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Fluorescence of pterin, 6-formylpterin, 6-carboxypterin and folic acid in aqueous solution: pH effects

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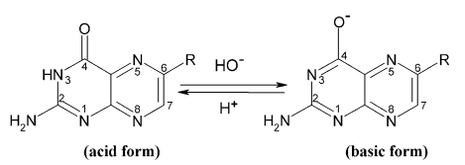
Received 28th February 2002, Accepted 23rd April 2002
First published as an Advance Article on the web 8th May 2002

Steady-state and time-resolved studies have been performed on four compounds of the pterin family (pterin, 6-carboxypterin, 6-formylpterin and folic acid) in aqueous solution, using the single photon counting technique. The fluorescence characteristics (spectra, quantum yields, lifetimes) of these compounds and their dependence on the pH have been investigated. Most pterins can exist in two acid–base forms over the pH range between 3 and 13. Emission spectra and excitation spectra were obtained for both forms of each compound studied. Fluorescence quantum yields (Φ_F) in acidic and basic media were measured. The Φ_F of folic acid (<0.005 in both media) is very low compared to those of pterin (0.27 in basic media and 0.33 in acidic media), 6-carboxypterin (0.18 in basic media and 0.28 in acidic media) and 6-formylpterin (0.07 in basic media and 0.12 in acidic media). The variation in integrated fluorescence intensity and fluorescence lifetimes (τ_F) was analysed as a function of pH. Dynamic quenching by OH^- was observed and the corresponding bimolecular rate constants for quenching of fluorescence (k_q) were calculated. The reported values for k_q (M^{-1} s^{-1}) are 3.6×10^9, $1.9 \times 10^9$and 1.1×10^{10} M^{-1} s^{-1} for pterin, 6-carboxypterin and 6-formylpterin, respectively.

1 Introduction

Pterins are a family of heterocyclic compounds present in biological systems. These compounds are derived from 2-amino-4-hydroxypteridine (pterin, Fig. 1). It is well-known that pterins



R	Compound
-H	Pterin (PT)
-COOH	6-carboxypterin (CPT)
-CHO	6-formylpterin (FPT)
(*)	Folic acid (FA)

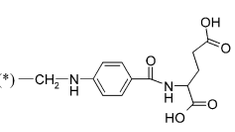


Fig. 1 Molecular structure of the pterins and the acid–base equilibrium in aqueous solution at pH 7–9.

participate in important biological functions,[1,2] *e.g.*, folic acid (pteroylglutamic acid), a conjugated pterin, is a vitamin of the B group and acts as a coenzyme in reactions related to the synthesis of purinic and pyrimidinic bases.[3]

It has been reported that some pterins are involved in different photochemical processes. The sensitivity of pterins to light has been known for several decades[4,5] and they have been found in photosensitive organs, such as the eyes, of different animals.[6] Some reports suggest that pterins may act as blue antennas in superior plants[7] and other organisms such as the fungus *Phycomyses blakesleeanus*.[8] Moreover, it has been suggested that pterins may play some role in photosynthesis.[9] 5,10-Methenyltetrahydrofolylpolyglutamate, a folic acid derivative, is the light-harvesting antenna of DNA photolyases, enzymes involved in DNA repair processes[10–12] that take place after UV irradiation. Recent studies have shown that pterin and some pterin derivatives are instrumental in the photosensitisation of DNA. Along this line, photoinduced cleavage of thymus calf DNA[13] and cleavage of plasmid DNA[14] have been reported.

In spite of the evident importance of pterins in photochemical processes that take place in biological systems, relatively few reports deal with the photochemistry[15–20] and photophysics[21–23] of this family of compounds. More basic studies on these topics are required in order to understand in detail the role of pterins in such photobiological processes.

