Photophysical and Photochemical Studies of Pyridoxamine

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The absorption and fluorescence emission of pyridoxamine were studied as function of pH and solvent properties. In the ground state, pyridoxamine exhibits different protonated forms in the range of pH 1.5 – 12. Fluorescence studies showed that the same species exist at the lowest singlet excited state but at different pH ranges. The phenol group is by *ca.* 8 units more acidic in the excited state than in the ground state. On the other hand, the pyridine N-atom is slightly more basic in the lowest excited state than in the ground state. Excitation spectra and emission decays in the pH range of 8 – 10 indicate the protonation of the pyridine N-atom by proton transfer from the amine group, in the ground and singlet excited states. Spectroscopic studies in different solvents showed that pyridoxamine in the ground or excited states exhibits intramolecular proton transfer from the pyridine N-atom to the phenol group, which is more favorable in solvents of low hydrogen-bonding capacity. The cationic form with the protonated phenolic group, which emits at shorter wavelength, is the dominant species in nonprotic solvents, but, in strong proton-donor solvents, both forms exist. The fluorescence spectra of these species exhibit blue shift in protic solvents. These shifts are well-correlated with the polarity and the H-donor ability of the solvent.

Introduction. – Pyridoxinic compounds are of biological interest because they constitute the different forms of the vitamin B_6 (pyridoxal, pyridoxal 5′-phosphate, pyridoxine, and pyridoxamine). They act as coenzymes in a wide variety of reactions involved in amino acid, carbohydrate, protein, lipid, and nucleic acid metabolisms [1]. These species are catalysts in numerous reactions involved in the normal metabolism, including transamination, de-aldolation, and elimination. All living organisms require vitamin B_6, and they must either synthesize it or, like humans, derive it from nutrients [2].

The pyridoxal 5′-phosphate (PLP) and pyridoxamine 5′-phosphate are active forms of vitamin B_6. In all PLP-dependent enzymes, the coenzyme binds to the protein to form an aldimine or *Schiff* base with the ε-amino group of a lysine residue of the polypeptide chain. The pyridoxamine is the reduced form of the *Schiff* base it meets in the protein. For example, the prosthetic group of all aminotransferasas is PLP, which is derived from pyridoxine. During transamination, PLP is transiently converted into pyridoxamine [3].

The spectral properties of the pyridoxinic compounds are very sensitive to the environment. In particular, they present several different ionization states depending on the pH of the medium. From the change of the absorption spectrum, the pK values of the different protonated forms of the ground state have been determined [3 – 5]. Also, strong changes of the absorption characteristics of these compounds with the solvent polarity have been detected [6][7]. These changes have been interpreted in terms of the different tautomeric forms present in the different media. Strong changes

with pH and solvent polarity have been observed also in the fluorescence properties of vitamin B_6 like compounds [8–11]. However, most of the studies refer to pyridoxal and its *Schiff* bases with compounds bearing the amine group. Pyridoxamine has received much less attention despite its important biological role. Even more, the reduced *Schiff* base, which has a structure similar to pyridoxamine, is used as a fluorescent probe to infer the physicochemical properties and stability of proteins [12–14].

Here, we report the spectroscopic characteristics of pyridoxamine as a function of the pH and medium properties. The p*K* values, fluorescence quantum yields, and emission lifetimes for the different ionization states were evaluated. We also studied the effect of the solvent on the properties of the ground and singlet excited states of the different species.

Experimental. – *Materials.* Pyridoxamine dihydrochloride, formamide, *N*-methylformamide (MF), and *N*,*N*-dimethylformamide (DMF) were purchased from *Sigma*. EtOH, MeOH, pentanol, heptanol, and 1,4-dioxane were from *Merck*. Dimethyl sulfoxide (DMSO) was from *Burdick & Jackson Laboratories*. All solvents were of spectroscopy grade and contained less than 0.02% of H_2O.

Methods. Absorption spectra were measured with an *HP8453* diode-array spectrophotometer. The pH measurements were carried out with an *Oyster* pH-meter, model *120000*, with the *Sensorex* epoxy body combination electrode. The pH was adjusted by NaOH or HCl addition. Solns. of $pH < 1$ were prepared according to the *Hammett* H_o acidity function of H_2SO_4 solns. [15].

Corrected fluorescence spectra were obtained on a *Spex Fluorolog* spectrofluorometer in air-equilibrated solns. at 20°. Bandwidths of 1.25 nm were used for excitation and emission slits. Solns. were prepared with absorbances 0.1–0.2 at the excitation wavelength. Fluorescence quantum yields were determined from the integrated area of the corrected fluorescence spectrum. Quinine sulfate monohydrate in 0.5M H_2SO_4 at 20° ($\Phi_F = 0.53$) [16] was used as the standard. The errors in the ionization constants (p*K*) measured either from the absorption or fluorescence intensity were estimated to be ± 0.1.

