pH-Dependent Fluorescence Spectroscopy. Part 12.[1] Flavone, 7-Hydroxyflavone, and 7-Methoxyflavone

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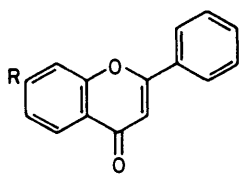
Flavone † is found to be non-fluorescent in neutral aqueous solution, but strongly fluorescent in sulphuric acid. The $pK_a(S_1)$ value of its conjugate acid was calculated from ground-state data and is found to be in poor agreement with the pK_a value obtained from a plot of fluorescence intensity *versus* Hammett acidity. This is interpreted in terms of rapid intersystem crossing to the triplet state, before a prototropic process can take place. Conversely, 7-hydroxyflavone is found to be fluorescent in its neutral, conjugate acid, and conjugate base form. Excited state pK_a values have been obtained from Förster–Weller calculations and from fluorescence titration curves, but are in poor agreement. 7-Hydroxyflavone undergoes adiabatic photodissociation in its S_1 state and forms an exciplex (or a phototautomer) in pH 3 to H_0 −3 solution, and similarly in acidified methanol. Fluorescence quantum yields are given for alkaline, neutral, and acidic solutions. Compared with 7-hydroxycoumarins, flavones are only weakly fluorescent in alkaline solution. This behaviour is explained by the small energy difference between the S_1 and T_1 states giving rise to more efficient intersystem crossing. To demonstrate the dynamic nature of excited state proton transfer processes and the involvement of water molecules in quenching processes, deuteriation experiments have been performed. Drastic changes are found in both fluorescence intensity and shape of the curves.

In continuation of our work on the pH-dependence of the fluorescence properties of natural products [1,2] we have now examined the model compounds flavone (I), 7-hydroxyflavone (II), and its methyl ether (III). Flavone, which is known to induce benzoyren hydroxylase activity,[3] is found in the farina of several *Primula* and *Dionysia* species [4,5] and acts as an efficient photosensitiser.[6,7] 7-Hydroxyflavone and 7-methoxyflavone were considered to be useful models for a possible fluorimetric assay of natural occurring and biologically active flavones,[8,9] most of which are oxygenated in the 7-position. Reports on the fluorescence properties of these flavones are scarce and, surprisingly enough, with one exception [10] limited to nonaqueous solvents.[11–13] We now present the first detailed study of the pH-dependent fluorescence spectra of the title compounds,



(I) R = H
(II) R = OH
(III) R = OCH_3

together with ground and singlet excited state dissociation constants, fluorescence quantum yields, and deuteriation effects thereon.

EXPERIMENTAL

Materials.—Flavone (I),[14] 7-hydroxyflavone (II),[15] and 7-methoxyflavone (III) [16] were synthesized according to the literature. Flavone was repeatedly recrystallized from spectroscopic grade light petroleum–cyclohexane. Compounds (II) and (III) were purified by preparative t.l.c. and recrystallized from absolute ethanol. Solvents and buffers were the same as described.[2] Deuterium oxide, sulphuric [$^2H_2$]acid, and [$^2H_4$]methanol were from Aldrich and were of 99.8, 99.5, and 99.5 atom % purity, respectively.

Absorption and Emission Spectra.—The absorption spectra were run on a Perkin-Elmer–Hitachi instrument at room temperature. They obeyed the Lambert–Beer Law in the experimental concentrations. The fluorescence spectra of freshly prepared non-degassed solutions were scanned in rectangular quartz cells at 25 °C using a Aminco SPF 500 spectrofluorimeter. In order to avoid fluorescence quenching by buffer ions (*e.g.* the fluorescence of flavone conjugate acid is strongly quenched by chloride) the following technique was applied to obtain the spectra. The flavone solution was pumped through a flow-through cell in the fluorimeter. The pH was adjusted externally by the addition of either dilute sulphuric acid or sodium hydroxide. The error resulting from volume changes was found to be negligible under proper working conditions and after some training. This method guarantees the measurement of fluorescence spectra, which are not affected by buffer ions.[17]

