pH-DEPENDENT FLUORESCENCE SPECTROSCOPY XVII: FIRST EXCITED SINGLET STATE DISSOCIATION CONSTANTS, PHOTOTAUTOMERISM AND DUAL FLUORESCENCE OF FLAVONOL

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Summary

3-hydroxy-2-phenyl-4*H*-1-benzopyran-4-one (flavonol) fluoresces from four excited state species, the anion A, the neutral molecule N, the cation C and a phototautomer. Two excited state pK_a values governing the A–N–C equilibria were estimated using the Förster–Weller equation. The pK_a^1 obtained by fluorescence titration is in poor agreement with the pK_a^1 obtained from the Förster cycle but is in good agreement with the pK_a^1 of the ground state, thus indicating deactivation prior to prototropic equilibration of the excited state. A pK_a^2 could not be obtained from the fluorescence titration curve which was flat and featureless.

Fluorescence quantum yields in alkaline, neutral and acidic solutions were found to be low, but intersystem crossing did not appear to be a major competitive deactivation path for the S_1 state owing to the lack of phosphorescence. The long wavelength (green) emission of flavonol in neutral aqueous and organic solvents is shown to result from an excited state intramolecular phototautomer. This conclusion is based on the concentration independence of the relative band intensities, the results of IR studies and the kinetic deuteration effects in neutral and acid solutions. These effects are discussed in terms of proton–deuteron transfer rates in the first excited singlet state.

1. Introduction

As part of our work on the pH-dependent fluorescence properties of natural products [1] we have examined [2] 3-hydroxy-2-phenyl-4*H*-1-benzopyran-4-one (3-hydroxyflavone), to be referred to here as flavonol (Fig. 1), which is the parent compound of a variety of natural products [3, 4], many of which are glycosidated [4, 5]. Flavonols are characterized by a green or yellow fluorescence which distinguishes them from other flavones [6]. Flavonol has been used as a fluorimetric reagent for the determination of certain metal ions [7]. The appearance of its bright green fluorescence

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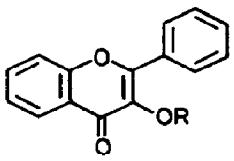

Fig. 1. Structural formula: 1, R ≡ H (flavonol); 2, R ≡ CH_3.

after the enzymatic hydrolysis of non-fluorescent flavone-3-diphosphate has been used for assays of both acidic and alkaline phosphatase [8].

The fluorescence properties of flavonol in organic solvents [9 - 11] and on cellulose plates [12] have been reported. In each case an unusual long wavelength emission was noted. Sengupta and Kasha [13] suggested that an intramolecular excited state tautomerization together with a rotational barrier of the phenyl ring was responsible for the large Stokes shift. Consequently, Salman and Drickamer [14] have studied the viscosity dependence of the flavonol fluorescence, whilst Woolfe and Thistlethwaite [15] have followed the kinetics of the proton transfer by picosecond techniques.

In this paper we present the results of emission spectroscopic studies in aqueous solutions of various acidities which were performed in order to obtain more insight into the prototropic dynamics of the excited state. The results should also be of significance with respect to fluorimetric detection and to the quantitative determination of flavonol-derived natural products.

2. Experimental section

2.1. Materials

Flavonol (1) was obtained from Eastman Kodak (Rochester). 3-methoxyflavone (2) was prepared by reacting 1.2 g (5 mmol) of 1 with 0.7 g (5 mmol) of methyl iodide and 0.5 g of K_2CO_3 in 25 ml of boiling acetone. After 14 h the colourless solution was filtered and concentrated. The residue was recrystallized twice from methanol to give colourless 2 with a melting point of 114 - 115 °C (literature value, 112 - 114 °C) [16]. Both compounds were crystallized repeatedly from a benzene–methanol solution to remove impurities which were detectable by fluorescence spectroscopy and thin layer chromatography.

The solvents were of the best available quality (Merck, spectroscopic grade). D_2O (purity, 99.8 at.%), D_2SO_4 (purity, 5 at.%) and methanol-d_4 (purity, 99.5 at.%) were obtained from Aldrich (Beerse, Belgium).