Fluorescence lifetimes were measured with an *Edinburg Instrument OB-900* time-correlated single-photon-counting fluorimeter. H_2 Gas was used in the flash lamp. Analysis of the fluorescence decays were carried out by a least-squares iterative convolution method based on the *Marquardt* algorithm by means of the analysis routine provided by *Edinburgh Instrument* (Edinburgh, UK). The quality of the fit was examined by χ^2 value together with the distribution of the residuals.

Results and Discussion. – *Effect of pH on the Absorption Properties of Pyridoxamine in Aqueous Solution.* The absorption spectrum of pyridoxamine presents different bands depending on the pH of the medium. These bands have been assigned to different protonated forms of this compound (*Scheme 1*) [5][8]. At low pH (<2), a single band centered at 294 nm, corresponding to the totally protonated form **I** of pyridoxamine was observed. As the pH increases, the absorbance decreases, and new bands appear at 326 and 254 nm, assigned to the cationic species **II** with a deprotonated phenol group [5][17][18]. At higher pH (6.5–9.4), the deprotonation of the pyridine N-atom takes place, and both absorption bands are blue-shifted, reaching maxima at wavelengths of 311 and 245 nm (*Table 1*). Changes of the absorbance and maximum wavelength absorption allowed the evaluation of macroscopic p*K* values of 3.1 and 7.9, which are similar to those reported from absorption titration [5][8] and NMR experiments [17]. At pH higher than 9.5, almost negligible changes in the absorbance or band position ($\Delta\lambda_{max} = 2$ nm) are observed. However, a third p*K* value between 10.3 and 10.6, which corresponds to the deprotonation of the amine group, has been reported from NMR experiments [17][18].

Scheme 1

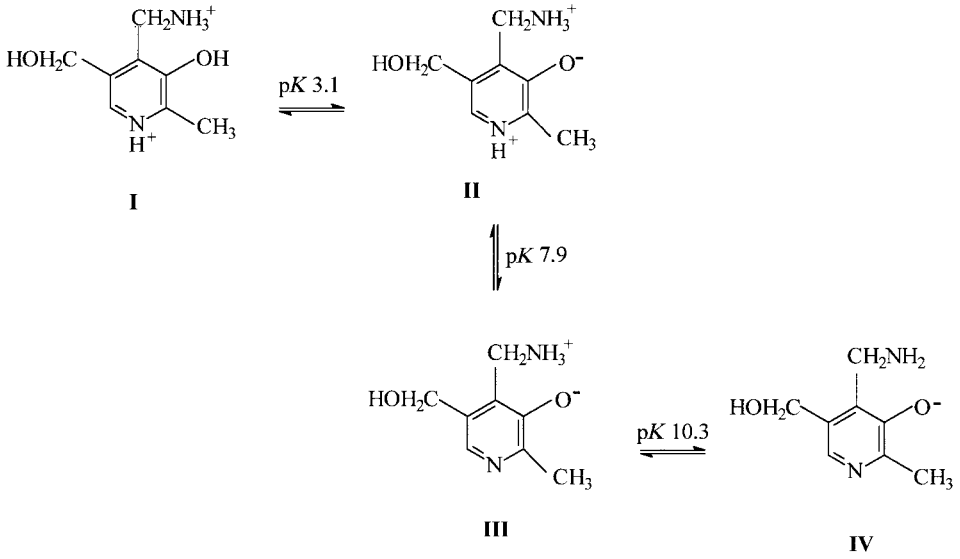


Table 1. *Absorption and Fluorescence Properties of Pyridoxamine in Aqueous Solutions*

pH/ H_o	Absorption	Fluorescence	
	λ_{max} [nm]	λ_{max} [nm]	Φ_F[a])
H_o – 6.6	286	336	–
pH 1.7	294	393	0.15
pH 6.2	326	393	0.15
	254		
pH 9.5	311	372	0.028
	245		
pH 12	309	365	0.08
	245		

[a]) Φ_F was obtained with an experimental error of ±0.01.

Effect of pH on the Fluorescence Properties in Aqueous Solution. The fluorescence of the pyridoxamine is strongly dependent on the pH. *Fig. 1* shows fluorescence spectra at selected pH values. These spectra were recorded on excitation at the wavelength of the absorption maximum at each pH. Fluorescence quantum yields and maximum emission wavelengths at several pH are given in *Table 1*. These values show that the species present at pH 6.2, where the pyridine N-atom is protonated (*i.e.*, **II**), is the more fluorescent species in aqueous medium and is the same species that emits at pH 1.7. The fluorescence quantum yield is similar to that reported for pyridoxal 5′-phosphate [10]. However, it is higher than that obtained for the *Schiff* base of the pyridoxal with isoleucine [9], indicating that substituents influence the different deactivation pathways of the excited species.