The digital output of the fluorimeter was processed using a Hewlett–Packard 9815 A computer and software provided by the American Instrument Comp. to give corrected emission spectra. In the case of very diluted solutions the blank solvent spectrum was subtracted from the sample spectrum. The nm-linear readout of the instrument was converted to a wavenumber-linear readout by the computer. The integration of the curves thus obtained was used for the determinations of the fluorescence quantum yields, relative to quinine sulphate in 1M-sulphuric acid (ϕ_f 0.546 [18]).

pK_a Values were determined from the inflection points of the titration curves, which were obtained from plots of extinction or fluorescence intensity *versus* acidity (pH or H_0). In some cases pK_a values were also calculated using equation (1) [19] where E is either the absorption or the fluorescence intensity. For the $pK_a(S_1)$ determinations excitation was done with a bandpass of 10 nm at the isosbestic wavelength of absorption to provide constant light absorp-

$$pK = pH - \log[(E_x - E_{BH})/(E_B - E_x)] \quad (1)$$

† The IUPAC name for flavone is 2-phenyl-4*H*-1-benzopyran-4-one. The trivial name will be used throughout this paper.

tion. All the changes in the spectra with pH or H_0 were found to be fully reversible.

RESULTS

Absorption and Fluorescence Spectra.—The fluorescence spectra of flavone in various concentrations of sulphuric acid are depicted in Figure 1 and compiled, together with u.v. absorption data in Table 1. Flavone is found to be non-fluorescent in neutral aqueous solution at room temperature, as it is in tetrachloromethane [20] or ethanol.[21] We noticed, however, the development of a fluorescence band at 400—420 nm in aqueous, alcoholic acetonitrile or dioxan solution within a few minutes. The new band is found in addition to a strong Raman scatter peak at *ca.* 370 nm (excitation at 305 nm) and steadily increases with time. Its excitation spectrum, having maxima at 340 and 265 nm and a minimum at 295 nm (in methanol) is not consistent with the absorption spectrum of flavone. The formation of this apparent new species may be the result of a photochemical transformation.[22] A similar observation has already been made for flavone in alcohol and hydrocarbon solution.[12]

Governed by a pK_a of —1.33 [23] flavone is protonated to form its strongly fluorescent conjugate acid, having $\lambda_{max.}^{flu}$ 404.5 nm (Figure 1).

On the other hand, both 7-hydroxy- and 7-methoxyflavone fluoresce in alkaline, neutral, and acidic solution (Table 1). The fluorescence spectrum of (III) peaks at 416 nm in neutral and slightly alkaline or acidic aqueous solution, but is shifted to 436.5 nm on changing to 60% sulphuric acid (H_0 —4.46).

There are three species evident in the absorption spectra of (II), namely its conjugate base (anion), its neutral form, and its conjugate acid (cation). All three have distinct fluorescence maxima. The anion emission peak is at 528 nm, the neutral molecule emissions are at 425 (rather weak) and 530—539 nm, depending upon the pH, and finally the cation emission is at 452 nm (at H_0 —4.46). The absorption and fluorescence curves are shown in Figure 2 and the corresponding maxima are compiled in Table 1. According to the absorption spectra compound (II) is uncharged in the pH 6—1 range. The two fluorescence bands observed at low pH values have identical excitation spectra which coincide with the absorption spectrum. The existence of two emission bands points to two deactivation pathways.

Similar behaviour is found for (II) in dry methanol solution. Two fluorescence bands are observed, having maxima at 421.5 and 537 nm and with identical excitation spectra. After the addition of sulphuric acid the

TABLE 1

Longwave u.v. absorption, excitation, and fluorescence maxima of (I)—(III) in aqueous solutions of various pH, in sulphuric acid of various H_0, and in methanol at 25 °C. Concentrations from 18.2 to 66.2μM for absorption spectra and 18.2 to 36.4μM for fluorescence spectra. Ten-fold dilution does not affect the fluorescence maxima