2.2. Absorption and emission spectra

The absorption spectra were measured using a Zeiss PMQ II spectrophotometer at room temperature. The uncorrected fluorescence spectra obtained from freshly prepared non-degassed solutions in rectangular cells were scanned at 25 °C using an Aminco SPF 500 spectrofluorometer. The digital readout of the spectrofluorometer was processed using a Hewlett–Packard

9815 A computer and software provided by the American Instrument Company. In the case of very dilute solutions the blank solvent spectrum was subtracted from the sample spectrum. The computer was used to convert the wavelength linear readout of the instrument into a wavenumber linear readout. The integral of the curves thus obtained was used to determine the fluorescence quantum yields relative to quinine sulphate in 1 M H_2SO_4 (ϕ_f = 0.546 [17]).

The $pK_a(S_1)$ values, which were determined from the inflection points of the titration curves, were obtained from plots of the fluorescence intensity at several wavelengths in the spectral range of maximum change *versus* the acidity (pH or H_0 where H_0 is the Hammett acidity [18]). The excitation was performed at the isosbestic wavelength of absorption to ensure constant light absorption.

3. Results

3.1. Absorption and emission

The absorption and emission spectra of 1 at pH 7, pH 10 and pH 13 are shown in Fig. 2. Figure 3 shows the spectra obtained at various concentrations of H_2SO_4. The data are given in Table 1.

Three species are evident in the absorption spectra which show maxima at 402 nm (pH > 10), 342 nm (pH 9 - 1) and 378 nm (H_0 from −3 to −8). Two isosbestic points appear in the long wave region of the absorption spectrum, one at 366 nm for pH 13 - 5 and the other at 349 nm for pH 7 to H_0 −5.8. At H_2SO_4 concentrations of more than 70% ($-H_0 > 5.8$) deviations of the absorption spectra from the isosbestic points are observed.

In contrast, four distinct fluorescence bands are observed in the fluorescence spectra when the acidity of the solution is increased stepwise from pH 13 to H_0 −7.4. These bands have maxima at 508, 406, 514 and 430 nm.

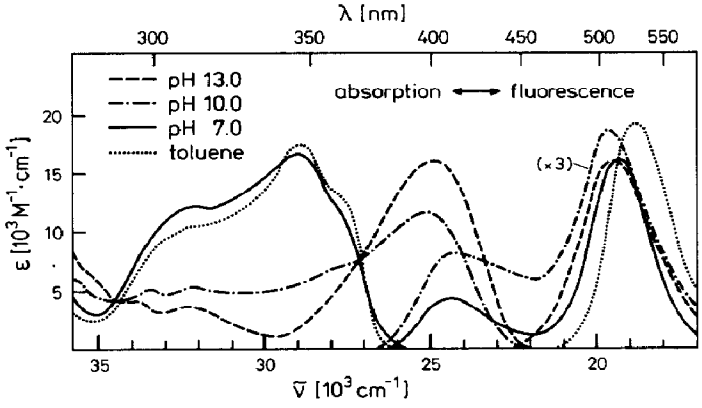

Fig. 2. Absorption and fluorescence spectra of flavonol in aqueous solutions of pH 7.0, pH 10.0 and pH 13.0 and in toluene. The flavonol concentrations were 29.3 μM in water and 18.0 μM in toluene.

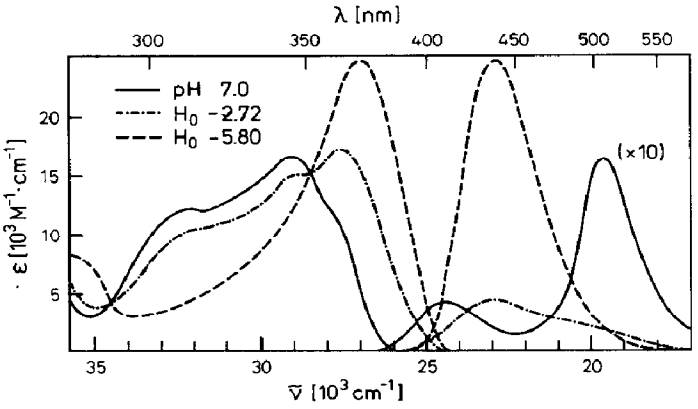

Fig. 3. Absorption and fluorescence spectra of flavonol in neutral water and in various H_2SO_4 concentrations. The flavonol concentration was 25.5 μM.

