** Excited-state pK_a (see the text).

†† Metzler & Snell (1955).

‡‡ Albert & Hampton (1954).

Fig. 2. Variation of (a) fluorescence intensity and of (b) fluorescence (Fl.) and excitation (Exc.) wavelengths with pH and H_0 of 2-hydroxypyridine (●—●), 2-methoxypyridine (○—○) and 2-hydroxy-*N*-methylpyridine (×—×) (concentration of each 5 μg./ml.). H_0 is the acidity function of Hammett (see the text). Intensity values are arbitrary, that of the 3-hydroxypyridine anion at pH 11 being taken as 100.

cation (IX) diminishes to a small value, which is still observable in 10*N*-hydrochloric acid ($H_0 = 3.7$). This fall in fluorescence may be due to quenching by Cl^- or H^+ ions.

The possible forms of *N*-methyl-2-pyridone occurring in solution at various pH values are structures (XI)–(XIV). The cation could be (XI) or the protonated pyridone (XII) (Albert & Phillips, 1956). Because of excited-state ionization, the fluorescence of the cation (345 mμ) is observable only in concentrated acid (36*N*-sulphuric acid).

The fluorescence wavelength changes at about $H_0 = 3.8$ to 365 mμ, whereas the excitation remains at 290 mμ until $H_0 = 0.3$ (1.2*N*-hydrochloric acid) is reached. The excitation then changes over the range $H_0 = 0.3$ to pH 2.0, where structure (XI) [or (XII)] ionizes normally to structure (XIII) [or (XIV)]. The pK_a for this change, estimated from the excitation curve, is about 0.5 (see Table 2). It seems probable that the fluorescence at 365 mμ is due to structure (XIII) resonating with structure (XIV). Above pH 10.8, the fluorescence and excitation wavelengths shorten to the values for the anion of 2-pyridone, probably because of alkaline demethylation of *N*-methyl-2-pyridone.

2-Pyridone shows fluorescence characteristics that are similar to its *N*-methyl derivative (see Fig. 2). The cation (XV) (pyridone form not shown here) shows excited-state ionization and its fluorescence (345 mμ) is detectable only in 36*N*-sulphuric acid. The fluorescence wavelength changes to 360 mμ at $H_0 = 1.95$ (5.5*N*-hydrochloric acid), but the excitation does not change until pH about 0 is reached. The excitation change occurs where the cation (XV) ionizes normally to structure (XVI) [and (XVII)]. The pK_a of this change, estimated from the excitation-wavelength curve, is 0.6 (see Table 2). There are two changes in the intensity curve (see Fig. 2), one at pH 0 and the other at pH 10, these corresponding to the normal ionizations of (XV) to (XVI) [and (XVII)] and of (XVI) to (XVIII) [and (XIX)] respectively. The fluorescence at 360 mμ is probably due to the dipolar ion (XVI) in resonance with the pyridone (XVII). In ethanol, the pyridone (XVII) fluoresces maximally at 370 mμ ($\lambda_{\text{exc.}}$ 320 mμ). At pH about 11 and above, 2-pyridone becomes intensely fluorescent, the wavelength of which shortens to 351 mμ ($\lambda_{\text{exc.}}$ 300 mμ). This is presumably due to the formation of the anion (XVIII), which is more than twice as

Table 2. *Fluorescence of 2-hydroxypyridine and its methyl ethers*The concentration of each compound was 5 μ g./ml.

Compound	Molecular species	Wavelength (m μ)			Relative fluorescence intensity $\dagger$		pH range of max. fluorescence	p <i>K</i> _a				Literature values
		Of max. absorp- tion*	Of max. excita- tion	Of max. fluores- cence	Observed	Corrected		Int. §	Exc. §	Fl. §		
2-Methoxypyridine	Cation	279	290	325	32.1	48.8	pH 2.5 (peak)	3.2	3.65	3.4	3.28¶	
	Neutral form	269	281	315	6.9	13.2	pH 6-14	—	—	—	—	
	Cation	279	290	345	4.8	7.3	H ₀ -8.6	—	0.5	—	0.32¶	
<i>N</i> -Methyl-2-pyridone	Cation (excited)	—	290	365	5.3	8.2	H ₀ -4 to pH 0	—	—	—	—	
	Dipolar ion and/or pyridone	297	305	365	9.0	9.2	pH 2-10.4	—	—	—	—	
	Anion by de- methylation?	(291)	300	352	—	—	(Occurs in alkali solution)	—	—	—	—	
2-Hydroxypyridine	Cation	277	290	345	4.3	6.5	H ₀ -8.6	—	0.6	—	0.75¶	
	Cation (excited)	—	290	360	4.3	6.5	H ₀ -4 to pH 0	—	—	—	—	
	Dipolar ion and/or pyridone	293	310	360	8.6	8.2	pH 2-10	—	11.0	11.1	11.62¶	
	Anion	291	300	351	258.7	215.2	about pH 15 (10 <i>N</i> -NaOH)	—	—	—	—	
	Pyridone	300†	320	370	—	—	(In ethanol)	—	—	—	—	

* Mason (1959).