Compound	Solvent pH	Solvent H_0	$\lambda_{max.}^{abs}$/nm	ϵ/l mol^{-1} cm^{-1}	$\lambda_{max.}^{exc}$/nm	$\lambda_{max.}^{flu}$/nm
(I)	7.0		304, 310sh	17 500		
	0.00		306, 320sh, 330sh	17 800	325	405
		0.31	310, 330sh, 350sh	17 850	330	405
		1.01	324br, 340sh	18 050	330	405
		2.41	345br	24 850	330	404.5
		4.46	348br	25 560	325, 350sh	404.5
		7.05	348br, 350sh	25 900	320sh, 355	404.5
		9.85	355	27 200	356	404.5
(II)	9.80		360	14 600	358	528
	5.10		313.5, 335sh	18 270	313, 332sh	425, 530
	1.00		314, 335sh	18 600	316, 332sh	425, 530
	0.00		316, 335sh	19 100	330br	418, 539
		1.72	338	23 430	340.5br	472br, 535sh
		4.46	370	28 250	372	452
		9.85	368	28 500	371	449
(III)	7.00		312, 330sh	19 730	315	416
	1.00		313, 330sh	20 670	315, 330sh	432
		1.30	365	24 480	370	433
		4.46	371	26 630	373.5	436.5
		9.80	375	28 030	373.5	436.5
(II)	Methanol		309, 335sh	19 400	316, 330sh	421, 537
(III)	Methanol		309, 330sh	20 200	316, 330sh	404

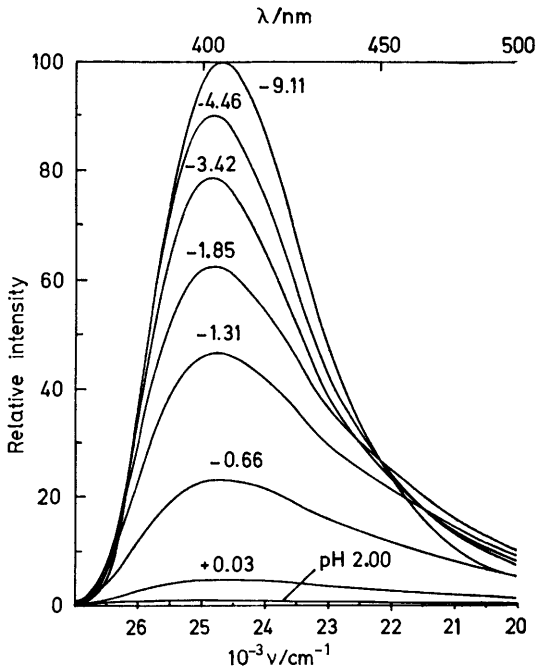


FIGURE 1 Fluorescence spectra of (I) in various concentrations of sulphuric acid at 25 °C. Excitation wavelength 319 nm (isobestic wavelength of absorption)

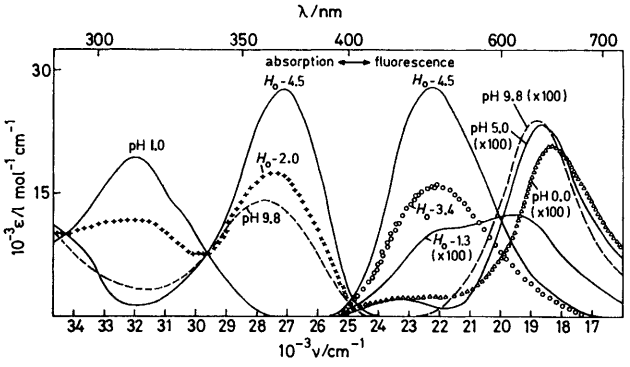


FIGURE 2 Absorption and fluorescence spectra of (II) in aqueous solutions of different pH or Hammett acidity at 25 °C. Concentration 20—28μM. The isosbestic points for the systems (II) anion–(II) and (II)–(II) cation are incidentally coincident. Excitation wavelength in all cases 338 nm at bandpass 5 nm

blue band disappears to leave a shoulder, and the green emission maximum is shifted to 544 nm. The excitation spectrum for the 544 nm band shows that both neutral (II) and its conjugate acid are excited (Figure 3).

