Spectroscopic Properties of 4-Pyridoxic Acid as a Function of pH and Solvent

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The photophysical properties and acid/base equilibria of 4-pyridoxic acid (= 3-hydroxy-5-(hydroxymethyl)-2-methylpyridine-4-carboxylic acid), the final product of the catabolism of vitamin B_6, have been studied in aqueous solutions. The ground state of 4-pyridoxic acid exhibits the different protonated forms **A** – **D** in the range of $H_0 = -6$ to pH 11.5. HMQC- and HMBC-NMR Studies allowed the pH-dependent assignment of the different C-atoms, and the evaluation of the deprotonation sequence. The 3-OH group in the ground state has a 'pK_a' of $H_0 = -0.64$, which is much lower than that found for other vitamin B_6 related compounds. The pK_a value of the 4-COOH group is 5.4. Fluorescence studies showed that the same species exist at the lowest excited singlet state, but in different pH ranges. The 3-OH group is four pH units more acidic in the lowest excited singlet state than in the ground state. Excitation spectra and emission decays in the pH range of 8 to 11.5 indicate that the pyridine N-atom is more basic in the excited singlet state than in the ground state. The emission spectra are red-shifted in protic solvents, in agreement with an intramolecular H-bond between the ionized 3-OH group and the nonionized 4-COOH group.

Introduction. – Pyridoxinic compounds are of biological interest because they are constituents of vitamin B_6. This group is composed of three naturally occurring compounds, pyridoxine, pyridoxamine, and pyridoxal. A crucial stage in the metabolism of these compounds is their conversion to the active form, pyridoxal 5-phosphate (PLP) mediated by a single kinase enzyme present in brain, liver, and erytrocytes. Pyridoxine 5-phosphate and pyridoxamine 5-phosphate are converted to PLP by a flavin-dependent oxidase [1]. Thereby, PLP acts as a cofactor in enzymes involved in transamination reactions, and is required for the synthesis and catabolism of amino acids, as well as in glycogenolysis, as a cofactor for glycogen phosphorylase [2]. An excess of pyridoxal in the blood is regulated by the liver, which is the main site of degradation to the water-soluble, urine-excretable 4-pyridoxic acid (= 3-hydroxy-5-(hydroxymethyl)-2-methylpyridine-4-carboxylic acid) [1][3].

One characteristic of pyridoxinic compounds is that their spectral properties are sensitive to pH, since these compounds may exist in different ionization states. From the changes of the absorption spectrum, the pK_a values of different protonated forms have been determined [4][5]. Also, strong changes with pH have been observed in the fluorescence characteristics of vitamin B_6 related compounds [6][7]. However, most of these studies refer to pyridoxal and *Schiff* bases with different amines. Recently, we carried out a detailed photophysical study of pyridoxamine to characterize the acid/base properties of its excited singlet state [8]. However, studies on 4-pyridoxic acid are scarce, in spite of its biological role.

Pyridoxinic compounds can react with nucleophilic groups of proteins, particularly lysyl residues, to form stable amide linkages. This makes the pyridoxyl moiety a suitable

chromophore to be used as a fluorescent probe sensitive to the structural properties of protein microenvironments. Thus, the fluorescence of 4-pyridoxic acid has been used to detect fast motions of proteins [9], and a reduced *Schiff* base has been used to study the stability of PLP-dependent enzymes [10]. The fluorescence of the pyridoxyl group, when linked as pyridoxamine, has been used to study the binding of several substrates and ligands to phosphoenolpyruvate carboxykinases [11].

In this paper, we report the spectral properties of 4-pyridoxic acid in different media. The pK_a values, fluorescence quantum yields, and fluorescence lifetimes for different equilibrium forms were evaluated, and NMR studies were carried out to derive the deprotonation sequence of the different protic groups as a function of pH.

Experimental. – *General*. 4-Pyridoxic acid and DMF were purchased from *Sigma*, EtOH, MeOH, and MeCN from *Merck*, and dimethylsulfoxide (DMSO) from *Burdick & Jackson*. All solvents were of spectroscopic grade and contained less than 0.02% of H_2O. pH Measurements: *Oyster* pH meter, model *120000*, with a *Sensorex* epoxy body combination electrode; pH values were adjusted by addition of NaOH or HCl; solns. of pH < 1 were prepared by addition of H_2SO_4, following the *Hammett* acidity scale [12]. UV Spectra were recorded on an *Hewlett-Packard 8453* diode array spectrophotometer.