TABLE 1

Long wavelength UV absorption, excitation and fluorescence data for flavonol in aqueous solutions of different acidities and in organic solvents

Solvent	λ_{max}^{abs} (nm)	ϵ (M^{-1} cm^{-1})	λ_{max}^{exc} (nm)	λ_{max}^{fl} (nm)
Aqueous solvent				
pH 13	402	15640	402	508
pH 10	402	11670	400[a], 348	408, 509
pH 7	342	16880	342, 310[b]	409, 514
pH 3	342	16880	342, 310[b]	406, 514
pH 0	344	17100	342, 312[b]	406, 440[c], 510
H_0 −0.31	345	17320	342, 314	510.5
H_0 −1.72	359.5	18270	355	430
H_0 −3.38	373	24870	380	430
H_0 −5.80	378	25150	380	430
Organic solvents				
Ethanol (95%)	344, 352[d]	16350	350, 310	405, 531
Methanol	345, 354[d]	15600	344.5, 308	409, 531
Diethyl ether	342, 355[c]	17205	342, 358[c], 308	401, 532
Toluene	345, 357[c]	16560	357[c], 344, 310	530
Cyclohexane	340, 352	16940, 13440	352, 342, 308	527

Flavonol concentration, 16.0 - 32.0 μM; the ratio of the intensity of the green band to that of the violet band is 3.6 in water (pH 7), 3.4 in 95% ethanol, about 10 in ether and infinity in toluene and cyclohexane.

[a] For the 509 nm emission only.

[b] Excitation maxima for both the violet and the green fluorescence band.

[c] Shoulder.

[d] Inflection.

In both strongly alkaline and strongly acidic solutions only one band is observed. Two fluorescence bands are observed in the pH 10 to H_0 −0.31 range as well as in ethanol and methanol. Their excitation spectra coincide, indicating that they originate from the same ground state species and that there are two pathways for deactivation. The ratio of the intensity of the blue band (λ_{max}^{fl} = 406 nm) to that of the green band (λ_{max}^{fl} = 514 nm) is dependent upon the solvent (Table 1). In pure hydrocarbon solvents only the green band is observed. In order to facilitate the assignment of the fluorescence bands of neutral flavonol we also measured the fluorescence and excitation spectra of 2 in methanol. However, this compound was found to be extremely photosensitive. Even in 2.5 mM solution only a weak fluorescence (λ_{max}^{fl} = 394 nm) was observed with λ_{max}^{exc} = 330 nm. After a few minutes this band was masked by a new band located at 430 nm (λ_{max}^{exc} = 333 nm) whose intensity steadily increased and which may have been the emission from the photoproduct of 2 [19].

3.2. First excited singlet state pK_a values

The direction of the pK_a shifts in the excited state was estimated using the Förster–Weller equation [20, 21]:

$$pK_a(S_1) = pK_a(S_0) - \frac{0.625}{T}(\tilde{\nu}_{BH} - \tilde{\nu}_B)$$

In the calculations the mean frequencies of the absorption and fluorescence maxima were assumed to be those of the 0–0 transitions. The results (Table 2) show that the acidity of the hydroxy group of flavonol in the S_1 state in-

TABLE 2

Ground state and first excited singlet state pK_as of flavonol and its conjugate acid at 25 °C

Flavonol species	$pK_a(S_0)$	*Förster–Weller calculations*				pK_a *by fluorescence titration*
		λ_{max}^{abs}	λ_{max}^{fl}	$\tilde{\nu}$ [a]	$pK_a(S_1)$	
Anion		24876	19685	22280		
Neutral molecule	9.6[b]	29240	24631[c]	26935	−0.16	9.8 ± 0.4
Cation	−2.88[d]	26455	23256	24855	+1.48[e]	—[f]

[a] $\tilde{\nu}$ is the frequency of the 0–0 transition.
[b] Value reported by Tyukavkina and Pogodaeva [22]. The following values have also been reported: 10.25 [23], 10.34 (in 50% dioxane) [24], and 9.53 and 9.60 [25].
[c] The 409 nm band was used for the calculations rather than the 514 nm phototautomer band.
[d] Value reported by Tyukavkina and Pogodaeva [26].
[e] A value of −3.48 is reported in ref. 27. Such a low excited state basicity would prevent phototautomerization.
[f] No reasonable pK_a value could be extracted from the unusually flat titration curve obtained in strong sulphuric acid.

creases by about 9.8 exponential units and that the basicity of the carbonyl group increases by about 4.4 exponential units. Frolov *et al.* [27] have calculated $pK_a(S_1)$ for the flavonol conjugate acid using the Förster–Weller equation. Apart from an incorrect value for the ground state pK_a (ref. 8 of their paper gives a pK_a value of −2.88 rather than −2.70) they used the long wave (phototautomer) emission for the determination of the 0–0 transitions. This leads to the unusual prediction that S_1 state flavonol should be a weaker base than ground state flavonol. No such decrease in basicity has been observed in the many carbonyl compounds investigated to date [28]. Such a low excited state basicity would prevent phototautomerization.