First Excited Singlet State Dissociation Constants.—In order to estimate the direction and the order of magnitude of the pK_a shifts in the S_1 state we have applied the Förster–Weller equation,[24] which, at 25 °C, is (2). For the calculations the means of the frequencies of the absorption and

$$pK_a(S_1) = pK_a(S_0) - 0.0021(\nu_{HB} - \nu_B) \qquad (2)$$

fluorescence maxima have been taken as O–O transitions.

On the other hand, pK_a values have been determined by fluorescence titration. Table 2 summarizes the results. As there were doubts regarding the ground state pK_a values reported for compound (II) in the literature,[25] we have redetermined it spectrophotometrically and obtained the value 7.39 ± 0.04 at an ionic strength I of 0.04. The excited state pK_a of (II) was obtained from a plot of the anion emission intensity *versus* pH and is estimated as 1.6 ± 0.2 in unbuffered solution.

The results obtained from ground state data using the Förster–Weller equation show all the flavones to become more basic in their S_1 state by 6.47—9.92 units. From a plot of cation fluorescence intensity *versus* pH or Hammett acidity we tried to obtain the p$K_a(S_1)$ of (I) and (II) (Figure 4). However, within experimental error the pK_a values were identical with the ground state values.

The ground state pK_a of the conjugate acid of (III) was determined from changes in absorption at 375 nm *versus* H_o to be −0.75 ± 0.08. The p$K_a(S_1)$ value calculated from the curve in Figure 4 is +0.06. The increase in basicity is significant, but in no way as big as predicted by the Förster–Weller calculation. In highly concentrated sulphuric acid the fluorescence of (II) is partially quenched, but its $\lambda_{max.}$ remains unchanged. The calculated p$K_a(S_1)$ values of (II) (−2.26) is lower by 9.65 units than the p$K_a(S_0)$ value and there is poor agreement with the p$K_a(S_1)$ value obtained by fluorescence titration (1.6). The latter is probably not too accurate, since the intensity of the anion emission is falsified by the appearance of the phototautomer band at 539 nm.

An anomalous titration curve is found for (II) in strong sulphuric acid (Figure 4). A pK_a value of −3.3 may be read from the curve. This would indicate a decrease in the carbonyl group excited state basicity, which appears not to be meaningful.

Deuteriation Effects.—In order to demonstrate the suggested dynamic nature of proton transfer processes prior to the fluorescence we conducted some deuteriation experiments, the most characteristic results of which are shown in Figure 5. On changing from H_2O to D_2O solution the intensity of the neutral band (the blue emission at 425 nm) remains unchanged, whereas the intensity of the green emission (at 530 nm) is increased by 48%. No such effect

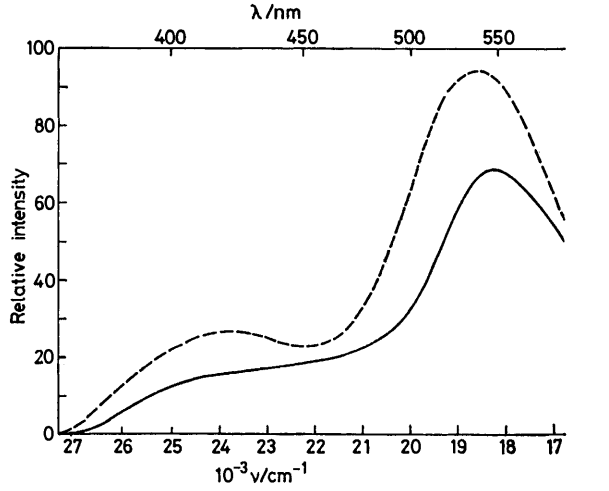


FIGURE 3 Fluorescence spectra of (II) in methanol (– – – – –) and in acidified methanol (——). In pure methanol the excitation spectra for the 421 and 537 nm bands are coincident ($\lambda_{max.}^{exc}$ 332 nm). After the addition of H_2SO_4 the excitation spectrum at λ^{flu} 544 nm indicates, that both neutral (II) and its conjugate acid are excited, but emission occurs mainly from the exciplex

