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Phototautomerism of Warfarin Cation in Lowest Excited Singlet State *via* an Intramolecular Hydrogen Bridge

G. J. YAKATAN*, R. J. JUNEAU, and S. G. SCHULMAN▲

Abstract □ The pH and Hammett acidity dependence of the fluorescence spectra of 4-hydroxycoumarin and warfarin were studied. Warfarin demonstrates an anomalous long wavelength emission in moderately concentrated sulfuric acid solutions. The emission properties of methylated derivatives of 4-hydroxycoumarin and warfarin were employed to show that the anomalous fluorescence of warfarin is due to a protonated zwitterionic excited species, formed by intramolecular proton transfer in the excited state of the warfarin cation from the hydroxyl group to the acetonyl oxygen atom. The long wavelength of the warfarin fluorescence in sulfuric acid may be useful in the selective fluorometric determination of warfarin in the presence of other 4-hydroxycoumarin derivatives.

Keyphrases □ Warfarin—pH and Hammett acidity dependence of fluorescent spectra, phototautomerism □ 4-Hydroxycoumarin—pH and Hammett acidity dependence of fluorescent spectra □ Phototautomerism—warfarin cation in the lowest excited singlet state □ Spectrophotofluorometry—analysis, warfarin and 4-hydroxycoumarin

Coumarin and several of its hydroxylated derivatives have found wide applications in analytical biochemistry and pharmacy. 7-Hydroxycoumarin (umbelliferone) has been employed as a fluorescent indicator in fluorometric titrations and as a fluorogenic substrate in the study of the kinetics of hydrolytic reactions catalyzed by phosphatase and sulfatase (1–4). Several derivatives of 4-hydroxycoumarin, notably warfarin and dicumarol (bishydroxycoumarin), are used extensively as anticoagulants in the prevention and therapy of thromboembolic disease.

Studies of the pH dependence of the fluorescences of 4-methylumbelliferone (hymecromone) and some related model compounds (5) have recently been employed to show that the red shifting of the blue fluorescence of the umbelliferone anion in the region near pH 2 is due to the formation of a zwitterion in the lowest excited singlet state, a species that is not measurably present in the ground electronic state of umbelliferone.

Solvent and acidity dependence studies of the fluorescences of drugs and related molecules are important from the standpoints of both the analytical chemistry and the photochemistry of pharmaceutically important compounds. From the analytical point of view, solvent and acidity studies are useful for the determination of the optimal emission wavelengths at which to carry out fluorometric analyses. Because the acidity dependence of fluorescence wavelengths frequently depends upon the thermodynamic parameters (*e.g.*, dissociation constants) of the excited molecules, rather than (or as well as) the ground state thermodynamic parameters, it is important to determine the excited state dissociation constants (pKa*) as well as the ground state dissociation constants (pKa) of drug molecules (having dissociable protons or basic groups capable of accepting protons). Due to the differences in electronic distributions between ground and electronically excited molecules, the pKa* values are generally quite different from the pKa values; thus, the pH regions in which the fluorescence spectra change are generally different from the pH regions in which the absorption spectra change as one prototropic form is converted to another. The knowledge of pKa* values of molecules is critical to the selection of the optimal solution acidity in which to carry out fluorometric determinations.

From the photochemical point of view, the shelflives of drugs and the phototoxic actions of some drugs are dependent upon the action of light on these compounds. While the photochemical reactions leading to decomposition and phototoxic action of drugs are probably primarily the results of the reactivities of the excited

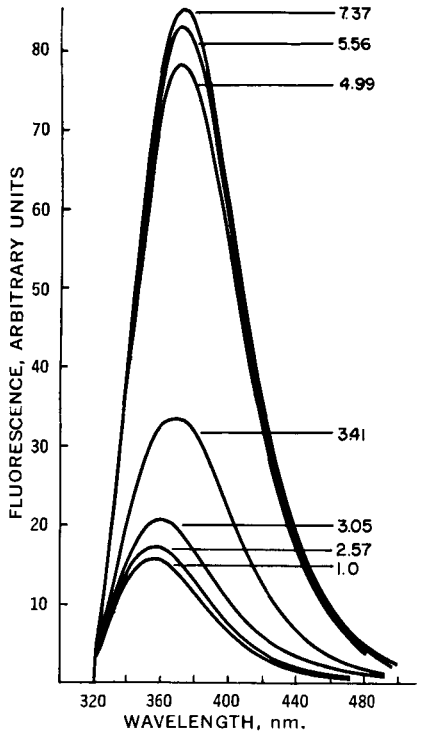


Figure 1—*Fluorescence spectra of ~10^{-4} M 4-hydroxycoumarin in the 1–7 pH range. The curves are labeled as to the pH value in which each spectrum was obtained.*