† In ethanol (Mosher, 1950).

‡ 3-Hydroxypyridone anion at pH 11 = 100.

§ See Table 1.

|| Excited-state pK_a .

¶ Albert & Phillips (1956).

fluorescent as the corresponding anion of 3-hydroxypyridine (see Table 2). It is possible for two anions to exist, namely (XVIII) and (XIX), the second being formed by deprotonation of the nitrogen atom of the pyridone (XVII). The existence of the anion (XIX) may explain the high fluorescence of 2-pyridone in alkaline solution.

4-Hydroxypyridine. Neither 4-hydroxy- nor 4-methoxy-pyridine was fluorescent at any pH. 4-Hydroxypyridine occurs in aqueous solution almost entirely as the pyridone (XX), which is non-aromatic (Albert & Phillips, 1956). This could explain its lack of fluorescence. 4-Methoxypyridine

(XXI), like its 3-isomer, is non-fluorescent and, presumably, the methoxyl group in the 3- or 4-position is not sufficiently powerful to counteract the deactivating effect of the pyridine nitrogen atom. 2-Methoxypyridine, as shown above, is weakly fluorescent.

Pyridoxol. This compound fluoresces like 3-hydroxypyridine, for fluorescence maxima can be distinguished for the cation (XXII), dipolar ion (XXIII) and anion (XXV). Each of these species is more fluorescent than the 3-hydroxypyridine anion, the decreasing order of fluorescence intensity being: (XXIII), (XXV), (XXII). The cation also

Table 3. *Fluorescence of pyridoxol and its derivatives*The concentration of each compound was 5 $\mu\text{g./ml.}$

Compound	Molecular species	Wavelength (mμ)			Intensity of maximum fluorescence†		pH range of maximum fluorescence	pK _a				Literature values
		Of max. absorption*	Of max. excitation	Of max. fluorescence	Observed			Int.†	Exc.‡	Fl.‡	pK _a	
					Corrected	Corrected						
Pyridoxol (pyridoxine)	Cation	291	302	345	7.5	10	H ₀ -3.7 to -2.8	5.0	4.7	—	—	5.0 ¶
	Cation (excited)	—	302	400	130	150	pH 2.5	—	—	—	—	—
	Dipolar ion	324	332	400	238	165	pH 6.5-7.5	—	9.6	9.4	—	8.96¶, 8.97 , 8.72
	Anion	310	320	380	168	144	pH 12-14	—	—	—	—	—
	Neutral form	286	296	335	—	—	(In ethanol or dioxan)	—	—	—	—	—
Pyridoxal hemiacetal	Cation	288	300	350	11	13	H ₀ -3.7 to -3.4	3.8	3.9	—	—	4.20*, 4.23¶
	Cation (excited)	—	300	382	125	145	pH 0	—	—	—	—	—
	Dipolar ion	317	330	382	207	150	pH 6	—	—	—	—	—
	Anion	302	310	365	283	270	pH 12	—	8.9	9.0	—	8.66*, 8.70¶
	Neutral form	280 (dioxan)	291	330	—	—	(In ethanol)	—	—	—	—	—
Pyridoxamine	Dication	292	306	350	70	95	H ₀ -4.9	3.0	2.8	—	—	3.31*, 3.4 , 3.54¶
	Dication (excited)	—	302	400	325	365	H ₀ -1.0 to -0.8	—	—	—	—	—
	Tripolar ion	326	337	400	370	245	pH 4.9-5.5	8.2	9.4	9.6	—	7.90*, 8.21¶
	Dipolar ion	—	—	None	0	0	pH 15	—	—	—	—	—
	Anion	310	320	370	410	345	(In ethanol)	—	—	—	—	—
Neutral form	287 (dioxan)	309	361	—	—	—	—	—	—	—	—	