The value of pK_a^1 obtained from the fluorescence titration curves does not agree with the value obtained from the Förster cycle but is identical, within experimental error, with the ground state pK_a^1. We were unable to determine a reasonable value of $pK_a^2(S_1)$ from the plot of the fluorescence intensity at 430 nm *versus* the Hammett acidity because the curve obtained was flat and featureless even though radiation of the isosbestic absorption wavelength (349 nm) was used for excitation. Similar curves were obtained when the fluorescence excitation was measured at wavelengths near the isosbestic point. Thus this flat curve cannot be due to a lack of correspondence between the spectral responses of the photometer and the fluorometer. The spectra obtained in 24% H_2SO_4 are entirely different (Fig. 4). Emission is now observed from the phototautomer [13, 15] and the cation. The blue emission of the cation is enhanced in D_2SO_4 solutions.

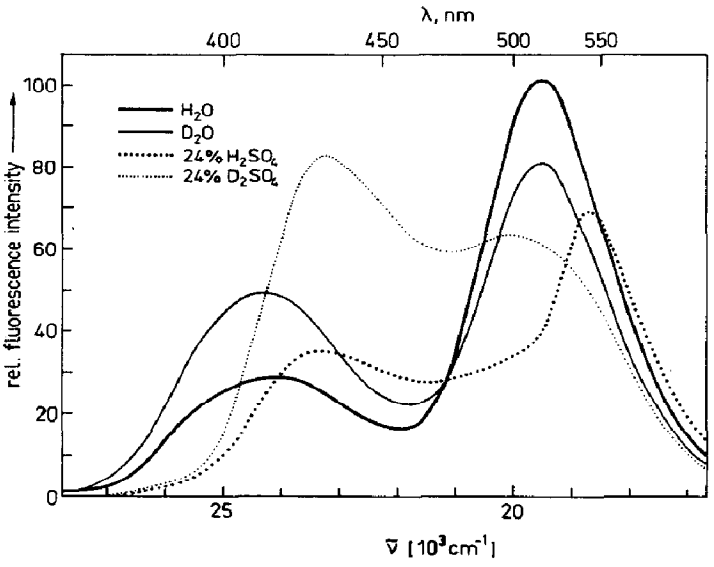

Fig. 4. The effects of deuteration on the fluorescence spectra of flavonol in pure water and in 24% H_2SO_4 (H_0 — 1.29).

3.3. Fluorescence quantum yields

The fluorescence quantum yields ϕ_f are strongly dependent on the acidity. The values obtained from alkaline, neutral and acidic solutions are listed in Table 3. They were rather low in the alkaline solution but were much higher in H_2SO_4. This result, together with similar observations for other hydroxyflavones [29], is fundamentally different from the behaviour of the hydroxycoumarines whose fluorescence intensity is known to be highest in alkaline solutions [30, 31].

The relatively low fluorescence quantum yields led us to look for other deactivation pathways. As has been demonstrated elsewhere [29], prototropic processes play an important role in the excited state dynamics of hydroxyflavones, giving rise to adiabatic and diabatic reactions. The rates of these processes are usually slowed down by changing from protic to deuterated solvents. Indeed, the shapes of the fluorescence spectra of flavonol in deuterated protic solvents differ strongly from their shapes in aqueous solutions. On changing from an aqueous to a D_2O solution, the intensity of the 514 nm band is reduced in favour of the 406 nm band (Fig. 4). The shapes of the spectra obtained in 9% H_2SO_4 and 9% D_2SO_4 are similar to those obtained in neutral solutions except that the ratios of the intensities of the green bands to those of the violet bands are shifted in favour of the green bands, probably because of the increased availability of the protons required for tautomerization. Another deactivation pathway may be via the triplet state. However, unlike other flavones [32], flavonol appears not to phosphoresce at liquid air temperature in ethanol [10]. Hermann [33], using flash photolysis, has recently detected a weak transient absorption by a flavonol triplet. The transient showed an absorption maximum at about 405 nm and decayed with a first-order rate constant of $10^4\ s^{-1}$ at $-150\ °C$ in ether–isopentane–ethanol solution.