Pterins behave as weak acids in aqueous solution, where several acid–base equilibria may be present. As reported by Albert[24] for several pterin derivatives, the dominant equilibrium in the pH range 4–12 involves the amide group (acid form) and the phenolate group (base form) (Fig. 1). The pK_a of this equilibrium is around 8 for the various pterin derivatives studied.[19,24–26] Other functional groups of the pterin moiety (*e.g.* 2-amino group or ring nitrogen atoms) have pK_a values lower than 2.[24] The photochemistry, as well as the photophysical behaviour, of the different acid–base forms of each compound presents significant differences, as reported in previous studies by us[18] and other groups.[15,16,21]

In the context of our investigations on pterin derivatives,[14,18,19,25,26] we have performed photophysical studies on a group of four compounds in aqueous solution: pterin (PT), 6-carboxypterin (CPT), 6-formylpterin (FTP) and folic acid (FA) (Fig. 1). We report here results of measurements of steady-state and time-resolved fluorescence and discuss the dependence of the emission and excitation spectra, fluorescence quantum yields (Φ_F) and lifetimes (τ_F) on the pH.

2 Experimental

The pterins (Shircks Laboratories) were used without further purification. pH measurements were performed using a pH-meter Schott CG 843P with a pH-combination electrode Blue-Line 14pH (Schott). The pH of the aqueous solutions was adjusted by adding drops of HCl or NaOH from a micro-pipette. The concentrations of the acid and base used for this purpose ranged from 0.1 to 2 M. For experiments at pH lower than 11 the ionic strength was held constant at 10^{-3} M; for experiments at higher pH the ionic strength was of the same order as the HO^- concentration. UV–visible spectra were recorded on a Cary 5 (Varian) spectrophotometer.

Steady-state and time-resolved fluorescence measurements were performed using single photon counting equipment EAI-FS/FL900 (Edinburgh Analytical Instruments, UK). The quartz measurement cell (1 cm path length) was thermo-regulated at 23.9 ± 0.2 °C. Corrected fluorescence spectra were recorded between 350 and 650 nm at different excitation wavelengths using a high pressure Xe lamp (419 W). The excitation spectra of the compounds studied were recorded between 200 and 500 nm, monitoring the fluorescence intensity at 450 nm.

A N_2 excitation lamp (1.2 bar, operated at 6.3 kV and a frequency of 40 kHz) was employed for time-resolved studies. The single photon counting range of the equipment is 500 ps–500 μs, but the selected counting time window for the measurements reported in this study was 0–100 ns. The emission decays were monitored at 450 nm after excitation at 350 nm. Lifetimes were obtained from the monoexponential decays observed after deconvolution from the lamp background signal, using the software provided by Edinburgh Analytical Instruments. Our method of analysis of steady-state and time-resolved data has previously been described in detail.[27,28]

The fluorescence quantum yields were determined from the corrected fluorescence spectra using quinine bisulfate (Riedel-deHaen) in 0.5 M H_2SO_4 as a reference[29] (Φ_F = 0.546[30]). In order to avoid inner filter effects, the absorptions of the solutions, at the excitation wavelength, were kept below 0.10.

3 Results and discussion

3.1 Absorption and emission spectra

The absorption and fluorescence characteristics of pterin (PT) and three pterin derivatives (CPT, FPT, FA) have been investigated in the pH range 4–13. Under these conditions, the acid–base equilibrium to be considered involves the amide group in the acid form and the phenolate group in the base form[24] (Fig. 1). In the pH ranges 4.9–5.5 and 10.0–10.5, pterins are present at more than 99% in the acid and base forms, respectively. Although not of direct interest for biological systems, a large pH range was chosen for our investigations, because knowledge of the photophysical properties of the "pure" acid and base forms appeared to be of fundamental interest for understanding the photophysics of these compounds under less harsh pH conditions.

The absorption spectra of pterin derivatives are highly sensitive to the pH[18,19,25,31] (Fig. 2, compare solid and dashed–dotted spectra).

It should be noted that the absorption spectra of PT and CPT are similar, both in acidic and alkaline media, suggesting that the electronic distribution on the pterin moiety is only slightly affected by the presence of the –COOH group at position 6. In contrast, interactions between the substituent at position 6 and the pterin moiety affect considerably the absorption spectra of FPT (formyl) and FA (relatively long chain substituent). As can be observed in Fig. 2, a new absorption band centered at 310 nm appears in the spectrum of FPT in its acid form, and the band centered at approximately 350 nm has a higher relative intensity than in the case of PT and CPT, in both acidic and alkaline media. The differences that can be observed in the absorption spectra of FA are even more striking.