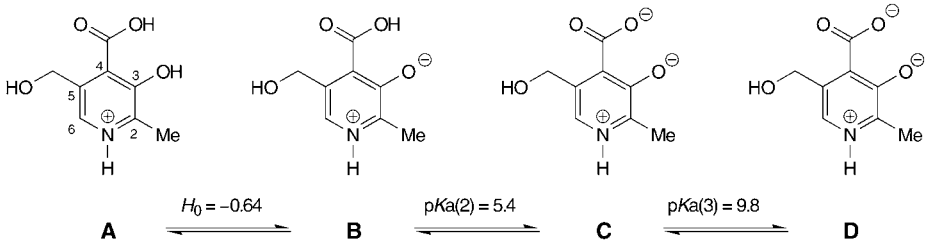
Fluorescence Spectra. Baseline-corrected fluorescence spectra were recorded on a *Spex-Fluorolog* spectrofluorometer in air-equilibrated solns. at 20°. Bandwidths of 1.25 nm were used for excitation and emission slits. Fluorescence quantum yields Φ_F were determined by integration, quinine sulfate monohydrate in 0.1M aq. H_2SO_4 soln. at 20° being used as a standard ($\Phi_F = 0.53$) [13]. Fluorescence lifetimes were measured with an *Edinburgh-Instrument OB-900* time-correlated single-photon-counting fluorimeter (H_2 lamp). Analysis of fluorescence decay was carried out by a least-squares iterative convolution based on the *Marquardt* algorithm, using the analysis routine of *Edinburgh Instrument* (Edinburgh, UK). The quality of the fit was assessed by means of χ^2 values, together with the distribution of residuals.

NMR Spectra. 1D- and 2D-NMR spectra were recorded on a *Bruker Avance-400* instrument (400 and 100 MHz for ^1H and ^{13}C, resp.), equipped with a broadband inverse probe and gradient system. The spectra were recorded in D_2O soln. at different pD values at 25°. The selected pD was adjusted by addition of NaOD or D_2SO_4. The actual pD values of the solns. were calculated from the known relation pD = pH + 0.40 [14]. HMBC Spectra were calibrated by means of a 1,4-dioxane external capillary, and chemical shifts δ (in ppm) are given rel. to $SiMe_4$ (= 0 ppm). The number of scans in each experiment was dependent on the sample concentration (*ca.* 10^{-2} M). The ge-2D HMQC (heteronuclear multiple-quantum coherence) and ge-2D HMBC spectra resulted each from a 128×1024 matrix.

Results and Discussion. – 1. *Absorption Properties of 4-Pyridoxic Acid*. The UV spectrum of 4-pyridoxic acid in H_2O at pH 7 showed a band at 316 nm, with a molar extinction coefficient of 5100 l mol^{-1} cm^{-1}. Generally, pH changes affected the absorption spectrum of 4-pyridoxic acid significantly. This behavior is similar to that of other 3-hydroxypyridine derivatives – a consequence of the different ionization forms of these compounds [5]. In the case of 4-pyridoxic acid, the forms **A** – **D** can be expected to occur in solution (*Scheme*).

The wavelengths of the absorbance maxima of 4-pyridoxic acid at different pH values are given in *Table 1*. The absorption spectrum remained unchanged in the pH range of 1.7 to 8.0, although the dissociation of the 4-COOH group is expected to occur in this pH range. On increasing the pH from 8 to 11.5, the band at 316 nm showed a blue-shift of 9 nm, and the intensity slowly increased. From the plot of the wavelength of the absorbance maximum *vs.* pH, a pK_a(3) value of 9.8 was obtained, which is in agreement with that reported by *Bridges* et al. [15]. A similar pK_a value was obtained from the increase of the absorbance at 307 nm. These changes were assigned to the deprotonation of the pyridinium HN^+ group of species **C** (see the *Scheme*), as in the

Scheme



case of other 3-hydroxypyridine derivatives [4][5]. In strongly acid solution ($H_0 = -1.5$), the absorption spectrum showed a band that was red-shifted by 6 nm (relative to that found at neutral pH), which can be assigned to the totally protonated form **A**. From the changes in wavelength and absorption intensity at 316 nm, an estimated '$pK_a(1)$' value of $H_0 = -0.64$ was found for the deprotonation of the 3-OH group.