TABLE 3

Fluorescence quantum yields ϕ_f of flavonol in aqueous solutions

Solvent	ϕ_f	*Excitation wavelength* (nm)
Borate–NaOH buffer (pH 11.0)[a]	0.0029 ± 0.0003	305, 308, 311, 314, 317, 320
Phosphate buffer (pH 7.0)[a]	0.0046 ± 0.0003	335, 338, 342, 345, 348, 351
60% H_2SO_4 (H_0 −4.46)[b]	0.039 ± 0.002	351, 354, 357, 360, 363, 366, 369, 372

[a] Containing 10% methanol.
[b] Corrected for the refractive index.

4. Discussion

4.1. Absorption and emission spectra

The absorption spectra indicate the existence of three flavonol ground state species in aqueous solutions, namely the anion A, the neutral molecule N and the cation C. Their respective equilibria are governed by the ground state pK_a (Table 2 and Fig. 5). Three of the four excited state species evident in the fluorescence spectra can be assigned to these species: λ_{max}^{fl} = 508 nm is assigned to A, λ_{max}^{fl} = 406 nm is assigned to N and λ_{max} = 430 nm is assigned to C. The assignment of the neutral flavonol fluorescence band was facilitated by comparison with the spectrum of 2. The green emission at 514 nm has been ascribed [13, 15] to an intramolecular phototautomer whose resonance structures are shown in Fig. 6.

The fair agreement of the pK_a^1 determined from fluorescence titrations with the ground state pK_a^1, together with the low quantum yield, indicates a predominantly diabatic deactivation process, *i.e.* fluorescence occurs before an excited state equilibrium is established. However, it cannot be strictly excluded that part of the green fluorescence in neutral solution originates from the anion which may be formed by photodissociation. Unfortunately the tautomer band at 514 nm lies too close to allow a distinction between the two emissions to be made.

An average $pK_a^2(S_1)$ of 1.48 was found by Förster cycle estimates but the fluorescence titration curve showed no inflection points in the pH 0 - 3 range. This also suggests that the excited neutral molecule is diabatically deactivated before it is able to capture a proton from the solvent.

The existence of two emission bands for flavonol has caused some speculation about their origin. It has been proposed that they stem from the flavonol keto and enol tautomers [9]. Alternatively, Frolov *et al.* [10] have postulated that there are two associative forms in solvents with electron

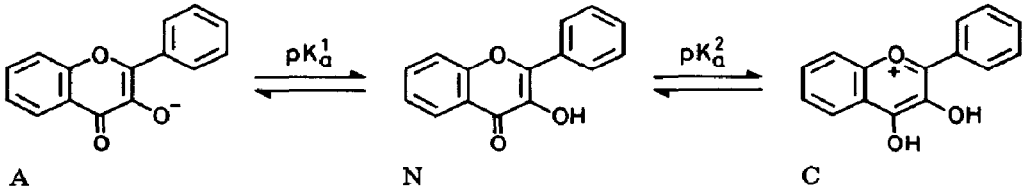

Fig. 5. Structures of A, N and C.

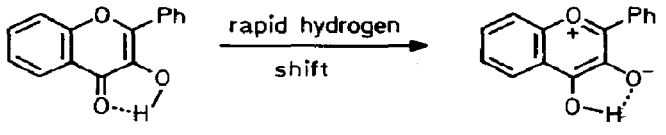

Fig. 6. The proton transfer of the intramolecularly hydrogen-bridged flavonol in hydrocarbon solution from the hydroxy group to the carbonyl oxygen during the lifetime of the excited state leads to the formation of the phototautomer whose green fluorescence is observed.

donor centres. Similarly, Tyukavkina *et al.* [11] have suggested that the violet emission is due to molecules associated with the solvent. Support for this assignment has been obtained from the results of IR investigations. Sengupta and Kasha [13] have studied the fluorescence spectra of flavonol in 2-methylpentane and methanol. A distinct dependence of the emission wavelength on viscosity and temperature was observed which was ascribed to a barrier to the rotation of the phenyl group. The green emission was assigned to a phototautomer. In a recent extensive study Woolfe and Thistlethwaite [15] have demonstrated by ultrafast time-resolved fluorescence spectroscopy, together with investigations of the effects of organic solvents and temperature, that this green emission is produced by an excited state phototautomer PT.

