FLUORESCENCE OF SOME SIMPLE UMBELLIFERONE DERIVATIVES

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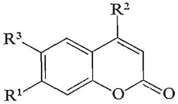
The absorption and fluorescence spectra of umbelliferone were studied; it was found that in the excited state the basicity of the α-pyrone part of the molecule increases to such an extent that it exceeds that of the phenolate oxygen of the molecule. This is responsible for the neutral non-dissociated form of umbelliferone being formed only in nonpolar medium which prevents the dissociation of the phenolic group in the excited state. In nonpolar medium, umbelliferone behaves as a substance with closely lying $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions. Hydrogen bonds affect the fluorescence of 7-methoxycoumarin so that the $\pi \rightarrow \pi^*$ transition is preserved as the lowest excited singlet state.

The cause of the great natural abundance of coumarins is not quite clear yet. One of their typical features is high photosensitivity; the excited singlet and triplet states can participate in processes involving energy transfer[1]. Coumarin itself fluoresces only slightly. In strongly alkaline medium the lactone ring opens and coumarin converts to the *trans-o*-hydroxycinnamic acid anion, giving rise to yellow-green fluorescence[2]. Much more important than coumarin itself are its 7-hydroxy derivatives, exhibiting intense blue fluorescence. The application of artificial fluorogenic substrates containing this substance made essentially more sensitive the determination of activities of some enzymes[3–6]. When a bulky alkyl substituent is attached to the ring, the hydrophobic character of the molecule becomes much more pronounced. The use of alkyl umbelliferone as the indicator enables a sensitive detection of the pH on the boundary of the hydrophobic and hydrophilic phases[7]. In strongly acidic medium the molecule gets protonized, and coumarins convert to positively charged fluorescing forms[8].

In the present work the excited state of some umbelliferone derivatives is studied and the results are confronted with known data.



EXPERIMENTAL

Methods and Apparatus

The dissociation constants of the umbelliferone derivatives were determined from the absorption spectra measured on a spectrophotometer Cary 118. The dissociation of the phenolic group was measured in Tris buffer (Tris = tris(hydroxymethyl)aminomethane), the dissociation constants were determined in the region of the anion absorption.

The dissociation constants of the conjugate acids were measured in sulfuric acid. In this medium the pH scale is replaced with the Hammett acidity function[9,10] H_0. The absorptivity of a conjugate acid cannot be determined directly; the dissociation constants were therefore calculated from the equation

$$\varepsilon h_0/(\varepsilon - \varepsilon_B) = \varepsilon_{BH} h_0/(\varepsilon - \varepsilon_B) - K_a \qquad (1)$$

by using the least squares method; here h_0 is a function defined by the relation $H_0 = -\log h_0$, ε is the experimental absorptivity, ε_B the absorptivity of the base, ε_{BH} that of the conjugate acid (the slope of the straight line), and K_a is the dissociation constant of the conjugate acid (the straight line intercept). The absorbances were read in the absorption region of the protonized form at several wavelengths; the values given in the subsequent section are average ones. The coumarins were stable in the sulfuric acid medium, the spectra did not alter even after 24 hours' standing. The umbelliferone solution in 85% sulfuric acid was allowed to stand for 1 h, then it was neutralized, extracted with chloroform and evaporated. The identity with the original sample was verified chromatographically.

The dissociation constants in the excited state can be calculated from the positions of both the absorption and the fluorescence peaks of the individual species. Since no correction was applied to the fluorescence spectra, the dissociation constants in the excited state were determined from the absorption spectra by employing the relation

$$\mathrm{p}K_a - \mathrm{p}K_a^* = 21\,187(\lambda' - \lambda)/\lambda\lambda' \,, \qquad (2)$$

where λ and λ' are the absorption maximum positions of the acid and the base, respectively (nm), and $\mathrm{p}K_a$ and $\mathrm{p}K_a^*$ are the dissociation constants in the ground state and the excited state, respectively.

The fluorescence spectra were measured on a spectrofluorimeter Aminco-Bowman. 4-Methyl-7-hydroxycoumarin (Lachema) was doubly recrystallized from 50% methanol. 4-Methyl-7-methoxycoumarin was prepared by using methyl iodide, recrystallized from 50% methanol, and purified chromatographically (Al_2O_3). Umbelliferone and 6-geranyl-7-hydroxycoumarin were obtained as described previously[11].