The high acidity of 3-hydroxypyridines is well-established [15][16]. However, our results indicate that 4-pyridoxic acid is a much stronger acid than other 3-hydroxypyridine derivatives of the vitamin B_6 group [8][16]. The low $pK_a(1)$ of 4-pyridoxic acid reflects the stabilizing influence of the 4-COOH group on the dissociation of the 3-OH group (see below).

Table 1. *pH Effects on the Photophysical Parameters of 4-Pyridoxic Acid in Aqueous Solution*

pH	Absorption	Fluorescence	
	λ_{max} [nm]	λ_{max} [nm]	Φ_F[a])
–6.5[b])	322	447	–
–1.5[b])	322	417	–
1.5	316	417	0.45
4.0	316	417	0.45
8.0	316	422	0.48
11	307	422	0.10

[a]) Experimental error: ±0.01. [b]) H_0 instead of pH value.

2. *NMR Assignments.* Further information on the deprotonation of the 3-OH and 4-COOH groups was derived from NMR spectra recorded at different pD values. 4-Pyridoxic acid gives rise to a simple ^{1}H-NMR spectrum. At pD 3.3, three sharp singlets are observed for H–C(6), the 2-Me group, and the aliphatic CH_2 group at δ_H 7.6, 2.14, and 4.47, respectively. The other protic H-atoms (two OH and one COOH) rapidly exchange in D_2O and are, thus, not observed. In *Fig. 1*, the ^{1}H-NMR titration curve of 4-pyridoxic acid is shown, which, considering the associated UV spectral changes, gave rise to a $pK_a(2)$ of 5.4 ± 0.1 for the 4-COOH group (see the *Scheme*). However, the chemical shifts of the different H-atoms were not those expected due to the deprotonation of the 4-COOH group, but rather to the presence of a charged *intramolecular* H-bond of the type COOH ··· $^-$O–C(3), *i.e.*, between the COOH and the deprotonated 3-OH group, which affects the chemical shifts of the aromatic

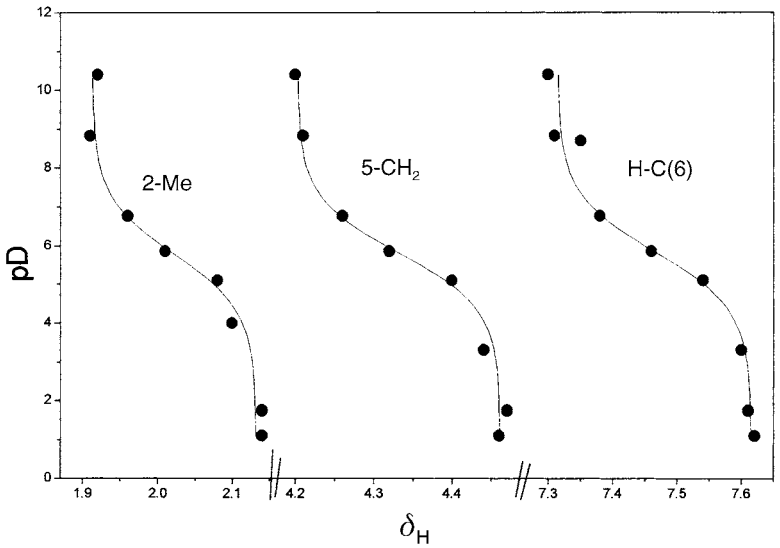


Fig. 1. *^{1}H-NMR Chemical shifts* δ_H (in ppm) *of pyridoxic acid as a function of pD*

C-atoms (shielding effect). Similar influences have been reported for other vitamin B_6 compounds [17].

The pK_a values of the 3-OH and 4-COOH groups were also determined by ^{13}C-NMR spectroscopy. A complete ^{13}C-NMR assignment was made by the concerted use of several gradient-enhanced techniques such as ^{1}H-, ^{13}C-, ^{1}H/^{13}C-2D-HMQC, and ^{1}H/^{13}C-2D-HMBC NMR experiments. The ^{1}H/^{13}C-2D-NMR correlations allowed the unambiguous assignment of C(2), 2-Me, C(3), 5-CH_2, and C(6). The 4-COOH C-atom was assigned from the corresponding ^{13}C-NMR spectrum since a 2D-NMR long-range $J_{C,H}$ correlation was not observed. Since the aromatic C(4)- and C(5)-atoms could not be assigned by 2D-NMR correlation, they were assigned according to the method of *Witherup* and *Abbott* [18].