The following arguments exclude the possibility that the green emission in aqueous solutions is due to the photodissociation of **1** to form its anion. The process should be accompanied by the appearance of the 508 nm emission band of the anion. Instead, however, a fluorescence band with a maximum at 514 nm is observed. Furthermore the ratio of the intensity of the green band to that of the violet band increases in favour of the green band with decreasing dielectric solvent strength (Table 1). In organic solvents of low dielectric constant (toluene and cyclohexane) the green band is the only emission observed at room temperature. Photodissociation to form two charged species is unlikely to occur preferentially in these solvents which do not stabilize protons and flavonol anions.

Phototautomerization can take place in either an intermolecular or an intramolecular fashion. Intermolecular phototautomerism can be excluded as the ratio of the intensity of the green band to that of the violet band remained unchanged on going from 0.17 mM to 0.85 μM solutions in both water and methanol. Additionally, there was almost no shift in the IR stretching frequencies of the carbonyl and hydroxy groups in the range from 21.0 mM (1620, 3063 and 3363 cm^{-1}) to 4.2 μM solutions (1620, 3060 and 3366 cm^{-1}). The latter was at the limit of detection in tetrachloromethane. These results further support the assumption that the green fluorescence of flavonol in neutral aqueous and organic solvents occurs from the excited intramolecular phototautomer. Excited state tautomerization would also be expected from the Förster cycle estimates which show that the hydroxy group becomes sufficiently acidic in the S_1 state (pK_a^1 −0.16) to protonate the carbonyl oxygen ($pK_a^2(S_1)$ +1.48 (see Table 2)).

In hydrocarbon solvents, in which flavonol forms strong intramolecular hydrogen bonds [34], phototautomerization appears to be the only process in the excited state (Fig. 6). In alcoholic and aqueous solutions flavonol is both intramolecularly and solvent hydrogen bridged and will undergo intramolecular single-proton transfer to form the green fluorescence as shown in Fig. 6. The external hydrogen-bridged fraction of flavonol requires double-proton transfer between solvent and solute to form a tautomer. This process appears to be too slow to be complete before emission takes place (Figs. 7 and 8). As a result emission in hydroxylic solvents is observed from both

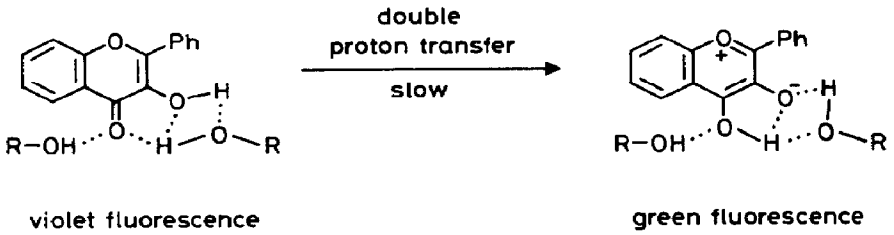

Fig. 7. Prototropic processes of externally hydrogen-bridged flavonol in hydroxylic solvents following photoexcitation. The double-proton transfer from the less acidic solvent hydroxy group to the hydrogen-bridged carbonyl oxygen is slow and gives rise to emission from both the externally bridged flavonol (violet fluorescence) and its phototautomer (green fluorescence).

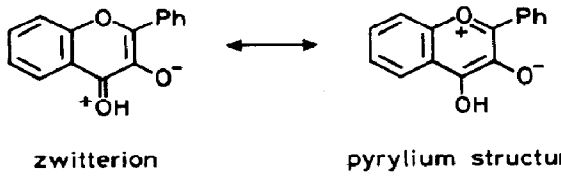

Fig. 8. Mesomeric structures of the phototautomer PT.

singlet excited flavonol (violet emission) and its phototautomer (green emission). Woolfe and Thistlethwaite [15] have shown that in methanol the blue fluorescence follows a single-exponential decay (lifetime, 69 ± 8 ps) whereas the fluorescence of the tautomer can only be fitted by the difference of two exponentials (decay time, 370 ± 25 ps).

Further evidence for this dual deactivation path is obtained from the results of the deuteration experiments shown in Fig. 4. The phototautomerization of both the intramolecular and the solvent hydrogen-bridged molecules is expected to be retarded by deuteration which results in a 20% decrease in the intensity of the green fluorescence. The double-proton transfer reaction of external hydrogen-bridged molecules is also expected to be retarded by deuteration. This results in a 71% increase in the intensity of the violet fluorescence.