RESULTS

The introduction of a hydroxy group on the coumarin ring results in a bathochromic shift of its nearest absorption maximum in the ultraviolet region. Dissociation of this phenolic group can be monitored in the absorption as well as fluorescence spectrum (Fig. 1). In the absorption spectrum, a new red-shifted peak appears. In the fluorescence spectrum the anion appears, however, at a considerably lower pH value than as would correspond to the dissociation constant of the phenolic group. This phenomenon has been described[12] in terms of dissociation in the excited state and it can be depicted as

$$\begin{tikzcd}
\mathrm{RH} \arrow[r] & \mathrm{RH}^* \arrow[r, shift left, "K_a^*"] & \mathrm{R}^{*(-)} + \mathrm{H}^{(+)} \arrow[l, shift left] \arrow[r] \arrow[d, "h\nu_2"] & \mathrm{R}^{(-)} + \mathrm{H}^{(+)} \arrow[r, shift right, "K_a"'] & \mathrm{RH}. \arrow[l, shift right] \\
{} \arrow[u, "h\nu_1"'] & & {} & &
\end{tikzcd}$$

A quantitative treatment of this process was the concern of Weller[12], who has calculated and experimentally verified Eq. (*2*).

Table I shows a comparison of the phenolic group dissociation constants for some coumarin derivatives in the ground and excited states. The table only gives the theoretical values, the experimental values cannot be measured directly from the fluorescence spectra, because umbelliferone and related derivatives take part in additional acid–base equilibria in the excited state.

The second acid–base centre of the umbelliferone molecule is the α-pyrone part. The protonized form shows intense fluorescence, and bathochromic shifts of both the absorption and fluorescence maxima are observed in sulfuric acid as compared with

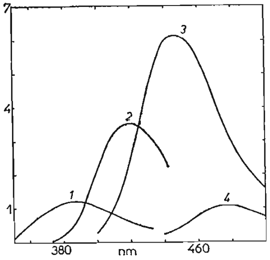


FIG. 1

Fluorescence Spectra of 7-Hydroxy-4-methylcoumarin

Concentration 6 μmol l^{-1}, for excitation wavelengths see Tables I and II. *1* ethanol, *2* 75% H_2SO_4, *3* 0·05M Tris, *4* 5% H_2SO_4.

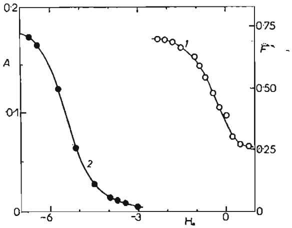


FIG. 2

Absorption and Fluorimetric Acid–Base Titration Curve of 7-Methoxy-4-methylcoumarin in Mixture H_2SO_4–Water

Concentration of the substance for photometric measurements 10 μmol l^{-1}, for fluorimetric measurements 6·7 μmol l^{-1}. *1* Excitation at 328 nm, fluorescence at 418 nm; *2* absorption at 370 nm.

TABLE I

Dissociation Constants of the Phenolic Group of Coumarins in the Ground (pK_1) and Excited (pK_1^*) States

λ_1 and λ_2 are the wavelengths of the maxima of the nonionized form and of the anion, respectively; the first row pertains to the absorption spectrum, the second to the fluorescence spectrum. The positions of the fluorescence maxima of the nonionized forms were measured in ethanol, the absorption spectra and the positions of the fluorescence maxima of the anions in 0·05M Tris buffer. The concentration of the substances for photometric measurements 40 μmol l^{-1}, for fluorimetric measurements 6 μmol l^{-1}.

Coumarin derivative			λ_1 nm	λ_2 nm	pK_1	pK_1^*
R_1	R_2	R_3				
OH	H	H	324 392	365 453	7·75	0·40
OH	CH_3	H	320 385	360 446	7·80	0·45
OH	H	geranyl	333 400	376 456	8·40	1·20
OCH_3	CH_3	H	319 380	— —	—	—

TABLE II

Dissociation Constants of the Conjugate Acids of Coumarin Derivatives in the Ground (pK_2) and Excited (pK_2^*) States

λ_3 is the wavelength of the maximum of the protonized form: the first row pertains to the absorption spectrum, the second to the fluorescence spectrum; FI is the relative fluorescence intensity of the protonized form. 10 μM solutions of the coumarins in 50—98% H_2SO_4 were used for both the photometric and fluorimetric measurements.