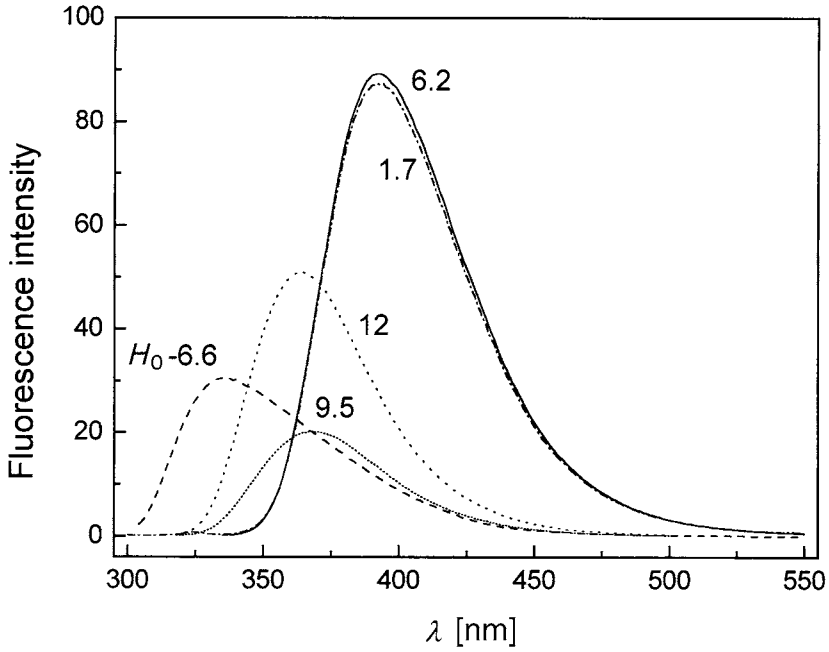


Fig. 1. *Fluorescence spectra of pyridoxamine in aqueous medium.* —: H_o −6.6; (·-·-·): pH 1.7; (—): pH 6.2; (····) pH 9.5; (···): pH 12. Excitation wavelength was used at maximum absorption of the lowest energy band at each pH.

In the pH range of 1.7–6.2, the fluorescence intensity at 393 nm with excitation at 326 nm increases with increasing pH, and decreases with the excitation at 294 nm. The p*K* value determined from the decrease or the increase of the fluorescence intensity was 3.2, which is in agreement with that obtained by absorption titration, indicating that pH-induced fluorescence-intensity changes reflect the ground-state acid-base equilibrium. However, the fluorescence quantum yield and the position of the fluorescence spectra remain unchanged from pH 1.7 to 6.2 (*Table 1*). The emission spectra were independent from the excitation wavelength 280 to 350 nm. At a given pH, the excitation spectrum mimics the absorption spectrum. The fluorescence profile decay, measured at pH 1.7 and 6.2, gave a monoexponential decay of 1.6 ns (*Table 2*). The fact that fluorescence behavior does not change in the pH range from 1.7 to 6.2 suggests that the singlet-excited species in this pH range is **II**, and the deprotonation of the phenol group of excited pyridoxamine occurs at pH lower than 1.7.

The fluorescence spectrum was measured in acid solution in the range $H_o = -2$ to -6.6. As the concentration of the acid was increased, the emission of the species **II** was quenched, and a new band appeared with a maximum at 336 nm (*Fig. 2*). A clear isosbestic point is observed at 350 nm, indicating the presence of two different species in the lowest singlet-excited state. The titration of both the fluorescence intensity decrease at 393 nm and the increase at 336 nm gave an apparent p*K** value of − 4.8. Since the absorption spectrum remains unchanged at these acid concentrations, this value would correspond to the protonation of the phenolic O-atom in the lowest

Table 2. *Fluorescence Lifetimes and Pre-exponential Factor* (a_1) *for Pyridoxamine in* H_2O *at Several pH or* Hammett *Acidity* (H_o) *Values Measured at Different Excitation and Emission Wavelengths*

pH/ H_o	λ_{exc} [nm]	λ_{em} [nm]	τ_1 [ns]	a_1 [%]	τ_2 [ns]	χ^2
H_o −6.6	286	336	0.50	100		1.07
H_o −4.8	293	326	0.72	68	1.52	1.02
H_o −4.8	293	412	1.1	100		1.63
pH 1.7	294	393	1.6	100		1.0
pH 6.2	326	393	1.6	100		1.2
pH 7.9	340	390	1.56	97	0.21	1.22
pH 9.0	311	382	1.61	30.8	0.31	0.96
pH 9.6	311	342	1.32	38	0.42	1.15
pH 9.6	311	400	1.49	40	0.30	1.36
pH 11.2	311	365	1.0	72	0.56	1.15
pH 12	311	365	0.86	100		1.19

excited singlet state of the pyridoxamine. However, fluorescence-decay measurements show that, at H_o of − 4.8, the emission lifetimes depend on the emission wavelength (*Table 2*). This indicates that the thermodynamic equilibrium is not attained for the totally protonated species **I** of pyridoxamine in the singlet excited state.