The deprotonation of the 3-OH and 4-COOH groups could be monitored by the differential ^{13}C-NMR chemical shifts of the corresponding C-atoms at different pD values (*Table 2*). The chemical shifts δ_C of all nuclei changed as a function of pD, in agreement with studies on other pyridoxinic compounds [19].

Under strongly acidic conditions (aq. D_2SO_4) up to a pD of 3.3, C(3), C(4), and 5-CH_2 experienced significant changes in chemical shift ($\Delta\delta_C$) as a result of the first deprotonation, that of the 3-OH group (p$K_a(1) = -0.64$). In the pD range of 3.3 to 8.7, significant differential shifts occured for the ^{13}C-NMR resonances belonging to C(2), COOH, C(5), and C(6). Thereby, $\Delta\delta_C$ of COOH and C(5) were attributed to the second deprotonation, that of the 4-COOH group (p$K_a(2) = 5.4$). The change in the chemical shifts of C(2) and C(6) probably resulted from the protonation/deprotonation of the pyridine N-atom.

The deprotonation sequence in 4-pyridoxic acid is different from that found for aromatic compounds containing a COOH group in *ortho*-position to a OH group. Thus, the first deprotonation in salicylic acid (=2-hydroxybenzoic acid) takes place at the

Table 2. *pD-Dependent ^{13}C-NMR Chemical Shifts* δ (in ppm) *and Chemical-Shift Changes* of *4-Pyridoxic Acid in Aqueous Solution*

C-Atom	D_2SO_4[a]) δ_1	pD 3.3 δ_2	pD 8.7 δ_3	$\delta_2-\delta_1$	$\delta_3-\delta_2$
C(2)	145.4	144.2	146.2	−1.2	+2.0
2-Me	12.9	14.5	16.0	+1.6	+1.5
C(3)	150.2	156.2	155.1	+6.0	−0.9
C(4)	121.4	130.7	129.8	+9.3	−0.9
4-COOH	170.0	170.0	172.3	0	+2.3
C(5)	140.6	139.0	135.5	−1.6	−3.5
5-CH_2	70.0	60.0	60.5	−10.0	+0.5
C(6)	125.5	128.0	132.0	+2.5	+4.0

[a]) $[D_2SO_4] = 11$M ($H_0 = -6.5$).

COOH rather than at the OH group [20]. The deprotonation of the COOH group occurs at a pH three units lower than that of the OH group in, *e.g.*, isocoumarin derivatives [21]. In 4-pyridoxic acid, the significantly higher acidity of the OH group relative to the COOH group is consistent with a resonance structure in which the protonated pyridinium N-atom withdraws electrons from the ionized 3-OH group.

3. *Fluorescence Properties.* 3.1. *Fluorescence at pH 1–8.* In *Fig. 2*, the pH dependence of the fluorescence-emission spectrum of 4-pyridoxic acid is shown. The corresponding wavelengths at the emission maxima are given in *Table 1*. As can be seen, the fluorescence spectra are insensitive to the H^+ concentration within pH 1 to 4. In this pH range, the emission spectra were independent of the excitation wavelengths