Coumarin derivative			λ_3 nm	FI	pK_2	pK_2^*
R_1	R_2	R_3				
H	H	H	321 435	0·2	−6·6 ± 0·3	−4·9
OH	H	H	350 428	4·0	−4·9 ± 0·6	−0·4
OH	CH_3	H	343 415	3·5	−4·9 ± 0·5	−0·5
OCH_3	CH_3	H	347 415	6·7	−5·0 ± 0·5	+0·4

the neutral form (Fig. 1). From this shift it is possible to calculate the difference between the excitation energies of the neutral and protonized forms, and thus also the difference in the basicity of the ground and excited states. Table II shows the determination of the dissociation constants of the α-pyrone part of the molecule in the ground and excited states. From the table it is apparent that in the excited state the basicity of the α-pyrone part increases. This implies that as the pH is lowered, the protonized forms appear first in the fluorescence spectrum, at the excitation wavelength corresponding to the neutral form. The determination of the experimental dissociation constant in the excited state is illustrated in Fig. 2 for 4-methyl-7-methoxycoumarin. The acid–base curve of this substance found from the absorption spectra is shown for a comparison, too. The experimental value $pK_2^* = -0{\cdot}40$ differs slightly from the theoretical value $pK_2^* = +0{\cdot}40$.

The basicities of the first basic centre of umbelliferone, *viz.* the phenolate oxygen, and the second centre, *viz.* the α-pyrone part, being close to each other in the excited state are responsible for the fact that protonation of the anion in the excited state need not lead to the neutral molecule, but also to the amphoion. Such a form has been, in fact, inferred[8] from the blue-green fluorescence at 480 nm. In the range pH 0, four forms of umbelliferone can thus occur in the excited state (Fig. 3). The dissociation constants K_3^* and K_4^* were calculated from the fluorescence spectra of 4-methyl-7-hydroxycoumarin in dependence on pH by using the least squares method. The calculations were carried out with values obtained from that fluorescence range

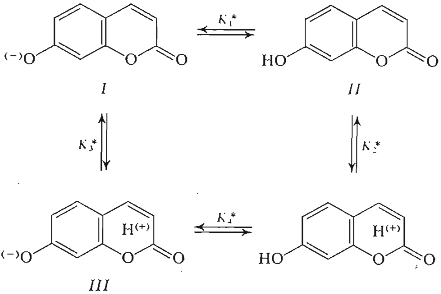


FIG. 3

Acid–Base Equilibria of Umbelliferone in the Excited State where the contribution from the forms *II* and *IV* is negligible. The equality $K_1^*K_2^* = K_3^*K_4^*$ holds true. Solving this system under the above conditions we obtain the straight line equation

$$F = -(1/K_4^*)\left[F(h_0 + K_2^*) + (K_1^*K_2^*/h_0)(F - F_0)\right] + F_R\,, \qquad (3)$$

where K_1^* to K_4^* are the dissociation constants of the various species in the excited state, h_0 is the function defined by $H_0 = -\log h_0$, F is the observed fluorescence, F_0 the fluorescence for the case that all the substance present occurs in the form *I*, and F_R is the fluorescence for the case that all the substance is present in the form *III* (the straight line intercept). The slope of the curve is the reciprocal of the negative dissociation constant K_4^*. The dissociation constants K_1^* and K_2^* were taken as given in Tables I and II. The values found are $pK_3^* = 1{\cdot}76$, $pK_4^* = -1{\cdot}82$ (Table III). Based on these calculated constants, the concentrations of the various forms of 4-methyl-7-hydroxycoumarin in the excited state were determined in dependence on the pH (Fig. 4). In the region pH greater than 3, only the anion *I* exists in the solution, in the region pH less than 0 the protonized form *IV* begins to prevail. The neutral form is present only in a very low concentration. In the range pH 0–3, the amphoion predominates. The neutral form can appear, however, in a medium in which its dissociation in the excited state is suppressed. Fig. 5 shows the fluorescence spectrum of 4-methyl-7-hydroxycoumarin in a mixture ethanol–water. As the ethanol concentration increases, the concentration of the neutral form *II* increases

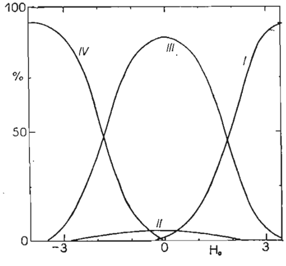


FIG. 4

Dependence of the Concentration of the Acid–Base Forms of 7-Hydroxy-4-methylcoumarin in the Excited State on the Acidity of the Medium

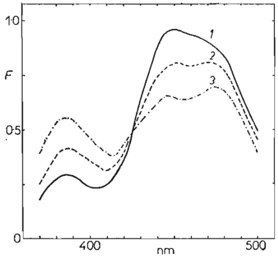


FIG. 5

Fluorescence Spectrum of 7-Hydroxy-4-methylcoumarin in Mixture Ethanol–Water

Concentration 8 µmol l^{-1}, excitation at 328 nm. *1* 85% Ethanol, *2* 92% ethanol, *3* 96% ethanol.

too. In addition, a peak appears in the spectrum at 475 nm, probably due to the fluorescence of the form *III*.