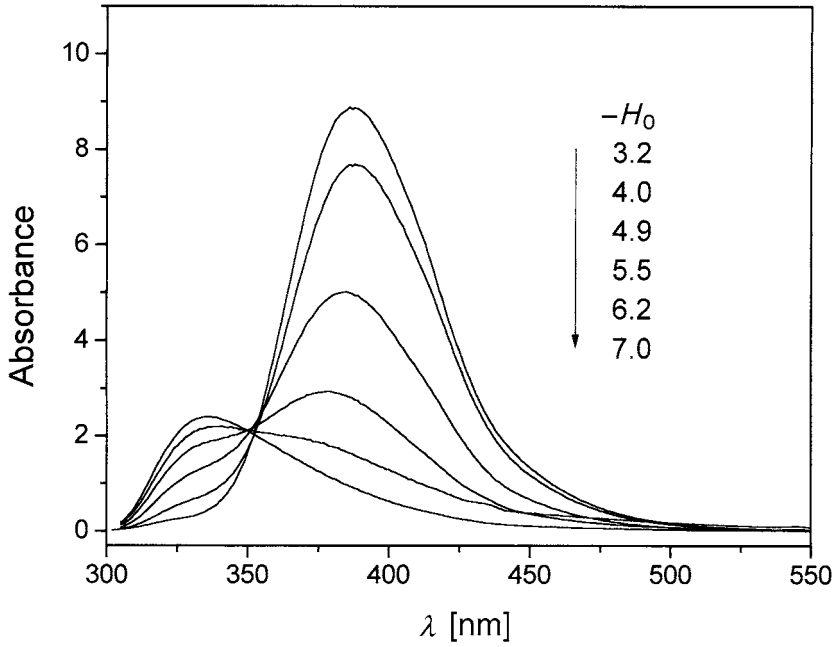


Fig. 2. *Absorption spectra of pyridoxamine in aqueous solutions as function of the* Hammett *acidity* H_o

Excited-state pK^* value was also estimated by *Föster*'s method using the intersection point of absorption and emission spectra, $E_{o,o}$, according to the procedure proposed by *Grabowski* and *Grabowska* [19]. The pK* value so obtained was −4.8, which is in excellent agreement with that obtained by fluorometric titration. Earlier

studies of *Bridges et al.* [8] on hydroxypyridine derivatives indicate that these molecules are very strong acids in the singlet excited state. More recently, *Echeverría et al.* [10] estimated that the phenol group in the pyridoxal 5′-phosphate is by 8.6 pH units more acidic in the excited state. Our results show that pyridoxamine is also a strong acid in the singlet excited state. The favorable deprotonation of the OH group in the excited state is consistent with a shift of electrons from the phenolic O-atom into the aromatic ring subsequent to excitation.

Fluorescence experiments were also carried out in the pH range 6.2–12. *Fig. 3* shows the maximum wavelength and the emission intensity measured at 376 nm as a function of pH. The changes in the fluorescence intensity with pH correspond to those of the ground state. The decrease of the fluorescence intensity in the pH region from 6.2 to 9.5 gave a pK value of 7.9, which is similar to that obtained by absorption titration and corresponds to the deprotonation of the pyridine N-atom. The increase of the emission between pH 9.5 and 12 gave the pK value of the deprotonation of the amine group in the ground state.