Pyridoxal 5-phosphate aldehyde hydrate	Cation	291	302	385	<1.0	<1.0	$H_0-2.5$ pH1	—	—	2.3	<2.5¶
	Cation (excited)	—	302	400	11	13	—	—	—	—	—
	Cation-phosphate	291	302	400	—	—	—	—	3.7	—	4.14¶
	monoanion	—	302	410	—	—	—	—	—	—	—
	Cation-phosphate monoanion	—	302	410	—	—	—	—	—	—	—
	Dipolar ion- phosphate	324	330	410	11	8	pH6	—	—	6.5	6.20¶
	monoanion	—	—	—	—	—	—	—	—	—	—
	Dipolar ion- phosphate	324	330	400	—	—	pH7.5-8.5	—	—	8.75	8.69¶
	dianion	—	—	—	—	—	—	—	—	—	—
	Trianion	305	315	370	17.5	20.5	pH12-14	—	—	—	—
	Trianion alde- hyde form?	388	400	525	about 2.0	—	pH12-14	—	—	—	—

*Metzler & Snell (1955).

† 3-Hydroxypyridine anion at pH11 = 100; see the text and Table 1.

‡ See Table 1.

§ Excited-state pK_a .

|| Lunn & Morton (1952).

¶ Williams & Neilands (1954).

Fig. 3. Fluorescence intensity of pyridoxol (5 μ g./ml.) in ethanol-water mixtures: ●—●, dipolar ion, λ_a 400 m μ (λ_{exc} 332 m μ); ×—× neutral molecule, λ_a 335 m μ (λ_{exc} 296 m μ).

Fig. 4. Variation of (a) fluorescence intensity and of (b) fluorescence (Fl.) and excitation (Exc.) wavelengths with pH and H_0 of pyridoxine (○—○) and pyridoxal (●—●) (concentration of each 5 μ g./ml.). H_0 is the acidity function of Hammett (see the text). Intensity values are arbitrary, that of the 3-hydroxypyridine anion at pH 11 being taken as 100.

shows excited-state ionization. The neutral molecule (XXIV) shows a weak fluorescence in ethanol, and in mixtures of ethanol and water the fluorescence of structure (XXIII) or (XXIV) or both can

be detected according to the concentration of ethanol. In water it fluoresces as structure (XXIII), but in pure ethanol as structure (XXIV) (see Fig. 3). The true fluorescence of the cation (XXII) is detectable in 6–10.05N-hydrochloric acid (H_0 –3.0 to –3.8), for above H_0 –3.0 the cation (XXII) shows excited-state ionization (see Table 3 for pK_a and fluoresces as structure (XXIII). This fluorescence persists from H_0 –3.0 to pH 9.0 (see Fig. 4), but the excitation corresponding to structure (XXII) persists to pH 4.3 and then changes to that of structure (XXIII) at pH 5. The pK_a of the ionization of the cation (XXII) is reported as 5.0 (see Table 3), whereas the value estimated from the excitation wavelength curve is 4.7 (see Fig. 4). Excitation and fluorescence wavelengths change together at pH 9.0–10.5 owing to formation of the anion (XXV). The pK_a for this ionization, estimated from the fluorescence- and excitation-wavelength curves (Fig. 4), are 9.4 and 9.6 respectively (see Table 3). The anion (XXV) is less fluorescent than the dipolar ion (XXIII), which is the reverse of the relationship for the corresponding species of 3-hydroxypyridine, and the most fluorescent form of pyridoxol is the dipolar ion (XXIII), at pH 7. The more intense fluorescence of the various forms of pyridoxol, as compared with 3-hydroxypyridine, may be due to the electron contribution of the methyl and hydroxymethyl groups (see Bridges & Williams, 1962). The dipolar

anion (XXIII) shows two peaks of fluorescence intensity, namely at pH 2.5 and 7.0, the latter being about twice as intense as the former. The probable explanation for this is that at pH 2.5 the excitation is at 302m μ whereas at pH 7 it is at 332m μ . At 330m μ the energy output of the xenon lamp is greater than at 300m μ and, further, the efficiency of the absorption of energy by the cation followed by emission of energy as the dipolar ion is less than that in which the dipolar ion is both the absorbing and emitting species.