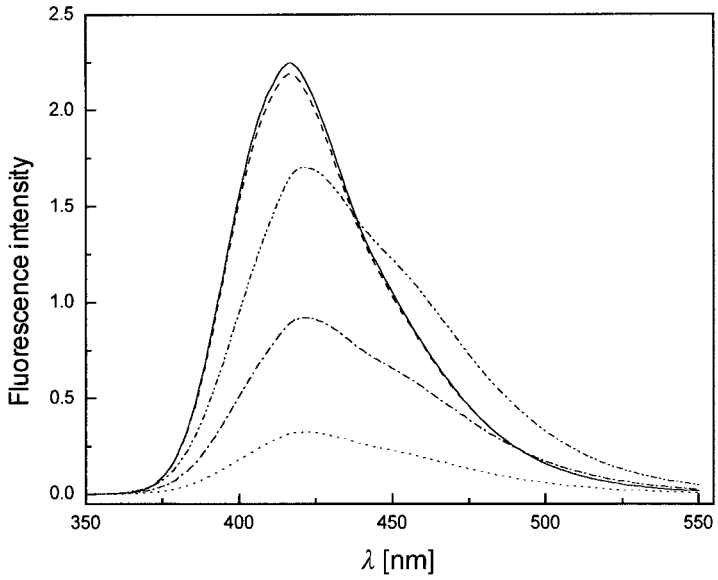


Fig. 2. *Fluorescence spectra of 4-pyridoxic acid in aqueous media as a function of pH*. The Spectra were recorded at pH 1.5 (—), pH 4.0 (– – –), pH 8.0 (– ·· – ··), pH 10.3 (– · –), and pH 11.0 (···). Excitation wavelengths at maximum absorbance were used at each pH.

within 280–340 nm, and the excitation spectra nicely mimicked the absorption spectra. The fluorescence showed a mono-exponential decay, with a lifetime τ of 4.7 ns (*Table 3*). These results indicate that the emitting species in the pH range 1.5–4.0 corresponds to **B** (see the *Scheme*), where the ionized 3-OH group is stabilized by an intramolecular H-bond to the 4-COOH group.

Table 3. *Fluorescence Lifetimes* τ (in ns) *of 4-Pyridoxic Acid as a Function of pH*. Values refer to the absorption/emission wavelengths of maximum absorbance and emission, resp., at each stated pH.

pH	τ_1	a_1[a])	τ_2	χ^2[b])
– 4.4[c])	4.8	0.39	0.25	1.09
– 4.4[d])	4.7	0.17	0.27	1.03
1.5	4.7	1.0	–	1.00
4.0	4.6	1.0	–	1.13
5.0	4.4	0.77	8.0	1.15
6.0	4.4	0.27	8.5	1.03
6.4	4.3	0.096	8.5	0.99
8.0	–	–	8.6	1.05
9.0	8.6	1.0	–	1.05
10.5	8.5	1.0	–	1.09
11.0	8.5	0.94	0.5	0.97
11.5	8.3	0.88	0.22	1.12

[a]) Pre-exponential factor. [b]) Deviation of measured value from expected value. [c]) Emission wavelength: 410 nm. [d]) Emission wavelength: 484 nm.

Upon increasing the pH from 4 to 8, a gradual decrease of the fluorescence intensity was observed, and the band was slightly red-shifted and markedly broadened (*Fig. 2*). This indicated the appearance of other chemical structures. From the decrease of the fluorescence intensity, a $pK_a(2)$ value of 5.5 ± 0.2 was determined, attributable to the deprotonation of the 4-COOH group, which results in species **C**. Such an assignment was further supported by fluorescence-lifetime measurements. The fluorescence decay could be nicely fitted with a double-exponential function (*Table 3*). The contribution of the component with the shorter lifetime decreased with pH. The decrease of the contribution of this component or the increase of the longer-lived species as a function of pH could be fitted to a sigmoidal function, with an apparent pK_a value of 5.5 ± 0.1, similar to that obtained from steady-state fluorescence and NMR experiments. This result suggests the existence of an equilibrium between species **B** and **C** in the ground state. The excitation of **B** then would lead to the fluorecent species with the shorter lifetime (4.6 ns), and the longer-lived component (8.5 ns) would correspond to **C**.

3.2. *Fluorescence at 8–11.5.* The fluorescence intensity of 4-pyridoxic acid decreased markedly upon raising the pH to highly basic conditions, but the wavelength at the maximum emission remained constant up to a pH of *ca.* 11.5, before it was shifted slightly towards shorter wavelength. From fluorescence-titration curves, a $pK_a(3)$ value of 9.9 ± 0.2 was obtained, in accordance with the one derived from UV measurements. This indicates that the pH-dependent intensity changes are determined by the nature of the absorbing species. In a pH range of 8–11, the fluorescence-emission decay could be nicely fitted to a mono-exponential function, with a lifetime of 8.5 ± 0.2 ns. This indicates that, below pH 11, the emitting species is **C**, bearing a pyridinium N^+-

atom. At higher pH, the decays became bi-exponential, with an important contribution of a very short-lived excited singlet state component, species **D**. Considering that **D** is in the ground state at pH 11, these results suggest that the pyridine N-atom in the lowest-excited singlet state becomes more basic than in the ground state, indicating an increase in charge density on the N-atom in the excited singlet state. This conclusion is somewhat different from that reached for other compounds of the vitamin B_6 group – only a small decrease in the basicity of the pyridine N-atom has been proposed for pyridoxal [22] and *Schiff* bases [6]. We found that excited singlet pyridoxamine is *ca.* one order of magnitude more basic in the excited state than in the ground state [8]. However, these differences can be rationalized in terms of donor/acceptor properties of the substituents in 4-position, which should also affect the charge density at the pyridine N-atom in the excited state and, hence, its basicity.