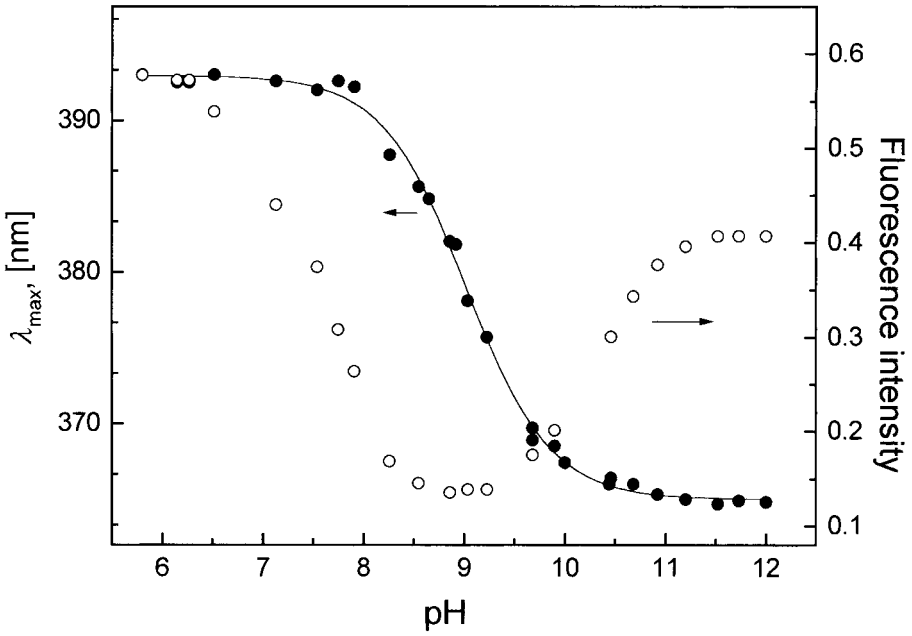


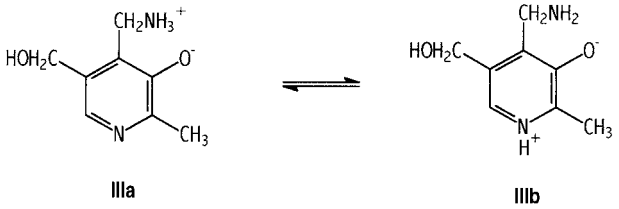
Fig. 3. *Variation of the emission maximum wavelength and fluorescence intensity as function of pH.* (●): Emission maximum wavelength; (○): fluorescence intensity at 376 nm. Excitation wavelength 314 nm.

The position of the fluorescence spectrum exhibits a different behavior (*Fig. 3*). At pH values in the range of 6.2–7.9, the position of the spectrum is unchanged and shows a maximum at 393 nm independent from excitation wavelength, and the fluorescence decay can be fit to a monoexponential decay of 1.6 ns. These observations suggest that the fluorescent species in the pH range 1.7 to 7.9 is **II**. Considering that the ground-state pK value is 7.9, these results suggest that the pyridine N-atom in the lowest singlet excited state becomes more basic in the excited state than in the ground state. This

conclusion is somewhat different from that reached for other compounds of the vitamin B_6 group, since a small decrease in the basicity of the N-atom has been proposed for pyridoxal [10] and *Schiff* bases [9]. However, these differences can be explained in terms of the donor–acceptor properties of the substituents in the 4-position, which should affect the charge density at the pyridine N-atom and, hence, its basicity.

In the pH range from 8 to 10.2, the emission spectrum progressively undergoes a blue shift when the pH is raised (*Fig. 3*). The position of the spectra depends on the excitation wavelength. The emission spectra are red-shifted with excitation at longer wavelengths (*Fig. 4,a*). The excitation spectra depend on the emission wavelength (*Fig. 4,b*). Furthermore, the fluorescence profiles show a biexponential decay (*Table 2*). All these observations indicate the existence of different species in the excited and ground states. These species could be ascribed to the form **II**, and to tautomers **IIIa** and **IIIb** (*Scheme 2*). This tautomeric equilibrium has been previously proposed on the basis of NMR experiments [17] and from the resolution of the absorption spectrum with log-normal curves [5].

Scheme 2



IIIa **IIIb**

Further evidence for the contribution of the tautomeric equilibrium was obtained from experiments carried out with 4-deoxypyridoxamine at pH 10, where only the corresponding species **III** is present. Under this condition, we did not observe changes in the excitation spectrum with emission wavelength. In this molecule, the CH_2NH_2 group at C(4) is replaced by a Me group, avoiding the proton transfer to the pyridine N-atom. The enhanced basicity of the pyridine N-atom due to the deprotonated phenol group could facilitate the proton transfer. The intramolecular proton transfer could also occur in the singlet excited state of pyridoxamine. It is reasonable to expect that the protonation of the pyridine N-atom gives a species, *i.e.*, **IIIb**, absorbing and emitting at a wavelength longer than that of tautomer **IIIa**. The results shown in *Fig. 4* are in agreement with this expectation. Moreover, the value of the longest-lifetime component at pH 9.6 is not that corresponding to species **II** or **IV**, indicating the presence of a new species. Favorable excited-state intramolecular proton transfer has been described in several molecules with acidic or basic moieties that become strong acids or bases in the excited states [20–22].

Fluorescence experiments were also carried out in alkaline solutions (pH 10–12). The fluorescence-lifetime measurements show that the amine deprotonation takes place in the excited state at higher pH. The decay profile at pH 11.2 showed two different lifetimes (*Table 2*), whereas, at pH 12, only one component appears, which