Pyridoxal. The fluorescence characteristics of pyridoxal at various pH values are shown in Fig. 4, and the pattern is similar to that of 3-hydroxypyridine, except that the latter is less fluorescent. Pyridoxal could exist in solution as a cyclic hemiacetal (XXVI), an aldehyde hydrate (XXVII) and a free aldehyde (XXVIII). These forms are distinguishable by absorption spectra, and Metzler & Snell (1955) have shown that the cyclic hemiacetals [e.g. (XXVI)] are the predominant forms in solution. The excitation wavelengths for the cation (300m μ), dipolar ion (330m μ), neutral molecule (291m μ) and anion (310m μ) correspond to the absorption data for the hemiacetal forms (Table 3) and therefore the fluorescences found are those of the hemiacetal forms (XXIX), (XXX), (XXXI) and (XXXII). These experiments suggest that each of the four forms (XXIX)–(XXXII) occurs, or occurs mainly, as a hemiacetal. The aldehyde group tends to diminish the fluorescence of aromatic systems (Bridges & Williams, 1962) because of its tendency to withdraw electrons from the ring. If pyridoxal occurred in the aldehyde form, then its fluorescence would probably be less intense than is found.

The fluorescence of the cation (XXIX) is found only in concentrated acid, since it shows excited-state ionization (see Fig. 4). The most fluorescent form of pyridoxal is the anion (XXXII) (at pH 12), which is more than 2.5 times as intense as the 3-hydroxypyridine anion (see Table 3). The fluorescence of the dipolar ion (XXX), which is 35 times as intense as that of the dipolar ion of 3-hydroxypyridine, shows two peaks of intensity, one at pH 0 and the other at pH 6 (cf. pyridoxol). The intensity

curve (Fig. 4) also shows an unexplained sharp peak at pH 8.5 that was repeatedly found. The rise in intensity at pH 8.8 is due to the formation of the anion (XXXII), and the fall in intensity above pH 12 could be due to some chemical change in alkali, possibly with the formation of the free aldehyde.

The pK_a values for pyridoxal determined from our fluorescence data are comparable with those reported in the literature (see Table 3).

Pyridoxamine. Four forms of pyridoxamine occur

Fig. 5. Variation of (a) fluorescence intensity and of (b) fluorescence (Fl.) and excitation (Exc.) wavelengths with pH and H_0 of pyridoxamine ($5 \mu\text{g./ml.}$). H_0 is the acidity function of Hammett (see the text). Intensity values are arbitrary, that of the 3-hydroxypyridine anion at pH 11 being taken as 100. The broken line shows intensities measured in H_2SO_4 .

at various pH values in aqueous solution, namely the dication (XXXIII), tripolar ion (XXXIV), dipolar ion (XXXV) and anion (XXXVI). Fluorescence peaks were found that corresponded to forms (XXXIII), (XXXIV) and (XXXVI) (see Table 3 and Fig. 5) but not to (XXXV). No fluorescence peak was found for the neutral form (XXXVII) in aqueous solution, but in ethanol it fluoresced at $361 \text{ m}\mu$. Because the cation (XXXIII) shows excited-state ionization its fluorescence was found only at $H_0 - 4.9$ (20N-sulphuric acid). The wavelength of fluorescence changes to that of structure (XXXIV) at $H_0 - 1$ (2.9N-hydrochloric acid) and this persists to pH 9.3. The excitation, however, changes at pH 2.6–3.0 and the pK_a estimated from this change is 2.8 (see Table 3). At pH about 9, the excitation and fluorescence wavelengths shorten together and they correspond to a change from structure (XXXIV) to (XXXVI). The pK_a for this change estimated from the excitation and fluorescence wavelengths is 9.4–9.6. This value is about half-way between the reported pK_a values for the ionizations of (XXXIV) to (XXXV) and of (XXXV) to (XXXVI), which are given by Williams & Neilands (1954) as 8.21 and 10.63 respectively. Again at pH 9.4, the fluorescence intensity falls to a low value. These observations suggest that the dipolar ion (XXXV) is virtually non-fluorescent. This could be due, in simple terms, to the combined effect of the heterocyclic nitrogen atom and the charged $\text{CH}_2\cdot\text{NH}_3^+$ group in deactivating the ring, and to interaction between the O^- group and the positively charged amino group, so that the ring-activating effect of O^- is diminished. The anion (XXXVI) is fluorescent with about the same intensity as the tripolar ion (XXXIV). Most of the fluorescent forms of pyridoxamine are more than three times as fluorescent as the 3-hydroxypyridine anion.

Fig. 5 shows also the effect of concentrated sulphuric acid and hydrochloric acid on the fluorescence of the pyridoxamine dication, which is more intense in sulphuric acid than in hydrochloric acid. This suggests that its fluorescence is more readily quenched by the latter.