The fluorescence emission spectra of the four pterins show a dependence on the pH (Fig. 3). The emission spectra of the base forms obtained by excitation at 350 nm are red shifted in comparison with the spectra of the acid forms obtained by excitation at the same wavelength. The wavelengths of the fluorescence maxima (λ_F) are listed in Table 1.

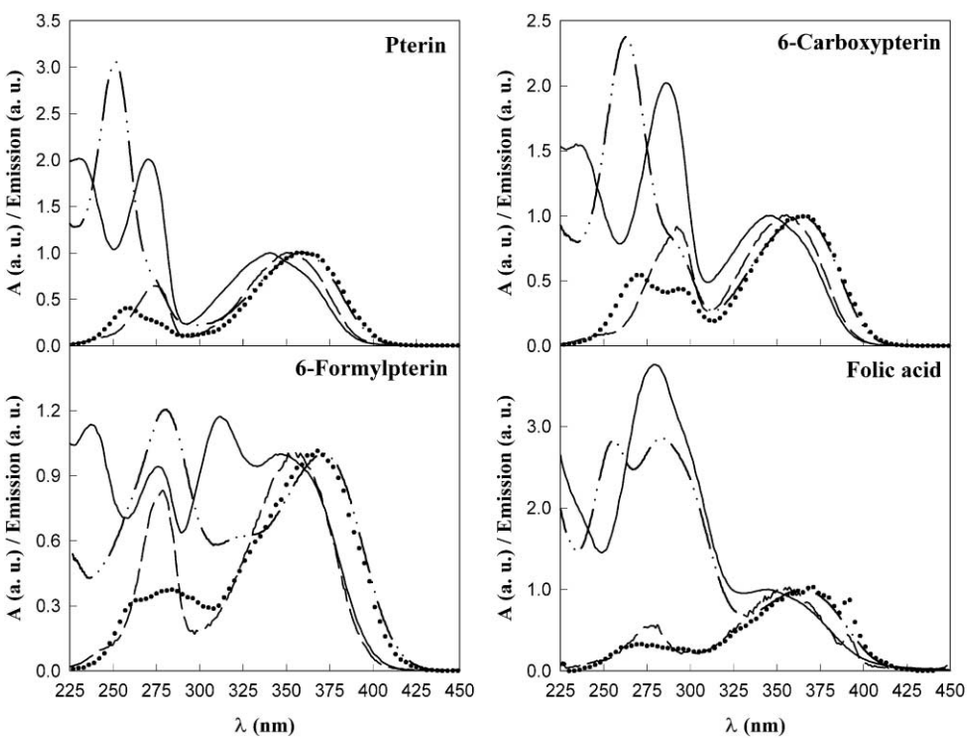


Fig. 2 Absorption and excitation spectra of air-equilibrated aqueous solutions of pterin derivatives; solid lines: absorption spectra of acid forms (pH 5.5); dashed lines: excitation spectra of acid forms; dashed–dotted lines: absorption spectra of base forms (pH 10.5); dotted lines: excitation spectra of base forms (excitation spectra obtained by monitoring the fluorescence at 450 nm; for comparative purposes, each spectrum was normalized relative to the maximum of the lowest energy band).

Table 1 Fluorescence quantum yields (Φ_F) in argon-saturated, air-equilibrated and oxygen-saturated aqueous solutions, wavelengths of fluorescence maxima (λ_F) and fluorescence lifetimes (τ_F) of the pterin derivatives[a]

Compound	Acid–base form	λ_F (±3)/nm	Φ_F (Ar) (±0.01)	Φ_F (air) (±0.01)	Φ_F (O_2) (±0.01)	τ_F (±0.4)/ns
Pterin	Acid	439	0.33	0.32	0.31	7.6
	Base	456	0.27	0.27	0.27	5.0
6-Carboxypterin	Acid	439	0.28	0.26	0.27	5.8
	Base	451	0.18	0.18	0.20	4.1
6-Formylpterin	Acid	446	0.12	0.12	0.12	7.9
	Base	454	0.07	0.07	0.07	2.2
Folic acid	Acid	445	<0.005	<0.005	<0.005	7.0
	Base	455	<0.005	<0.005	<0.005	3.5

[a] Measurements were carried out for the acid and base forms in the pH ranges 4.9–5.5 and 10.0–10.5, respectively (excitation wavelength: 350 nm; standard deviations are indicated in parenthesis).