3.3. *Fluorescence in Strongly Acidic Solutions.* Fluorescence spectra of 4-pyridoxic acid were also recorded in strongly acidic solutions in the range of $H_0 = -0.6$ to -8 (*Fig. 3*). As the pH was lowered, the contribution of the emission spectrum of **B** decreased, and a new, broad band appeared, with an emission maximum at 447 nm. The titration-induced change in fluorescence intensity at 419 and 447 nm gave rise to a H_0 value of -4.4 ± 0.2 (*Fig. 3*, inset). Since the corresponding absorption spectra remained almost unchanged, this H_0 value matched with the protonation of the ionized 3-OH group of the lowest-excited singlet state of 4-pyridoxic acid.

pK_a Values of excited molecules (designated as pK_a*) were estimated by *Föster*'s method, using the intersection point $E_{0,0}$ of absorption and emission spectra, as

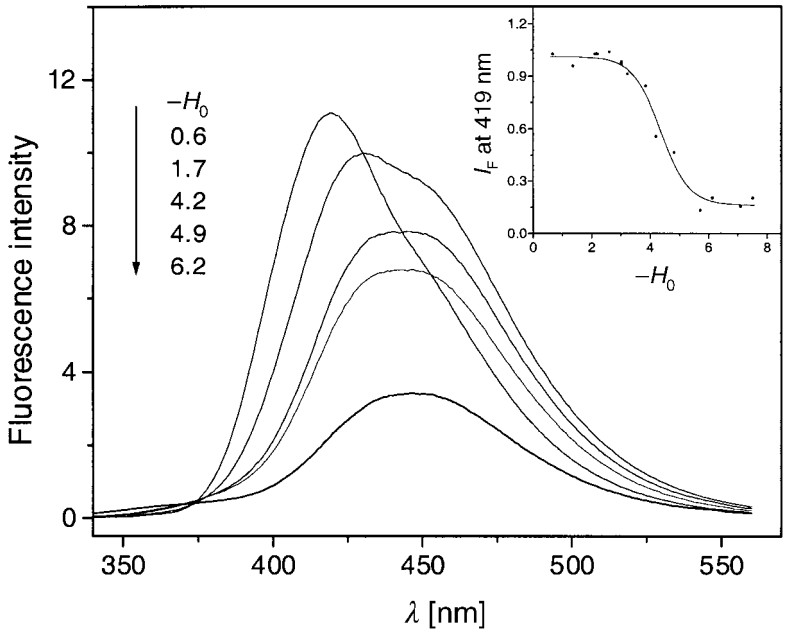


Fig. 3. *Fluorescence spectra of 4-pyridoxic acid in aqueous solutions as a function of the* Hammett *acidity scale* (H_0). Inset: titration curve of the fluorescence intensity (I_F) at an emission wavelength of 419 nm (excitation wavelength: 316 nm).

determined by the procedure of *Grabowski* and *Grabowska* [23]. For excited singlet state 4-pyridoxic acid, we obtained a pK_a^* value of -4.8, which was in agreement with that obtained by fluorimetric titration. Earlier studies of *Bridges et al.* [15] on hydroxypyridine derivatives have indicated that these molecules are very strong acids in their excited singlet states. More recently, *Echeverría et al.* [22] estimated that the aromatic OH group in pyridoxal 5′-phosphate is 8.6 pK_a units more acidic in the excited state than in the ground state. Studies on pyridoxamine also showed a strongly acidic aromatic OH group in the excited singlet state [8]. Our results indicate that 4-pyridoxic acid is a strong acid in its lowest excited singlet state as well. However, fluorescence-decay measurements showed that, at an H_0 value of -4.4, the emission lifetime depends on the emission wavelength (*Table 3*). This indicates that the thermodynamic equilibrium is not reached for the totally protonated species **A** of 4-pyridoxic acid in the excited singlet state. The favorable deprotonation of the 3-OH group in the excited state is consistent with a substantial decrease in charge density on the 3-OH O-atom upon electronic excitation, which results in a weakened O–H bond and, thus, in a more-acidic character.