TABLE 2

Ground state and first singlet excited state dissociation constants of (II) and of the conjugate acids of (I)—(III) at 25 °C

Species	p$K_a(S_0)$	$\nu_{max.}^{abs}$/cm^{-1}	$\nu_{max.}^{exc}$/cm^{-1}	$\nu_{max.}^{flu}$/cm^{-1}	abs/flu	p$K_a(S_1)$ O–O Transitions	exc/flu	p$K_a(S)_1$	p$\bar{K}_a(S)_1$	pK_a by fluorescence titration
(I)		32 895								
(I) conjugate acid	−1.33 [23]	28 169	26 596	24 722		8.59 [a]			8.59 [b]	−1.1 ± 0.2
(II) conjugate base		27 778	27 894	18 939	23 359		23 417			
(II)	7.39 [25]	31 898	31 949	24 039	27 969	−2.29	27 994	−2.22	−2.26	1.6 ± 0.4 [c]
(II) conjugate acid	−0.79 [23]	27 027	26 882	22 272	24 650	6.18	24 577	+6.39	+6.29	(−3.3)
(III)		32 051	31 746	24 039	28 045		27 892			
(III) conjugate acid	−0.75	26 954	26 774	22 910	24 932	5.78	24 842	5.66	5.72	0.06 ± 0.2

[a] The absorption and fluorescence maxima have been used rather than the O–O transitions. [b] Ref. 33 gives 6.75 using a ground state pK_a of −1.2. The fluorescence data given is this paper ($\lambda_{max.}^{flu}$ 372 nm) seem to be an artefact, possibly the first Raman scatter, since flavone is not strongly fluorescent, as stated. [c] The value is probably lower; the fluorescence titration curve is falsified by the appearance of a new fluorescent species at this part of the spectrum.

is found with compound (III). Similarly, in 9% sulphuric acid the intensity of both blue and green emissions is strongly enhanced (by 210 and 192%, respectively) on changing from H_2SO_4 to D_2SO_4.

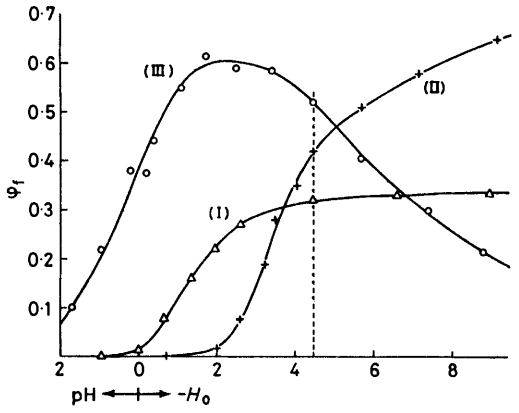


FIGURE 4 Plot of fluorescence quantum yields of (I)—(III) *versus* Hammett acidity or pH. The quantum yields have been determined at H_0 −4.46 and the other values were taken from the fluorescence spectra (wavenumber mode) and set relative to the H_0 −4.46 value

Fluorescence Quantum Yields.—Table 3 summarizes the measurements of quantum efficiencies of (I)—(III). Results on structurally related flavones [26] are added for comparison. It is evident that fluorescence of hydroxy-

TABLE 3

Fluorescence quantum yields of some flavones in aqueous or sulphuric acid solution containing 10% methanol. The sulphuric acid values are corrected for the refractive index; optical density in all cases smaller than 0.3. Temperature 25 °C

Compound	pH	H_0	ϕ_f	Excitation wavelengths (nm)
(I)		−4.46	0.32 ± 0.02 [a]	342, 346, 350, 354, 358
(II)	9.9		0.004 ± 0.001	350, 353, 356, 359, 362
(II)	6.6		0.004 ± 0.001	308, 311, 314, 317, 320
(II)		−4.46	0.42 ± 0.02	350, 354, 356, 359, 363
(III)	7.0		0.04 ± 0.01	308, 311, 314, 317, 320
(III)		−4.46	0.52 ± 0.03	340, 342, 344, 346, 348
3-Hydroxy-flavone	7.0		0.005 [b]	
3-Hydroxy-flavone		−4.46	0.042 [b]	
4′-Hydroxy-flavone	7.0		0.02 [b]	
4′-Hydroxy-flavone		−4.46	0.013 [b]	

[a] Ref. 32 gives 0.36 in H_2SO_4–AcOH at an unspecified temperature. [b] R. Schipfer and O. S. Wolfbeis, unpublished data.

flavones is greatest in strong sulphuric acid. This reflects a fundamental difference from the behaviour of, *e.g.* 7-hydroxycoumarins, whose fluorescence intensities are known to be very large in alkaline solution [27,28] and which are useful pH indicators.