Fluorescence spectra resulting from excitation at wavelengths shorter than 350 nm were also recorded for each compound in acidic and base solutions (results not shown). Wavelengths typically in the range between 230 and 280 nm were used for exciting the high energy band(s) of the pterins. In all cases, the fluorescence spectrum (normalized relative to the maximum emission value for comparative purposes) remained unchanged, irrespective of the excitation wavelength, suggesting that only one excited state contributes to the fluorescence. However, the fluorescence intensities decreased when exciting in the high energy absorption bands, *i.e.* at wavelengths shorter than 300 nm (for further discussion on this point, see Section 3.2).

3.2 Fluorescence quantum yields

For the four compounds investigated, the fluorescence quantum yields (Φ_F) were determined for both the acid and base forms in argon-saturated, air-equilibrated and oxygen-saturated solutions. The results for excitation at 350 nm are shown in Table 1. For PT and CPT, the values of Φ_F are relatively high, Φ_F(CPT) being slightly lower than Φ_F(PT). It is noteworthy that Φ_F(FPT) is lower by more than a factor of 2, both in acidic and alkaline media, whereas FA has very small Φ_F values (<0.005). Therefore, the nature of the substituent at position 6 on the pterin moiety affects the deactivation pathways of the singlet excited states considerably (as is the case for the absorption spectra, Section 3.1). In particular, the long chain substituent at position 6 on the pterin moiety of FA might act as an "internal quencher", thus enhancing the radiationless deactivation of the singlet excited state. The values of Φ_F for the acid forms of PT, CPT and FPT (0.33, 0.28 and 0.12, respectively) are higher than Φ_F for the corresponding base forms (0.27, 0.18 and 0.07). The differences between the Φ_F values determined in the presence or in the absence of O_2 were not significant, indicating that quenching of the singlet excited states by O_2 is negligible for the four compounds. To our knowledge, Φ_F of pterins have not been reported previously, except in the case of PT in buffered aqueous solution at pH 10.[21] The very low value reported by the authors (0.057) probably results from fluorescence quenching by the buffer components.[32]

The excitation spectra of the compounds studied in acidic and basic air-equilibrated solutions are shown in Fig. 2, together with corresponding absorption spectra. In both series of spectra, the wavelengths of the band maxima are similar. However, the intensities of the high energy bands relative to that of the lowest energy band are much lower in the excitation spectra. Fluorescence quantum yields obtained by exciting at different wavelengths ($\Phi_{F(\lambda)}$) were calculated from the excitation spectra and from $\Phi_{F(350)}$ (Table 1), using the following equation:

$$\Phi_{F(\lambda)} = \Phi_{F(350)} (I_{(\lambda)}/I_{(350)})[(1 - 10^{-A(350)})/(1 - 10^{-A(\lambda)})] \quad (1)$$

where $I_{(350)}$ and $I_{(\lambda)}$ are the emission intensities monitored at 450 nm obtained by exciting at wavelengths 350 nm and λ, respectively; $A_{(350)}$ and $A_{(\lambda)}$ are the corresponding absorbances ($A \leq 0.10$).

Table 2 Fluorescence quantum yields (Φ_F) calculated from analysis of the excitation spectra shown in Fig. 2, using eqn. (1)[a]

Compound	Acid–base form	λ_{an}/nm	Φ_F (±0.01)
Pterin	Acid	270	0.10
	Base	251	0.03
6-Carboxypterin	Acid	286	0.04
	Base	263	0.04
6-Formylpterin	Acid	276	0.10
		312	0.03
	Base	281	0.02

[a] Measurements for the acid and base forms were carried out in the pH range 4.9–5.1 and at pH 10.0, respectively [fluorescence monitoring wavelengths (λ_{an}) corresponded to the maxima of the high energy bands].