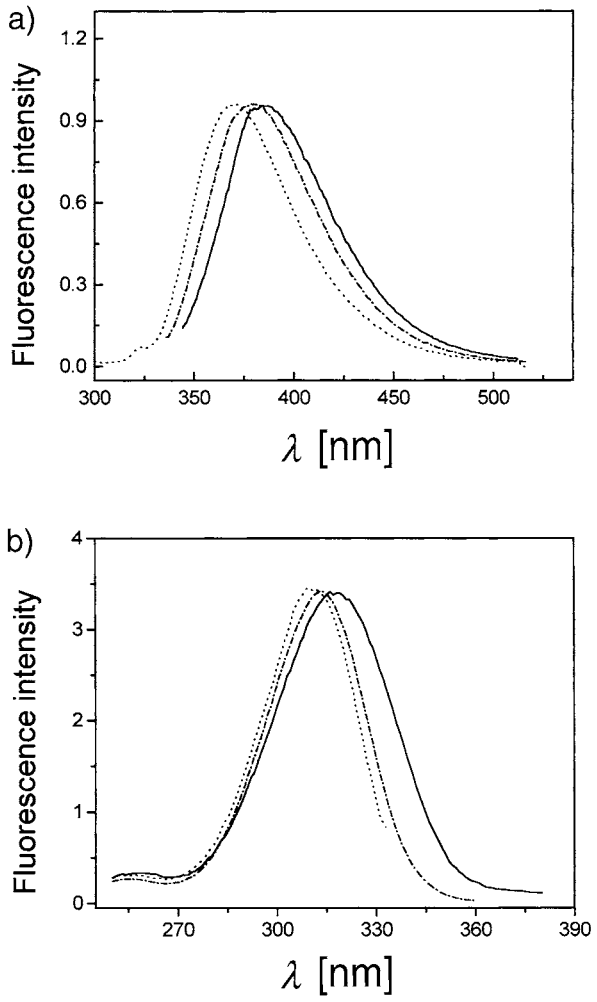


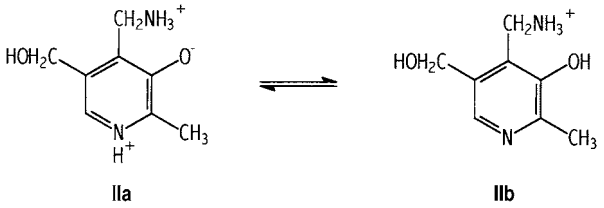
Fig. 4. *Effect of the emission and excitation wavelength on the excitation and emission spectra of pyridoxamine at pH 9.6. a*) Normalized emission spectra with λ_{exc}: (⋯): 292 nm; (-·-·-): 328 nm; (—): 335 nm. *b*) Normalized excitation spectra monitored at λ_{emis}: (⋯): 340 nm; (-·-·-) 350 nm; (—) 415 nm.

can be assigned to species **IV** in the singlet excited state. This suggests that the amine group becomes more basic when pyridoxamine is excited to the singlet state.

Solvent effect on absorption spectra. The absorption spectrum of pyridoxamine was recorded in different solvents, and absorption band maxima are listed in *Table 3*. *Fig. 5* shows absorption spectra in some selected solvents. These spectra show two well-defined bands, with wavelength maxima and relative absorbances highly dependent on the solvent. As reported for other compounds of the vitamin B_6 group [6][7][23], the band at *ca.* 333 nm can be assigned to the cationic form **IIa**, with the deprotonated

phenol group. The band at the short wavelength corresponds to the cationic form **IIb**, resulting from the tautomeric equilibrium shown in *Scheme 3*.

Scheme 3



IIa **IIb**

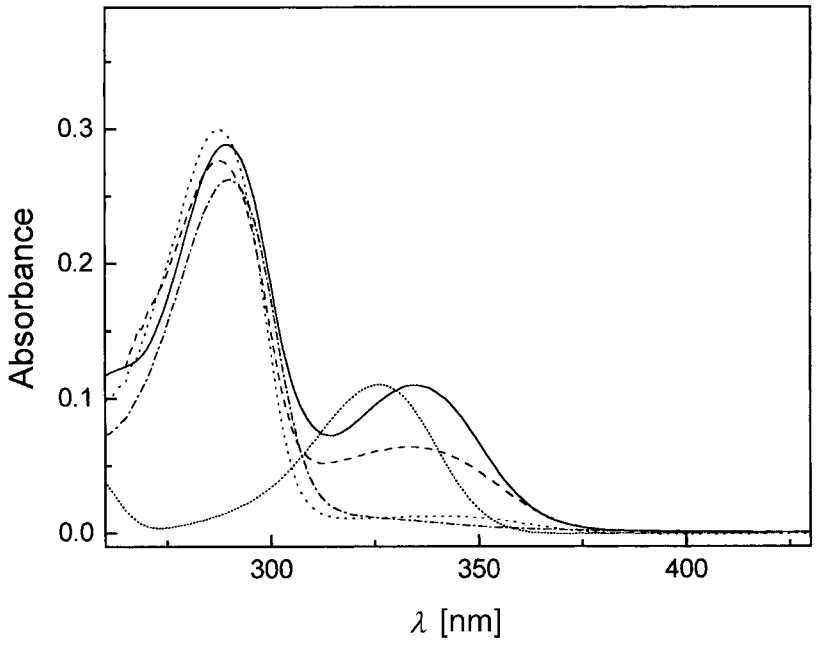


Fig. 5. *Absorption spectra of pyridoxamine in different solvents.* Spectrum in: H_2O (···); EtOH (—); methylformamide (---); DMF (· · ·); 1,4-dioxane (-· – · – ·).