Pyridoxal 5-phosphate. The fluorescence characteristics of this compound are shown in Fig. 6. It is immediately apparent that pyridoxal 5-phosphate is but weakly fluorescent, being only about 10% as fluorescent as the 3-hydroxypyridine anion. According to Williams & Neilands (1954), pyridoxal phosphate (aldehyde hydrate form) shows four pK_a values, namely <2.5, 4.14, 6.20 and 8.69, which correspond to the equilibria between structures (XXXVIII)–(XLII). The curves shown in Fig. 6 contain evidence for all these changes. The wavelength shift in the fluorescence from 400 to 410 mμ at pH 2.0–2.5 suggests the conversion of (XXXVIII) into (XXXIX), the wavelength shift in excitation from 302 to 331 mμ at pH 3.0–4.6 suggests the conversion of (XXXIX) into (XL), the wavelength shift in fluorescence from 410 to 400 mμ at pH 6.0–7.5 suggests the conversion of (XL) into (XLI), and the wavelength shifts in excitation (331 to 315 mμ at pH 8.5–10.0) and in fluorescence (400 to 370 mμ at pH 8.5–10.0) suggest the conversion of (XLI) into (XLII). These changes are also supported by fluorescence-intensity changes. Approximate pK_a values can be estimated from these changes and they are evaluated as 2.3, 3.7, 6.5 and 8.8 (see Table 3).

The cation (XXXVIII) shows excited-state ionization, since its true fluorescence wavelength maximum is in the region of 385 mμ, which shows up at H_0 –2.5 (6.7N-hydrochloric acid). When the cation (XXXVIII) undergoes excited-state ionization it probably forms the dipolar ion (XLIII), in which the phosphate group is not ionized and which

Fig. 6. Variation of (a) fluorescence intensity and of (b) fluorescence (Fl.) and excitation (Exc.) wavelengths with pH and H_0 of pyridoxal 5-phosphate (5 μg./ml.). H_0 is the acidity function of Hammett (see the text). Intensity values are arbitrary, that of the 3-hydroxypyridine anion at pH 11 being taken as 100.

fluoresces at 400 mμ. It appears also that structure (XXXIX) (cation-monophosphate anion) undergoes excited-state ionization, for at pH 2.5 the fluorescence (410 mμ) of structure (XL) appears whereas the corresponding excitation does not change completely until pH 4.6 has been reached. The change from structure (XL) to (XLI) involves a small change in fluorescence but not in excitation wavelength, and in fact structures (XL) and (XLI) have the same maximum absorption peaks (Metzler & Snell, 1955). The conversion of structure (XLI) into (XLII) involves changes in fluorescence and

Fig. 7. Variation of (a) fluorescence intensity and of (b) fluorescence (Fl.) and excitation (Exc.) wavelengths with pH and H_0 of pyridoxal acid (●—●) and pyridoxal acid lactone (×—×) (concentration of each 5 $\mu\text{g./ml.}$). H_0 is the acidity function of Hammett (see the text). Intensity values are arbitrary, that of the 3-hydroxypyridine anion at pH 11 being taken as 100.

excitation wavelengths that would be normally expected.

No fluorimetric evidence was found for the existence of the free aldehyde form of pyridoxal 5-phosphate below pH 12. At low pH values the excitation wavelength 302 m μ corresponds to the absorption maximum of the hydrated form of the cation of pyridoxal 5-phosphate, i.e. 291 m μ , and not to that of the free aldehyde form, i.e. 342 m μ (see Metzler & Snell, 1955). However, at high pH values, i.e. 12–14, the solution turns yellow and exhibits two excitation maxima and two corresponding fluorescence maxima. One is recorded in Fig. 6, i.e. λ_a 370 m μ and λ_{ex} 315 m μ . The other, which was considerably weaker, occurred at λ_a 525 m μ and λ_{ex} 400 m μ . This may be due to the trianion of the free aldehyde form (XLIV), since Williams & Neilands (1954) have recorded an absorption peak at 390 m μ for pyridoxal phosphate in alkaline solution that may be due to the free aldehyde form (Metzler & Snell, 1955).