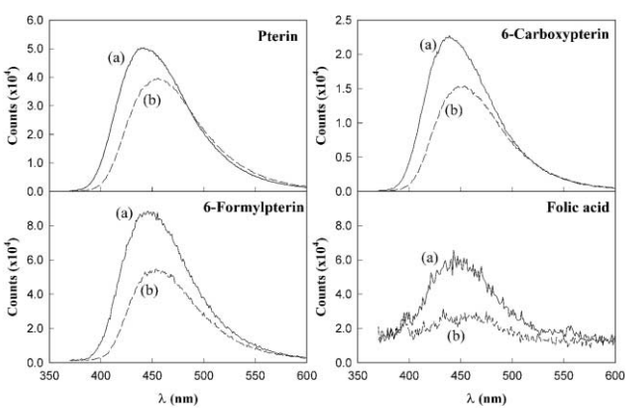


Fig. 3 Corrected fluorescence spectra of the acid forms (a) and of the base forms (b) obtained by excitation at 350 nm (spectra were recorded using solutions of equal absorbance (<0.12) at the excitation wavelength).

Fluorescence quantum yields calculated from the analysis of the excitation spectra, using eqn. (1), are listed in Table 2. The values of Φ_F for the high energy bands are lower by a factor of at least 3 than those corresponding to the low energy bands (Tables 1 and 2). These results suggest that only a fraction of the energy of the upper excited state(s) (S_2, S_n) is dissipated through internal conversion to the lowest singlet excited state (S_1). Therefore, intersystem crossing to the triplet manifold or photochemical reactivity should occur from an upper singlet excited state.

3.3 Influence of the pH on fluorescence emission

Fig. 4 shows the variation of the integrated fluorescence intensities as a function of pH in the range 4–10.5. The behavior observed is due to the previously mentioned equilibria between the acid and the base forms (Fig. 1). The pK_a values for these equilibria were determined from the changes in fluorescence

Table 3 Values of K_a and pK_a for the acid–base equilibrium of the pterins shown in Fig. 1 [a]

Compound	K_a [b]	pK_a [b]	K_a [c]	pK_a [c]
Pterin	$(1.23 \pm 0.06) \times 10^{-8}$	7.9	$(1.0 \pm 0.2) \times 10^{-8}$	8.0
6-Carboxypterin	$(1.3 \pm 0.1) \times 10^{-8}$	7.9	$(1.8 \pm 0.4) \times 10^{-8}$	7.7
6-Formylpterin	$(4.7 \pm 0.2) \times 10^{-8}$	7.3	$(9.8 \pm 2.0) \times 10^{-8}$	7.0
Folic acid	$(8.6 \pm 0.1) \times 10^{-9}$	8.1		

[a] Standard deviations are indicated in parenthesis. [b] Literature values obtained from spectrophotometric titration.[19,25,26] [c] Values obtained in this work by fitting the integrated experimental fluorescence intensities to eqn. (2).

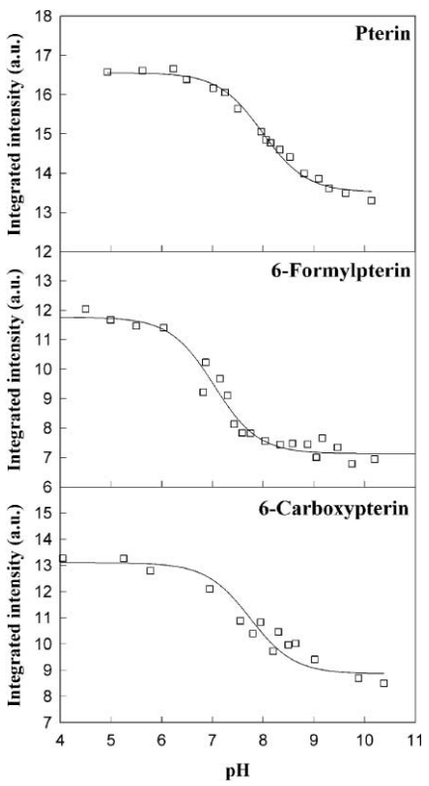


Fig. 4 Variation of the integrated fluorescence intensities corrected for absorbance (squares) as a function of pH (excitation wavelength: 350 nm; fluorescence intensities calculated by fitting the experimental data to eqn. (2) are shown as a solid line).

intensities (I_F) integrated between 360 and 650 nm and corrected for absorbance. The experimental variation of I_F as a function of pH at a given excitation wavelength could be fitted by eqn. (2),

$$I_F = I_a + (I_b - I_a)\,[K_a/(K_a + [H^+])] \qquad (2)$$

where, I_a and I_b are the integrated fluorescence intensities of the acid and base forms of the species involved in the acid–base equilibrium and K_a is the dissociation constant.