Table IV shows how the methylation of the phenolic group of 4-methyl-7-hydroxycoumarin affects the fluorescence of its form *II*. The solvents are ordered according to the *Z* constant, essentially correlating with the dielectric constant[13]. In the case

TABLE III

Determination of the Dissociation Constant of 4-Methyl-7-hydroxycoumarin in the Excited State (pK_4^*)

6·7 μM Solutions in mixtures of H_2SO_4 and water, excitation of the spectra at 326 nm.

Wavelength nm	Fluorescence intensity for the acidity function H_0									pK_4^*
	2·08	1·90	1·64	1·42	1·22	1·06	0·48	0·28	5·00	
440	73	65	50	38	30	25	14	13	105	−1·81
450	82	75	70	49	41	36	25	25	114	−1·83
460	83	80	67	59	53	50	42	41	102	−1·73
470	78	79	70	66	59	62	56	56	87	−1·72
480	67	70	64	64	63	62	62	60	72	−1·99

TABLE IV

Dependence of the Fluorescence Intensity of 4-Methyl-7-hydroxycoumarin and 4-Methyl-7-methoxycoumarin on the Polarity of the Medium

λ Position of the fluorescence maximum (nm), FI relative fluorescence intensity. Concentration of the substances 8 μmol l^{-1}, excitation wavelength 328 nm.

Medium	R_1 = OH		R_1 = OCH_3	
	λ	FI	λ	FI
Octanol	383	1·05	380	0·17
Butanol	385	0·76	380	0·20
Ethanol	385	0·69	380	0·20
Methanol	386	0·72	380	0·20
Glycerol	394	0·66	390	0·20
85% Ethanol	—	—	380	0·37
80% Ethanol	—	—	380	0·45
70% Ethanol	—	—	380	0·53
Water	—	—	382	1·10

of the derivative containing the phenolic group, the fluorescence is blue-shifted in nonpolar solvents and the fluorescence intensity increases. The viscosity, as apparent from the fluorescence in glycerol, does not have an essential effect. After the methylation of the phenolic group, the effect of polarity upon the fluorescence of the substance is not very marked. If, however, the series of aliphatic alcohols is replaced by a series of mixtures ethanol–water, which in polarity partly overlaps the alcohol series, the fluorescence intensity of the methoxy derivative is seen to increase several times on addition of water.

DISCUSSION

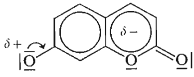
The acidity of the phenolic part in umbelliferone is higher than in naphthol[8]. The substitution by a methyl group in the position 4 does not affect the dissociation constant value. The bulky alkyl group of 6-geranyl-7-hydroxycoumarin lowers the acidity of the phenolic group. In the case of this substituent, which is essential to the biological activity of the substance[11,14], the steric effect may play a role, too. In the excited state, all quantum chemical parameters of the molecule are altered; the dissociation constants in the excited state differ therefore from those in the ground state too. The dissociation constant of the phenolic group in umbelliferone derivatives increases in this case by 7 orders of magnitude.

The α-pyrone part, on the other hand, has the character of a weak base. This basicity is enhanced by the introduction of the phenolic group in the position 7, and is not affected by the successive methylation in the position 4 or that of the phenolic group. The excited state features an increase of basicity in this case.

Although the presence of the phenolic group is not prerequisite for the formation of the fluorescing cation in strongly acidic medium, its introduction results in a great increase of the quantum yield of fluorescence; the quantum yield is additionally raised by the methylation of this phenolic group (Table II). The results indicated, however, that there still is a difference between the fluorescence of the coumarin and umbelliferone cations, which is evident if the Stokes shifts of the two substances are compared (this shift is proportional to the difference between the excitation and fluorescence wavelengths): for umbelliferone derivatives it makes $4000-5000\,\text{cm}^{-1}$ – for the anion, neutral form as well as cation – whereas for the coumarin cation it attains almost $8000\,\text{cm}^{-1}$. So high a Stokes shift is peculiar to molecules where the excited state energy is partly consumed for the process occurring during the excitation[15]. Thus, *e.g.*, the dissociation of the umbelliferone phenolic group in the excited state is accompanied by a Stokes shift of $8000\,\text{cm}^{-1}$. (The values given are not quite accurate, as the spectra have not been corrected.) An explanation of the enormously high Stokes shift for the coumarin cation may lie in successive protonation of the cation to the diprotonized form in the excited state.