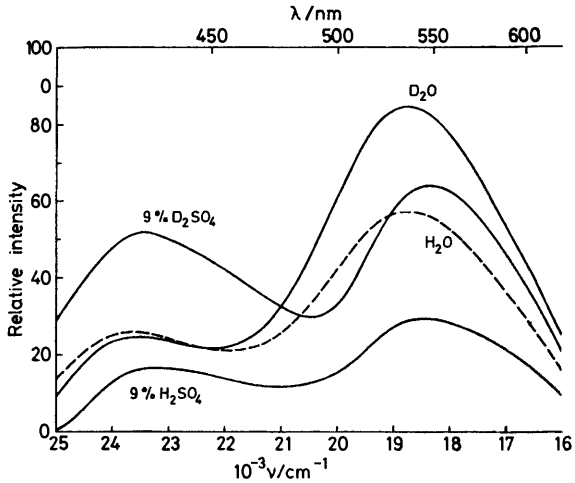


FIGURE 5 Deuteriation effects on the fluorescence of (II) in water (pH 5) and in 9% sulphuric acid, both containing 20% CD_3OD. No such effect is observed with (III) in water solution. Excitation wavelength 319 nm. Concentration 80.5μM

DISCUSSION

The finding, that flavone, like chromone [29,30] or xanthone,[31] is non-fluorescent in neutral solution is in contradiction to reports which claim it to be fluorescent [32] or even strongly fluorescent.[33] The lack of fluorescence in ethanol has been explained on the basis of the El Sayed selection rule by efficient intersystem crossing to the well characterised flavone triplet state,[6,12,32,34,35] which may be responsible for its photoreactivity.[22] It is evident from the lack of fluorescence and the identity of the pK_a values obtained by absorption and fluorescence measurements, that the rate of adiabatic protonation of S_1 flavone is too slow to compete successfully with intersystem crossing.

Protonation at the carbonyl oxygen blocks the n–π^* transition and suppresses intersystem crossing to closely lying n–π^* triplet states.[12] In fact the conjugate acid is found to be strongly fluorescent. From an analytical point of view, flavone clearly can only be assayed fluorimetrically at fairly high sulphuric acid concentrations, where variation of the fluorescence intensity with H_0 is small (Figure 4).

The introduction of a hydroxy- or methoxy-group into flavone increases the energy of the n–π^* transitions, so that the n–π^* (T_1) level comes to lie above the π–π^* (S_1) level.[12] This results in a suppression of intersystem crossing and both (II) and (III) become fluorescent. But the low fluorescence quantum yields still indicate significant contributions of other deactivation processes.

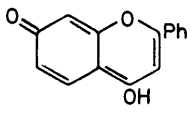
The green emission of 7-HF in alkaline solution is assigned to occur from its conjugate base (anion). The weak blue and the strong green luminescence which have been detected in the pH 7 to H_0 −2 range point to two independent deactivation paths. The blue fluorescence can be assigned to excited neutral (II), since (III) fluoresces with $\lambda_{max.}$ 416 nm in pH 7 solution.

The green emission of (II) in the pH 7—2 range is assumed to be the result of adiabatic photodissociation

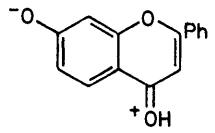
to form the anion. This is to be expected from the Förster–Weller calculations. The $pK_a(S_1)$ obtained by fluorescence titration (1.8) is in poor agreement with the one obtained by the calculations (−2.26). It should, however, be noted, that these two pK_a values do not correspond to each other. The calculated $pK_a(S_1)$ refers to (II), whereas the fluorimetric $pK_a(S_1)$ refers to a unusual excited state species, as will be demonstrated later.