The corresponding fit for FA could not be carried out due to the very low fluorescence of this compound and the large experimental error in the titration. The pK_a values determined in this work, as well as literature values obtained from spectrophotometric titration,[19,25,26] are listed in Table 3. No significant difference can be observed between either group of experimental data for PT and CPT. Two explanations may be suggested: 1) the pK_a of the equilibria between the excited singlet states are the same as the corresponding pK_a of the ground states; 2) deactivation of the excited singlet state of the acid (base) form is much faster than its deprotonation (protonation). In the case of FPT, however, the observed difference between the pK_a values strongly suggests the existence of an acid–base equilibrium in the excited state, resulting in more acidic behavior than the ground state.

In all the cases studied, a strong decrease of the fluorescence intensity upon excitation at 350 nm was observed at a pH value higher than 11, although the wavelength of the emission maximum was not altered. The decrease of the fluorescence intensity as a function of the concentration of HO^- followed Stern–Volmer behavior (Fig. 5a). The corresponding Stern–Volmer constants (K_{SV}) (Table 4) were compared to the K_{SV} values obtained by analysis of the lifetime dependence on the concentration of HO^- (Section 3.4).

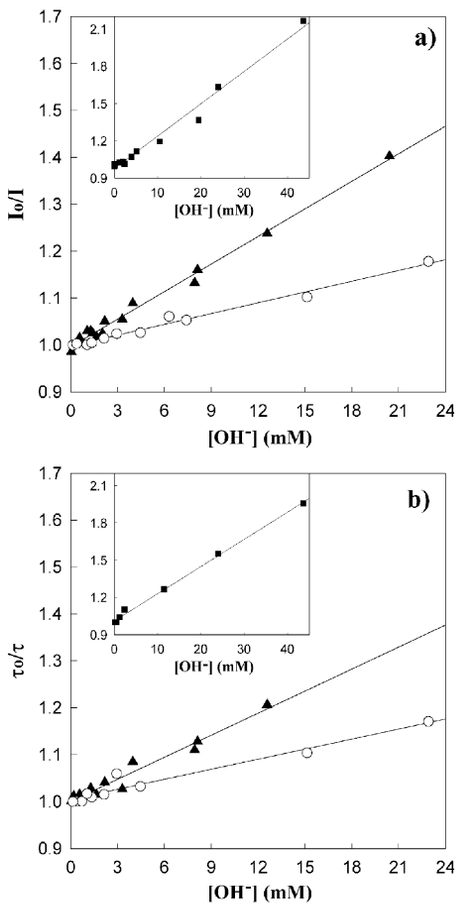


Fig. 5 Quenching of the fluorescence of pterin derivatives by HO^- (■: FPT, ▲: PT and ○: CPT): a) Stern–Volmer plots of the integrated fluorescence intensities of the base forms and b) Stern–Volmer plots of the fluorescence lifetimes (excitation wavelength: 350 nm; lifetimes were determined by monitoring the emission at 450 nm).

3.4 Time-resolved study

Fluorescence decays were analyzed for both the acid and base forms of the compounds studied. A first-order rate law was observed for all the decays. A typical trace recorded for FPT is shown in Fig. 6. Fluorescence lifetimes (τ_F) were obtained by averaging at least three values taken in the pH range 3.0–6.2 (for the acid forms) and in the pH range 9.0–11.0 (for the base forms). The results are shown in Table 1.

Table 4 Stern–Volmer analysis of the fluorescence quenching of the base forms of the pterins by HO^-

Compound	K_{SV} (±1)/M^{-1} (fluorescence intensity)	K_{SV} (±1)/M^{-1} (lifetimes)	k_q (±0.1)/M^{-1} s^{-1}
Pterin	20	16	3.6×10^9
6-Carboxypterin	7.7	8.1	1.9×10^9
6-Formylpterin	26	23	1.1×10^{10}