Pyridoxic acid and its lactone. The changes in fluorescence intensity and in excitation and fluorescence wavelengths with pH of pyridoxic acid and its lactone are shown in Fig. 7 (see also Table 4). In strongly acidic solutions (H_0 0 to -8.5, i.e. 1.08–34.5 N-sulphuric acid) the fluorescence characteristics of the acid and lactone are similar, but above pH 0 they differ considerably. The fluorescent forms of pyridoxic acid that could be expected to occur in solution are the cation (XLV), dipolar ion (XLVI), tripolar ion (XLVII) and dianion (XLVIII) (other forms can occur but they are disregarded to avoid complicating the picture). Below pH 0, the changes are difficult to explain (see below). The dipolar ion (XLVI) appears to fluoresce intensely at 420 m μ (λ_{ex} 320 m μ), its maximum intensity occurring at pH 2–4. This intensity is nearly 8 times that of the 3-hydroxypyridine anion. The pK_a of the ionization of (XLV) into (XLVI), estimated from the intensity curve, is 0.4. At pH 5 both excitation and fluorescence wavelengths and fluorescence intensity change slightly with the ionization of the carboxyl group, i.e. with the conversion of (XLVI) into (XLVII). The pK_a values estimated from the intensity, excitation and fluorescence curves are 4.8, 5.25 and 5.25 respectively [Huff & Pealzweig (1944) give pK_a 5.5]. At about pH 9 the intensity falls, but there is no change in excitation or fluorescence wavelength until pH 12 is reached. This fall in intensity is interpreted as being due to the formation of the weakly fluorescent dianion (XLVIII) by deprotonation of the ring nitrogen atom. From the change in the intensity curve, it can be estimated that the pK_a for the conversion of (XLVII) into (XLVIII) is about 10.8. The weak fluorescence of the dianion (XLVIII) is probably due to the combined ring-deactivating effect of the uncharged ring nitrogen atom and the CO_2^- group.

Pyridoxic acid lactone (see Fig. 7) shows a completely different picture from the acid, since its main fluorescence occurs at pH 8–13 at 430 m μ (λ_{ex} 360 m μ). This fluorescence is very intense, being 20 times that of the 3-hydroxypyridine anion. The forms of the lactone that might be expected are the cation (XLIX), dipolar ion (L), neutral form (LI) and anion (LII). The dipolar ion (L) is

very weakly fluorescent compared with the corresponding dipolar ion (XLVI) of the acid. The dipolar ion (L) fluoresces at 423 m μ (λ_{exc} , 365 m μ) with an intensity of 35 compared with 770 for the dipolar ion (see Table 4). Here the formation of the lactone ring appears to have suppressed fluorescence intensity, a fact that is not easy to explain in view of the fluorescence of the anion (LII). The pK_a of the conversion of (XLIX) into (L), estimated from the excitation curve, is 1.6. At pH 5, the dipolar ion (L) ionizes to the intensely fluorescent anion (LII), the pK_a of the change, estimated from the fluorescence-intensity curve, being 6.95. Pyridoxic acid lactone is a derivative of the aza-isobenzofuran, furo[4,3-c]pyridine. The isobenzofurans (cf. LIV) are highly fluorescent compounds, presumably because of their ability to form free radicals (see Adams & Gold, 1940), and it is possible that an aza derivative such as the anion of pyridoxic acid lactone (LIII) is fluorescent for similar reasons. Several resonance structures for (LIII) which could contribute to its fluorescence can be drawn.

The fluorescent-wavelength curves suggest that three fluorescent forms of the acid and lactone occur below H_0 or pH 0. These forms (for the lactone) have fluorescent wavelengths of approx. 412, 451 and 423 m μ , the excitation wavelength being 320 m μ in each case. In 34.5N-sulphuric acid both pyridoxic acid and its lactone are strongly fluorescent at 412 m μ (see Fig. 7). This may be due to a hydrogen-bonded cation of the acid or lactone (LV), which undergoes excited-state ionization to a form fluorescing at 451 m μ with a much lower intensity and in which the hydrogen bond is ruptured. This second form then ionizes to the dipolar

ion (L). Hydrogen-bonding is a factor that could tend to increase fluorescence (see Lippert, 1957).

Excited-state pK_a . Förster (1950) and Weller (1952) showed that the excitation by ultraviolet light of certain weak acids, such as α - and β -naphthol, enhanced their protolytic dissociation, with the result that the dissociation constants of the acids in the excited state (pK_a^*) were greater than those in the ground state (pK_a) by a factor 10^6 – 10^7 . Weller (1952) derived a relationship between pK_a and pK_a^* from the Förster cycle (see Jaffé, Beveridge & Jones, 1964) for absorption and fluorescence, which is:

$$pK_a - pK_a^* = \frac{\Delta E - \Delta E'}{2.303RT} \quad (1)$$

where ΔE and $\Delta E'$ are the transition energies from the ground state to the first excited singlet-state level of the acid and anion (or conjugate base) respectively, R is the gas constant and T is the absolute temperature. If R is replaced by k , the Boltzmann constant, the equation then applies to one molecule.