The relative absorption of these bands is highly dependent on the solvent. Thus, in dioxane, an aprotic nonpolar solvent, mainly the short-wavelength band appears, while, in H_2O, only the long-wavelength band is observed. In general, it has been proposed that species **IIa** prevails in polar media, and the **IIb** tautomer prevails in non-polar media [6][7][23]. Since we found that the molar extinction coefficients of the tautomers at the maximum wavelength differ only by 10%, a relative proportion of these species was estimated from the absorbance of one tautomer relative to that of both species. The fraction (f) of the absorbance of the short-wavelength band with respect to that of both bands at their respective maximum is shown in *Table 3*. These data, although they have large errors due to the assumption that the molar extinction

Table 3. *Absorption and Emission Maxima of Pyridoxamine, and Relative Absorbance (f) of Species* **IIb** *in the Ground State*

Solvent	$(\lambda_{max})_{abs}$ [nm]	f[a])	$(\lambda_{max})_{emis}$ [nm]
H_2O (pH 6.2)	326	–	393
MeOH	290, 333	0.68	325, 408
EtOH	290, 336	0.70	325, 409
BuOH	291, 336	0.73	325, 409
Hexanol	291, 336	0.73	325, 409
Octanol	291, 337	0.74	325, 409
t-BuOH	292, 337	0.75	325, 406
2,2,2-Trifluoroethanol	289, 321	0.45	320, 399
Formamide	–	–	404
N-Methylformamide	288, 334	0.80	333, 409
DMF	288, 342	0.96	332, 412
DMSO	288, 345	0.95	332, 411
Dioxane	292, 343	0.98	412

[a]) Estimated errors in f are $\pm 10\%$.

coefficient of the tautomers is independent of the solvent and, due to the superposition of the bands in some of the solvents (*e.g.*, 2,2,2-trifluoroethanol), show that f is not directly correlated with the solvent polarity. Comparing DMSO, DMF, and MeOH, solvents of similar polarity it is observed that, in DMSO and DMF, the band at 288 nm appears almost exclusively, whereas, in MeOH, a protic solvent, the intensities of both bands are comparable. An inspection of the f values in different solvents shows that, in solvents of low acceptor number, a parameter that measures the proton-donor capacity of the solvent [24][25], the species **IIb** is formed almost exclusively. In solvents of intermediate proton-donor capacity, like alcohols and methylformamide, both **IIa** and **IIb** species co-exist. The origin of this behavior must be the solute–solvent intermolecular H-bond. The fact that the proton-donor capacity of the solvent is the main factor governing the tautomeric equilibrium is expected from the stabilization of the negatively charged phenolic O-atom by the H-bond with the solvent, leading to an unfavorable proton transfer from the pyridine N-atom. Also the absorbance of both bands was measured in a series of aliphatic alcohols, from MeOH to octanol. The f values almost do not show changes on moving from MeOH to octanol. This indicates that the H-bond is the dominant factor, even in long-chain alcohols, where the bulk polarity is reduced.

Data in *Table 3* also show that the position of the short-wavelength absorption band is almost unaffected by the solvent, whereas the long-wavelength band is blue-shifted in protic solvents. Due to the presence of oppositely charged groups in the aromatic ring, species **IIa** is expected to be more sensitive to changes in the solvent properties than the species **IIb**, which contains only one positive center at the amine moiety.

Solvent Effect on the Fluorescence Properties. Fig. 6 shows fluorescence spectra of pyridoxamine obtained with excitation at 290 and 340 nm in EtOH and DMF. The spectrum in EtOH with excitation at 290 nm shows two well-resolved bands with maxima at 325 and 409 nm, the intensity being almost two times higher for the long-wavelength band. This band matches fairly well that obtained upon excitation at

340 nm, where mainly the tautomer **IIa** absorbs. If it is considered that the species that prevails at 290 nm is **IIb**, these results indicate that the intramolecular proton transfer occurs also in the singlet excited state. Furthermore, the excitation spectrum of the species emitting at 325 nm gave a maximum at 289 nm, in agreement with the absorption of the species **IIb** in the ground state. The excitation spectrum at 409 nm gave a main band centered at 335 nm with a shoulder in the region of 290 nm. Similar observations are found in other alcohols of different chain lengths (*Table 3*).