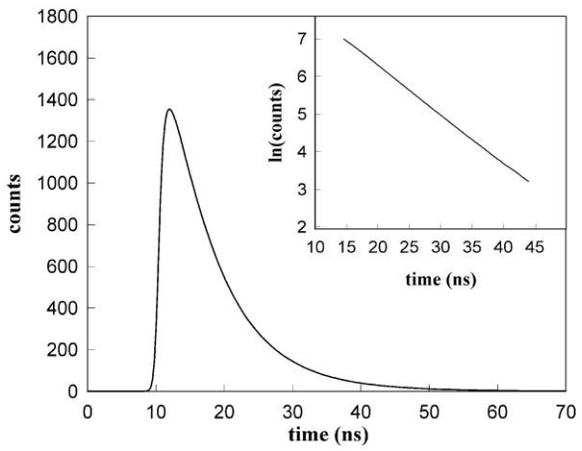


Fig. 6 Fluorescence decay of FPT in aqueous solution (pH 6.0; excitation wavelength: 350 nm, monitoring wavelength: 450 nm).

The fluorescence lifetimes of the base forms also decreased at pH values higher than 11. The dependence of this parameter on the HO^- concentration showed Stern–Volmer behavior. The corresponding K_{SV} (Table 4) are comparable to those obtained from fluorescence intensity measurements. These results reveal that quenching of pterin fluorescence by HO^- is a purely dynamic quenching. Knowledge of the K_{SV} and τ_F values permitted calculation of the bimolecular rate constants for the quenching of fluorescence by HO^- (k_q). An average of the values obtained from plots of I_{Fo}/I_F *vs.* HO^- and of τ_{Fo}/τ_F *vs.* HO^- was used for K_{SV}. These results are also shown in Table 4. The values of k_q decrease in the order k_q(FPT) > k_q(PT) > k_q(CPT).

Assuming that no acid–base equilibrium occurs in the excited state (Section 3.3), the dependence of the fluorescence lifetimes on the pH in the range 4–13 results from the reaction scheme shown in Fig. 7.

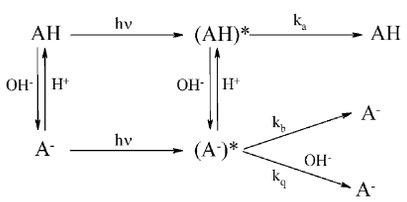


Fig. 7 Simplified reaction scheme for the interpretation of the influence of the pH on the fluorescence of pterins (k_a and k_b: rate constants of fluorescence decay for the acid and base forms, respectively).

If the steady-state hypothesis is applied to the excited species, the following expression relating τ_F to $[H^+]$ can be deduced:

$$1/\tau_F = \{(k_a[H^+] + k_bK_a)/([H^+] + K_a)\} + \{k_qK_w/[H^+]\} \quad (3)$$

where k_a and k_b are the rate constants of fluorescence decay for the acid and base forms, respectively; the other parameters have the same meaning as previously defined.

Since all the constants in eqn. (3) have been determined for PT, FPT and CPT (Tables 1, 3 and 4), the evolution of $1/\tau_F$ as a function of pH may be predicted using this equation. The experimental and predicted values for $1/\tau_F$ are in good agreement, as shown in Fig. 8. Therefore, this result supports the mechanism proposed in Fig. 7.

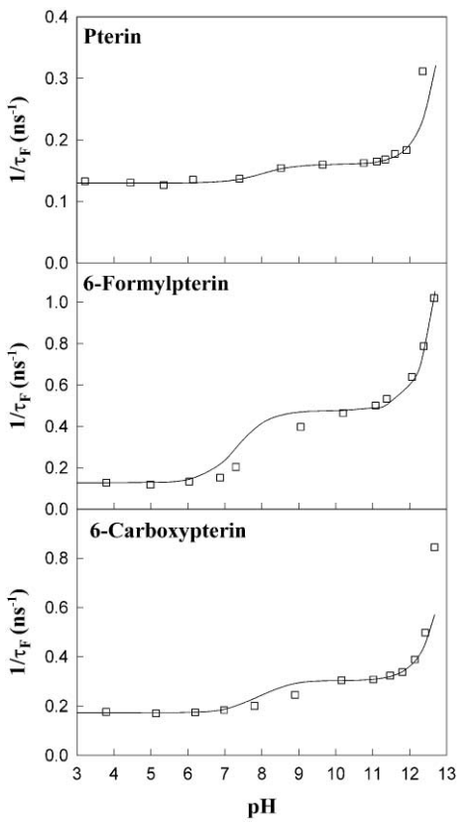


Fig. 8 Experimental variation of $1/\tau_F$ as a function of pH (□) and predicted values calculated using eqn. (3) (continuous line).