4.2. The fluorescence quenching of the flavonol cation

The unusual fluorescence titration curve of the flavonol cation, which prevented the calculation of a reasonable value for $pK_a^2(S_1)$, may result from several processes and interactions. There is a steady increase in the viscosity of the solution on going from low to high H_2SO_4 concentrations. It should also be remembered that the inflection points of luminescence titration curves are not strictly pK_as, but rather are functions of the excited state rate, the equilibrium constants and the lifetimes of the excited state. Changes in the hydration of flavonol molecules may also play an important role. As mentioned above, the absorption spectra obtained in highly concentrated H_2SO_4 suffer a bathochromic shift with a loss of isosbestic points. Similar shifts have been observed for aromatic carboxamides [35] and other hy-

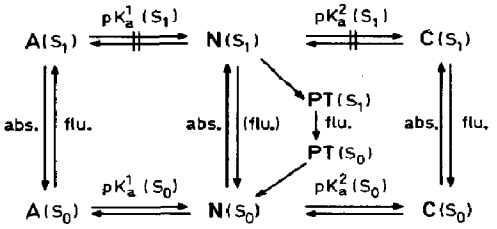

Fig. 9. Model of the prototropic processes and radiative and non-radiative deactivation paths of flavonol and its conjugates. Despite the predicted high basicity of $N(S_1)$ the excited state process $N(S_0) \rightarrow N(S_1) \rightarrow C(S_1) \rightarrow$ fluorescence plays no significant role in moderately acidic solutions. The sequence $N(S_0) \rightarrow N(S_1) \rightarrow PT(S_1) \rightarrow$ fluorescence becomes more important in non-hydroxylic solvents and is the only radiative deactivation path in hydrocarbons. The photodissociation of $N(S_1)$ to form fluorescent $A(S_1)$ predicted by Förster–Weller estimations occurs only to a very small extent, if at all.

droxy flavones [36]. They were interpreted in terms of changes in the solute hydration number [37]. We perceive that this H_0-dependent fluorescence variation might be, at least partially, the result of quenching by water molecules. This quenching should be dynamic rather than static as there is no evidence for a ground state association from the absorption spectra at $H_0 - 1$ to $H_0 - 4$ and the emission spectra are strongly affected by deuteration (Fig. 4). On changing from a 24% H_2SO_4 solution ($H_0 - 1.29$) to a 24% D_2SO_4 solution there is an increase of 133% in the intensity of the cation band which is accompanied by a simultaneous intensity decrease and hypsochromic shift of the phototautomer band.

We propose a model for the prototropic processes and deactivation paths of flavonol in aqueous solutions as depicted in Fig. 9. According to the calculations of the excited state pK_a the S_1 state of N should be sufficiently basic to pick up a proton and to form its conjugate acid $C(S_1)$. We assume three possible mechanisms for the proton transfer in moderate-to-strong acidic solutions.

(1) If the proton delivery to $N(S_1)$ is much faster than its decay time, only fluorescence from excited C is expected. Deuterons react more slowly, but deuteration should not strongly influence the fluorescence.

(2) If the rate of protonation and the fluorescence lifetime are comparable, fluorescence from both excited C and N should be observed. Deuteration should further decrease the fluorescence intensity from excited C in favour of fluorescence from excited N.

(3) If the rate of protonation is much slower than the fluorescence lifetime, only fluorescence from excited N should be observed. Deuteration should not have any effect.

The increase in the fluorescence intensity of C on deuteration certainly excludes mechanism (2) and renders mechanism (1) very unlikely. Because the excitation spectra of cation fluorescence in the pH 0 to $H_0 - 3.38$ range are very similar to the corresponding absorption spectra, it is concluded that mechanism (3) is operative in acid solutions. Thus the sequence $N(S_0) \rightarrow N(S_1) \rightarrow C(S_1) \rightarrow$ fluorescence does not play an important role. $C(S_1)$ is

almost exclusively populated by the direct excitation of $C(S_0)$. $C(S_1)$ can now be deactivated *inter alia* by both fluorescence and quenching by water molecules. A strong deuterium effect on the intensity of the cation band points to comparable rates of quenching and fluorescence decay in acid solutions.

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