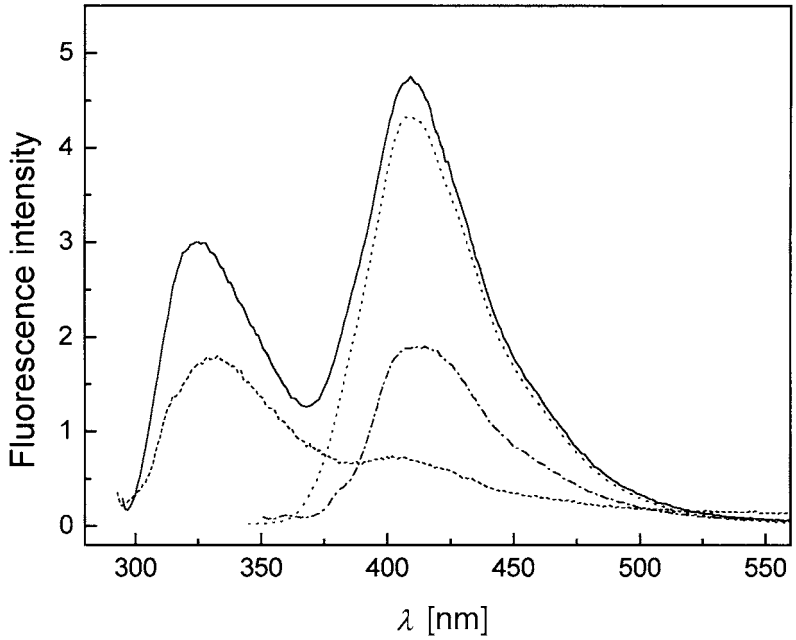


Fig. 6. *Fluorescence spectra of pyridoxamine in EtOH and DMF.* Spectra in EtOH with: (—): exc. 290 nm, (···): exc. 340 nm; in DMF: with (- - -): exc. 288 nm; (-···-): exc. 342 nm.

The fluorescence behavior of pyridoxamine in nonprotic solvents such as DMF is somewhat different. The fluorescence spectrum obtained upon excitation at 288 nm also shows two bands (*Fig. 6*), but the relative proportion of their intensities is different from that described in alcohols. The intensity of the long-wavelength band is much lower than that at short wavelengths. This implies that, in nonprotic solvents, the more favorable species is that with the protonated phenol group, **IIb**, in agreement with the lack of H-bonding capacity of the solvent.

The cationic form **IIb** is a very weakly fluorescent species. The fluorescence quantum yield is 0.006 in EtOH. On the other hand, the species **IIa** presents a fluorescence quantum yield of 0.22. This latter is higher than that measured in H_2O.

To gain further insight on the nature of the singlet excited state, fluorescence lifetimes of pyridoxamine in different solvents was measured (*Table 4*). The fluorescence decay in EtOH, measured at wavelength where mainly the species **IIb** absorbs (290 nm) and emits (325 nm) is very short. Measurements with an emission wavelength of 409 nm gave a monoexponential decay of 1.37 ns, independent of the

excitation in the range of 280–335 nm. Other nonprotic or protic solvents show a similar behavior (*Table 4*). The emission at 325 nm decays with a very short lifetime, and the emission at 409 nm has a longer lifetime, independent of the excitation wavelength.

Table 4. *Fluorescence Decays of Pyridoxamine in Different Solvents Measured at Different Excitation and Emission Wavelengths*

Solvent	λ_{exc} [nm]	λ_{em} [nm]	τ [ns]	χ^2
EtOH	290	325	0.37	1.1
	290	409	1.37	1.03
	335	409	1.37	1.04
BuOH	338	410	1.67	1.16
DMF	288	332	0.23	1.80
	342	412	2.1	1.26
Dioxane	288	409	1.73	1.80

Data in *Table 3* also show that the fluorescence maximum of both bands presents a moderate blue shift in going from nonpolar to polar protic solvents. To obtain information on the properties of the singlet excited state, the wavelength of the emission maximum of the band corresponding to the species **IIa** was correlated with solvent parameters. Spectral shifts do not show linear dependence with the classical orientation polarizability parameter (Δf). This indicates the existence of specific solute–solvent interactions. Then, the position of the maximum was correlated with the multiple-parameter *Kamlet–Taft* equation [26],

$$(\nu)_{flu} = (\nu_o)_{flu} + s\,\pi^* + a\,\alpha + b\,\beta \tag{1}$$

where π^* is the solvent polarity/polarizability, α H-bond donor acidity, and β the H-bond acceptor basicity. The best fit of the data corresponds to:

$$(\nu)_{flu} = 24224 + 377\,\pi^* + 374\alpha - 384\beta \qquad r = 0.966 \tag{2}$$

The positive numeric values of coefficients s and a reflect the decrease in the charge delocalization in the excited state with respect to the ground state. Also, *Eqn. 2* shows that the main parameters that influence the hypsochromic shift of the fluorescence of species **IIa** are the polarity and the H-donor ability of the solvent. Data in *Table 3* indicate that the same parameters would also influence the shift of the maximum emission of the species **IIb**.

Conclusions. – From the results of the effect of solvent properties on absorption and fluorescence maxima, fluorescence quantum yields, and fluorescence lifetimes, it can be concluded that pyridoxamine in the ground and singlet excited states exists in the form **IIa** or **IIb**, depending on the H-donor capacity of the solvent. In low proton-donor solvents, the cationic form with the protonated phenol group is the dominant species, but in high proton-donor solvents both forms exist. The hypsochromic shift of the

fluorescence band corresponding to the species **IIa** indicates higher stabilization of the ground state with respect to the singlet excited state.

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