4 Conclusions

The fluorescence properties of pterin (PT), 6-carboxypterin (CPT), 6-formylpterin (FTP) and folic acid (FA) have been studied in aqueous solution over the pH range 4–13. Under these conditions, the pterins participate in an acid–base equilibrium that involves the amide group (acid form) and the phenolate group (base form).

The fluorescence emission spectra of the four pterins showed a dependence on the pH, the emission spectra of the base forms being red shifted in comparison with the spectra of the acid forms. The normalized emission spectra remained unchanged, irrespective of the excitation wavelength, suggesting that only the lowest excited singlet state contributes to the fluorescence. The fluorescence quantum yields (Φ_F), however, depend on the excitation wavelength, decreasing at shorter wavelengths. Values of Φ_F (determined by exciting in the absorption band of lowest energy) and lifetimes (τ_F) for the acid forms were higher than the corresponding values for the base forms, and were considerably affected by the nature of the substituent at position 6 on the pterin moiety. In particular, FA showed very small Φ_F values suggesting that its relatively long chain substituent might act as an "internal quencher", enhancing the radiationless deactivation of the singlet excited state.

No significant differences were observed between the pK_a values of PT and CPT as determined from the changes in integrated fluorescence intensities and those obtained from spectrophotometric titration, suggesting that the pK_a values of the equilibria between the excited singlet states are the same as

the corresponding pK_a values of the ground states, or that deactivation of the excited singlet state of the acid (base) form is much faster than its deprotonation (protonation).

Above pH 11, the fluorescence of the pterins was efficiently quenched by hydroxide ions (HO^-). The Stern–Volmer quenching constants (K_{SV}) obtained from the analyses of fluorescence lifetimes and intensities were comparable showing that quenching of pterin fluorescence by HO^- is a dynamic process. The differences in the values of the bimolecular quenching rate constants k_q (Table 4) may be related to the different size and charge of the molecules. In fact, the rate constant for a diffusion-controlled process for charged species A^{Z_A} and B^{Z_B}, with diffusion coefficients D_A and D_B and apparent molecular radii r_A and r_B, can be expressed as[33]:

$$k/\mathrm{M}^{-1}\,\mathrm{s}^{-1} = -4\pi(D_{AB})Z_AZ_BN_0r_0/(1000[1 - \exp(Z_AZ_Br_0/R)]) \quad (4)$$

with $D_{AB} = D_A + D_B$, $R = r_A + r_B$ and $r_0 = 7.1 \times 10^{-10}$ m.

QSAR calculations allow the estimation of the size of the molecules investigated using the semi-empirical AM1 method: thus, we calculated value of 452.7, 519.2 and 509.2 Å^3 for PT, FPT and CPT, respectively. The apparent size of the HO^- anion was assumed to remain constant. Therefore, for a diffusion-controlled process, a lower size should lead to a smaller second-order quenching rate constant if the ionic species have identical charges. As PT has a smaller volume than FPT and the same charge of −1, it is expected to show a lower k_q value than FPT, as observed. In the case of colliding species having similar sizes, the charge difference becomes the dominant factor. Therefore, the k_q value for CPT, which has a formal charge of −2, is expected to be lower than that observed for the quenching of monovalent anions by HO^-.

Acknowledgements

This work was supported in part by a project grant from Agencia de Promoción Científica y Tecnológica de Argentina (ANPCyT Grant No. PICT 06-03531). A. H. T. wishes to thank Consejo Nacional de Investigaciones Cientificas y Tecnológicas de Argentina (CONICET) for a research graduate grant and Universidad Nacional de La Plata for a travel grant to Karlsruhe, Germany. C. L. wishes to thank Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC) for a research graduate grant. A. L. C, A. M. B. and E. O. are grateful to SETCiT (Argentina) and BMFB (Bundesministerium für Bildung und Forschung, Germany) for their support of the current cooperation program.

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