Energy (E) is related to light frequency (ν) by the relation:

$$E = h\nu$$

(h is Planck's constant), and, since $\nu = c/\lambda$ (c is the velocity of light and λ is the wavelength in cm.), then:

$$E = hc/\lambda$$

Substituting for ΔE and $\Delta E'$ in eq. (1) and expressing λ in m μ , we get:

$$pK_a - pK_a^* = \frac{10^7 hc}{2.303kT} \left(\frac{\lambda' - \lambda}{\lambda\lambda'} \right) \quad (2)$$

Table 4. *Fluorescence of pyridoxic acid and its lactone*The concentration of each compound was 5 $\mu\text{g./ml.}$

Compound	Molecular species	Wavelength ($m\mu$)			Relative fluorescence intensity†			pH range of maximum fluorescence	pK _a			Literature values
		Of max. absorption	Of max. excitation	Of max. fluorescence	Observed	Corrected			Int.	Exc.	Fl.	
Pyridoxic acid lactone	Cation 1*	315	320	about 412	about 800	820		H ₀ -8.8	-8.0	-	-7.9	
	Cation 2*	315	320	451	28.5	32		H ₀ -7.16 to -3.7	-	-	-2.1	
	Cation 2 (excited state)*	315	320	423	15	16		H ₀ -0.1 to pH2	-	-	1.6	
	Dipolar ion Anion	357 350	365 360	423 430	32 2150	19 1430		pH2.5-4.8 pH8.7-13	-	-	6.95	7.05†
Pyridoxic acid	Cation 1*	317	320	412	about 600	615		H ₀ -8.8	-7.9	-	-7.8	
	Cation 2*	316	320	446	38.5	42		H ₀ -7.1 to -3.7	-	-	-2.0	
	Cation 2 (excited state)*	316	320	420	36	39		H ₀ -0.1				
	Dipolar ion	316	320	420	770	810		pH1.5-4.0	0.5	-	-	
	Tripolar ion	307	315	425	650	690		pH6.1-9.4	4.85	5.2	6.0	5.5†
	Dianion	306.5	315	about 415	23	27		pH12-13	10.85	-	-	9.75†

* See the text.

† See the text and Table 1.

‡ Huff & Pealzweig (1944).

Table 5. *Excited-state dissociation constants of the cations of pyridoxal and related compounds*

Compound	pK^*_A of cation		$pK_A^\ddagger$	$pK_1^\ddagger$	$pK_A - pK^*_A$ (obs.)
	Observed*	Calc.†			
3-Hydroxypyridine	-3.0	-2.24	5.37§	5.10§	8.37
3-Hydroxy-N-methylpyridine	-3.0	-2.39	4.96	4.96	7.96
Pyridoxol	-2.2	-2.36	5.05§	5.00§	7.25
Pyridoxal (hemiacetal)	-3.3	-2.53	4.20§	4.20§	7.50
Pyridoxamine	-4.1	-4.25	3.32§	3.31§	7.42
Pyridoxal 5-phosphate (aldehyde hydrate)	< -2.5	-3.28	?	4.41¶	> 6.64
2-Hydroxypyridine	-3.3	-2.52	0.75	0.75	4.05
2-Hydroxy-N-methylpyridine	about -4.0	-3.48	0.32	0.32	4.32

* Estimated from fluorescence data.

† Calculated by Weller's (1952) equation from pK_A values except for pyridoxal 5-phosphate.

‡ See the text.

§ Metzler & Snell (1955).

|| Mason (1959).

¶ Williams & Neilands (1954).

where λ and λ' are the maximum absorption or fluorescence wavelengths of the acid and anion (or conjugate base) respectively. Substituting the known values for the constants h , c and k , and taking T as 293°A (20°C), eq. (2) becomes:

$$pK_a - pK^*_a = 21187 \left(\frac{\lambda' - \lambda}{\lambda\lambda'} \right) \quad (3)$$

Absorption wavelengths are more accurately measured than fluorescence wavelengths on present-day instruments, and can be determined with an accuracy of $1\text{m}\mu$. Therefore in Table 5 λ and λ' used in calculating pK_a^* are absorption wavelengths.