The shift of the longwave emission to *ca.* 540 nm after acidifying the aqueous or methanol solution requires further interpretation. By analogy with the behaviour of 4-methylumbelliferone (4-MU),[36,37] 7-hydroxylepidone,[38] and 2-methyl-7-hydroxychromone [39] this band may originate from a phototautomer. For 4-MU, Zinsli [40] as well as Beddard *et al.*[41] have alternatively postulated the existence of an exciplex between 4-MU anion and H_3O^+.

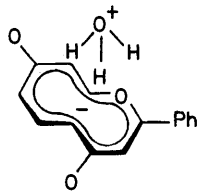
We propose structure (VI) for such an exciplex. Unlike the phototautomer, the formation of this species would not require a double proton transfer. It may rather be formed by a single proton transfer from singlet excited (II) to a water molecule along a hydrogen bridge within the solvent cage. Preliminary measurements of fluorescence decay times give 1.92 ns for the exciplex (in 1M-sulphuric acid), which is certainly enough time for prototropic processes to occur. Phototautomerisation



phototautomer (IV)



zwitterion (V)



exciplex (VI)

is also predicted to occur on the basis of the results of the Förster–Weller calculations. They show (II) in its S_1 state to be more acidic at its hydroxy-group than its conjugate acid in the ground state and in the S_1 state. It follows, that on acidimetric titration, the anion in its S_1 state is protonated at the carbonyl group rather than at the phenolate oxygen. The overall reaction in the pH 2 to H_0 −2 range is a proton release at the 7-hydroxy-group and simultaneous proton capture at the carbonyl oxygen. As this process is fully adiabatic, the emission from the phototautomer (or zwitterion or exciplex) at 540 nm is the predominant one.

The excitation spectra for the phototautomer band in acidified methanol indicate that both neutral and protonated (II) can be excited to give the 544 nm fluorescence. To do so neutral (II) has to pick up a proton from the solvent and to release one at the hydroxy-group. Protonated (II) has to release the hydroxy-group proton. Evidently, the conjugate acid of (II) is more acidic in its S_1 state.

Despite a broadband emission of (II) in methanol or acidic aqueous solution, which suggests its use in tunable lasers, the fluorescence quantum yields are probably too low as compared with coumarins,[42,43] which has a known adverse effect on laser dye properties.

In more concentrated sulphuric acid the strongly fluorescent cation becomes the only species evident in emission. The poor agreement in the pK_a values of the conjugate acid of (II) obtained by the two methods may be rationalised by uncertainties in both methods (*e.g.* neglect of entropy changes, neglect of weak shoulders in the absorption spectra *). Another reason may lie in the unusual titration curves in strong sulphuric acid. We assume that for (II) in concentrated acid an unusual quenching process is operative, possibly *via* a second excited state species as in the case of 8-methoxypsoralene.[44]

Quenching by water is also demonstrated by the results of the deuteriation experiments. Similar effects have been found with 7-hydroxyisoflavone.[1] A further reason for the disagreement in the calculated and measured pK_a values for the S_1 state of (II) results from the fact, that the calculated value is that for unchanged (II), whereas the value obtained by fluorimetry is the one for the phototautomer (or zwitterion or exciplex). From the deuteriation experiments it can be concluded, that (a) the photodissociation process proceeds much faster than fluorescence decay and (b) that both the phototautomer and the conjugate acid are quenched by water molecules. These processes are slowed in D_2SO_4 solution and lead to an increase in fluorescence intensity.

Surprisingly enough phototautomerisation, like photodissociation, seems not to be adversely affected by deuteriation, which again points to a very rapid process.

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* In the given case the absorption spectra of (II) indicate such a weak transition that we find a shoulder in its absorption spectrum in pH 4 or methanol solution. If this shoulder at *ca.* 329.5 nm is used in the calculations a $pK_a(S_1)$ of −0.66 is obtained for (II), and a $pK_a(S_1)$ of 4.55 for its conjugate acid.

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