An interesting question is, in which part of the coumarin molecule the protonation takes place. The phenolic group does not seem to be this basic centre, because the acidity of the responsible centre of the umbelliferone molecule drops in the excited state[2,8]. Yakatan and coworkers[8] assume the protonation at the carbonyl oxygen, similarly as is the case with carboxylic acids. However, the highest electron density in the α-pyrone part of the molecule of coumarin itself does not appear at the hetero atom, and, moreover, unlike aromatic carbonyls the coumarin carbonyl oxygen loses a part of its electron density in the excited state[16]. HMO quantum chemical calculations did not evidence an appreciable difference in the basicity of the hetero atom and carbonyl of the umbelliferone or coumarin molecule. Thus it seems to be appropriate to consider the basicity of the α-pyrone part rather than the basicity of the hetero atom or the carbonyl.

The dissociation constants of coumarin derivatives in the ground state have been measured by Yakatan and coworkers[8]. The constants obtained in the present work agree with them; a marked difference was found in the case of the coumarin cation, for which the authors[8] report the value $pK_a = -7{\cdot}4$. As can be seen from Table III, the basicity of the α-pyrone part of the molecule of 4-methyl-7-hydroxycoumarin in the excited state ($pK_3^* = 1{\cdot}67$) exceeds that of the phenolate oxygen ($pK_1^* = 0{\cdot}45$), although in the ground state the dissociation constants of the two centres differ by 12 orders of magnitude. As a consequence, the neutral species II is formed in the excited state in a negligible concentration only. This result is in a good agreement with the data published[8]. It should be noted that the dissociation constants K_3^* and K_4^* as found from Eq. (*3*) are not quite accurate, as the values of the K_1^* and K_2^* constants inserted involve an error. (It fact, inaccuracy of 1 nm in the position of the absorption band maximum is reflected by an error of 0·2 in the pK value for the excited state). After the disscociation of the proton from the phenolic group the stabilization of the anion formed is good. This is corroborated by the umbelliferone being more acidic than naphthol and also by HMO quantum chemical calculations of the electron densities showing a 40% drop of the density at the phenolic oxygen on the proton dissociation and a simultaneous slight increase of the density at the carbonyl oxygen. The HMO method is not suitable for calculations of the electron densities at atoms in a molecule in the excited state, still its results are not in contrast with the experimental finding that the basicity of the α-pyrone ring rises in the excited state. It thus seems to be appropriate to write the formula of umbelliferone in the excited state at least as



It is interesting that the amphoion *III* was observed also in mixtures ethanol–water. It is feasible that it is formed in this medium by proton transfer from the phenolic group to the α-pyrone part in the excited state[8]. Pohl[17] attributed the occurrence of this maximum to the excimer formation, he did not explain, however, why then its intensity is independent of the umbelliferone concentration.

The neutral form *II* appears in the excited state only in a medium preventing its dissociation. Its fluorescence intensity depends upon the polarity of the surrounding medium. The fluorescence of coumarins in nonaqueous medium can be characterized by closely lying $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions. The energy difference between the two transitions is affected by the substituent types as well as by the medium[18]. For substances with a $\pi \rightarrow \pi^*$ transition it is typical that the fluorescence maximum is usually red-shifted in polar medium with respect to that in nonpolar medium, whereas the reverse is true for substances with an $n \rightarrow \pi^*$ transition. The cause of this behaviour lies in the different solvation of the excited state[19]. As can be seen from Table IV, 4-methyl-7-hydroxycoumarin behaves as a substance with the lowest excited singlet of the $\pi \rightarrow \pi^*$ type. The methylation of the phenolic group obviously enhances the probability of the $n \rightarrow \pi^*$ transition. The dependence of the fluorescence on the medium polarity is then not very pronounced; the fluorescence intensity of this methyl derivative increases, however, on addition of water. This has been observed in the case of some quinoline derivatives too. The electron pair at the nitrogen atom facilitates the conversion of the excited singlet state to the triplet state and brings about an internal effective quenching of fluorescence. If this electron pair is hydrogen bonded, the transition between the levels is less probable and the fluorescence intensity increases rapidly[15]. This can be anticipated for 4-methyl-7-methoxycoumarin too. The electron density at the phenolic oxygen was found to lower in the excited state. This could be the first centre to cause the radiationless decay of the excited state. To what extent the electrons of the α-pyrone part are involved cannot be inferred from the above results. At any rate, the hydrogen bonds affect the molecule of the substance in such a way that they preserve the $\pi \rightarrow \pi^*$ transition as the lowest excited singlet state.

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