When the 3-hydroxypyridine cation ionizes, it can lose a proton from the hydroxyl group or the positively charged nitrogen atom thus:

The overall dissociation constant is pK_1 , and pK_A and pK_B are the constants for the ionizations shown above. These constants are related, for:

$$K_1 = K_A + K_B$$

The excited pK determined fluorimetrically is pK^*_A , since the neutral molecule is virtually non-fluorescent. pK^*_A can also be calculated by Weller's (1952) equation above from published values of pK_A . For most of the compounds given in Table 5, pK_1 and pK_A are almost identical because, in these cases, very little of the neutral molecule occurs in aqueous solution (Metzler & Snell, 1955). Table 5 gives pK^*_A values estimated from our fluorescence data compared with those calculated from Weller's (1952) equation. The observed values are of the right order and the agreement justifies the use of the acidity function, H_0 , for very strongly acidic solutions. The values for $(pK_A - pK^*_A)$ are all about 7-8 for the 3-hydroxypyridine compounds and this indicates that the behaviour of the cations is similar. The value for both the 2-hydroxypyridines is about 4.

Stokes shifts. Stokes (1852) showed qualitatively that the wavelength of fluorescence is longer than that of the corresponding excitation. The differ-

Table 6. *Stokes shifts for pyridoxol and related compounds*

Stokes shift in $\text{cm}^{-1} = 10^7 (1/\lambda_{\text{exc.}} - 1/\lambda_{\text{fl.}})$, λ being in $\text{m}\mu$. — means either that the form does not exist or that it is non-fluorescent.

Compound	Stokes shift (cm^{-1})				
	Cation	Cation ionizing in excited state	Dipolar ion	Neutral form†	Anion
3-Hydroxypyridine	5750	9830	6240	4520	6160
3-Methoxypyridine	4150	—	—	—	—
3-Hydroxy- <i>N</i> -methylpyridine	4690	9400	5910	—	—
Pyridoxol	5210	9420	4920	5430	5870
Pyridoxal	5980	8470	5300	5220	5560
Pyridoxamine	5510	9310	5730	—	5090
Pyridoxal 5-phosphate	*	9360	5860	5500	5760
Pyridoxic acid	7700	9530	8150	—	8370
Pyridoxic acid lactone	7700	9770	3870	—	4140
2-Hydroxypyridine	7110	8320	6230	6300	5870
2-Methoxypyridine	4880	—	—	5090	—
2-Hydroxy- <i>N</i> -methylpyridine	6850	8440	6160	—	—

* The fluorescence wavelength could not be measured.

† In ethanol.

ence between the wave number of the longest wavelength of the excitation maximum and that of the shortest wavelength of the fluorescence maximum is referred to as the 'Stokes shift' (Leemann, Stich & Thomas, 1963) and is a measure of the energy involved when a compound is raised to the excited state. It is also of structural significance (see Leemann *et al.* 1963). The Stokes shift, in cm^{-1} , can be calculated from the expression:

$$\text{Stokes shift} = 10^7(1/\lambda_{\text{exc.}} - 1/\lambda_{\text{fl.}})$$

where $\lambda_{\text{exc.}}$ and $\lambda_{\text{fl.}}$ are the maximum excitation and fluorescence wavelengths expressed in $\text{m}\mu$.

The Stokes shifts for the various species of the compounds studied in this paper are given in Table 6. The first nine compounds are derivatives of 3-hydroxypyridine, and, apart from pyridoxic acid and its lactone, the values in each column are similar. The Stokes shift for the cations, dipolar ions, neutral forms and anions of the first seven compounds is 4000–6000 cm^{-1} , whereas the value for the cation undergoing excited-state ionization is 8000–10000 cm^{-1} . For these seven compounds the similarity of the values in each column supports the view that the ionic species considered are similar, and supports our interpretation of the fluorescence data. The larger values for the cations undergoing excited-state ionization indicate that a process in addition to absorption and emission is involved. In fact, some of the absorbed energy is being used to bring about ionization in the excited state. The values for the cation, dipolar ion and anion of pyridoxic acid and its lactone do not agree

with the others or with one another except for the cations, and the interpretation of the fluorescence data may be more complicated than has been suggested.

The 2-hydroxypyridines give another series of Stokes shifts. The values for the dipolar ion and anion are similar to those of 3-hydroxypyridine and suggest similarity between these forms of the two isomers. Again, 2-methoxypyridine, which cannot occur as a pyridone, falls in line with the 3-methoxypyridine. The 2-hydroxypyridine cation and neutral form and 2-hydroxy-*N*-methylpyridine cation have Stokes shifts that are larger than those of the 3-hydroxypyridines, and this may be due to their ability to exist as pyridones. The Stokes shifts have not been extensively studied, but their possible uses have been discussed by Leemann *et al.* (1963).

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