3.4. *Fluorescence Quantum Yields.* The fluorescence quantum yields Φ_F of 4-pyridoxic acid were pH dependent (*Table 1*). In the pH range of 1.5–4.0, Φ_F was 0.45. Then, at pH 4.0–8.0, it slightly increased, and, at higher pH, it decreased again, reaching a value of 0.10 at pH 11. These results suggestes that species **B** and **C**, with deprotonated 3-OH groups and pyridinium N^+-atoms, are highly fluorescent, with a considerable lifetime of 8.5 ns. The low fluorescence quantum yield at pH 11 was attributed to the presence of species **D**, lacking the charge at the N-atom due to deprotonation. A similarly low value of Φ_F in highly basic media has been also reported for pyridoxal [22], its *Schiff* base [6], and pyridoxamine [8].

4. *Solvent Effects.* The deprotonation of the 4-COOH group in the excited state can be rationalized by the fluorescence properties of 4-pyridoxic acid in different solvents. In *Fig. 4*, the fluorescence spectra of 4-pyridoxic acid in solvents of different H-bonding strength, are shown. The spectra were blue-shifted, when the solvent was changed from MeOH (a strong H^+ donor) to DMF (moderately good H^+ acceptor) to MeCN (a weak H^+ acceptor). Moreover, the spectrum in MeCN matched fairly well with that obtained in H_2O at pH 4.0. The fluorescence in MeOH followed a bi-exponential decay, with values of $\tau = 7.5$ ns ($\Phi_F = 0.73$) and 2.4 ns ($\Phi_F = 0.27$). However, in DMF, the data could be better fitted to a mono-exponential function, with a lifetime of 6.4 ns, assigned to species **B**. These results are in agreement with the deprotonation of the COOH group in MeOH, the most-protic solvent, where species **B** and **C** dominate. Furthermore, the corresponding absorption spectrum was unaffected in these solvents, as expected from the similar absorption spectra of **B** and **C**.

Studies on the solvent-dependent absorption spectra of pyridoxyl compounds have revealed that the existence of different tautomeric forms depends on the polarity of the solvent [24]. These tautomers are due to intramolecular H^+ transfer from the protonated pyridinium HN^+ group to the 3-O^--atom, a process that is favored in protic media [8]. The lack of this tautomerization in 4-pyridoxic acid agrees with the proposed intramolecular H-bond.

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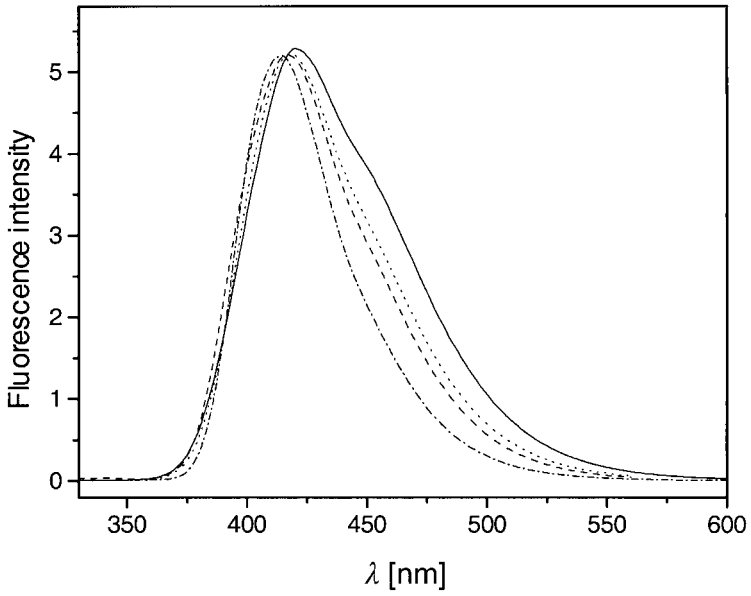

Fig. 4. *Solvent-dependent fluorescence spectra of 4-pyridoxic acid.* Media: H_2O (pH 8) (—), MeOH (···), DMF (– – –), and MeCN (· – · –). Excitation wavelength: 316 nm.

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