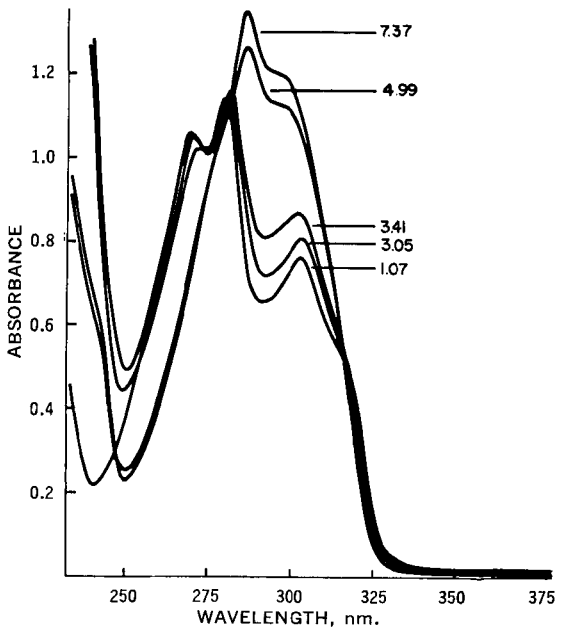


Figure 2—*Absorption spectra of ~10^{-4} M 4-hydroxycoumarin as a function of pH. The curves are labeled as to the pH value in which each spectrum was obtained.*

triplet states of these compounds, the triplet states are populated almost entirely from the lowest excited singlet states (the fluorescent states). The efficiency of singlet–triplet intersystem crossing (the population of the triplet state *via* the lowest excited singlet state) depends in large part upon the photochemistry of the lowest excited singlet state (*e.g.*, singlet–triplet separations and pKa* values). Knowledge of the photochemistry of the excited singlet state, by means of fluorescence spectroscopy, can lead to the technology that will provide the most light-stable package and dosage forms of potentially photolabile and phototoxic drugs.

Because of our interests in the analytical chemistry and photochemistry of anticoagulants derived from 4-hydroxycoumarin, the present study of the acidity dependence of the fluorescence spectra of 4-hydroxycoumarin and warfarin was undertaken.

EXPERIMENTAL[1]

4-Hydroxycoumarin[2] and warfarin[3] were obtained from commercial sources. The 4-methoxycoumarin and 4-methoxywarfarin derivatives were prepared by methylating the respective hydroxyl compounds with dimethyl sulfate and potassium carbonate in acetone. The methylated products had melting points that agreed with those in the literature (6, 7). All compounds were recrystallized from 95% ethanol prior to use.

Analytical reagent grade sulfuric acid[4] was purchased and used without further purification. Solutions of varying acidity for fluorometric and absorptiometric titrations were prepared by dilution of the sulfuric acid with distilled water. All other chemicals used in this study were of analytical reagent grade.

The corrected Hammett acidity scale of Jorgenson and Hartter (8) was employed for the concentrated sulfuric acid solutions.

Estimates of excited state dissociation constants (pKa*) were made by fluorometric titration and by employment of the Förster cycle (9):

$$\mathrm{pKa} - \mathrm{pKa}^* = \frac{Nhc}{2.3RT}\left(\frac{1}{\lambda_a} - \frac{1}{\lambda_b}\right) \qquad \text{(Eq. 1)}$$

where N is Avogadro's number, h is Planck's constant, c is the speed of light, R is the gas constant, T is the temperature, and λ_a and λ_b are the emission wavelengths of acid and conjugate base, respectively.

RESULTS

4-Hydroxycoumarin—The fluorescence spectrum of 4-hydroxycoumarin is invariant with pH in the region from 11 to 6. From pH 6 to 2, the fluorescence intensity varies with acidity and the fluorescence maximum shifts to the blue with increasing acidity (Fig. 1). The absorption spectra in the same pH range also vary with acidity (Fig. 2). As the acidity is increased still further, a red shift in the fluorescence emission occurs (Fig. 3).

The absorption spectra of 4-hydroxycoumarin also vary with acidity in the strong acid solutions (Fig. 4).

4-Methoxycoumarin—In contrast to the 4-hydroxy derivative, 4-methoxycoumarin does not fluoresce in the pH region from 0 to 11. The absorption spectrum does not vary with acidity in this range either. The neutral 4-methoxycoumarin molecule has an absorption spectrum very similar to the 4-hydroxy compound. As the acidity is increased in the Hammett range, the absorption spectra of the 4-methoxy compound change in a manner similar to those of the 4-hydroxy compound. Presumably, this is the result of protonation at the oxygen atom of the carbonyl group of the heterocyclic ring.

The protonated 4-methoxycoumarin molecule does fluoresce, the

[1] Fluorescence measurements were performed on a Perkin-Elmer model MPF-2A spectrophotofluorometer (Perkin-Elmer Corp., Norwalk, Conn.) whose monochromators were calibrated against the xenon line emission spectrum and whose output was corrected for instrumental response by means of a rhodamine-B quantum counter. The pH measurements were made on an Orion model 801 digital pH meter (Orion Research Inc., Cambridge, Mass.) equipped with a combination pH electrode (Sargent-Welch Scientific Co., Birmingham, Ala.). Absorption spectra were obtained using a Beckman DB-GT grating spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

[2] Aldrich Chemical Co., Cedar Knolls, N. J.

[3] K & K Laboratories, Plainview, N. Y.

[4] Mallinckrodt Chemical Works, Inc., St. Louis, Mo.

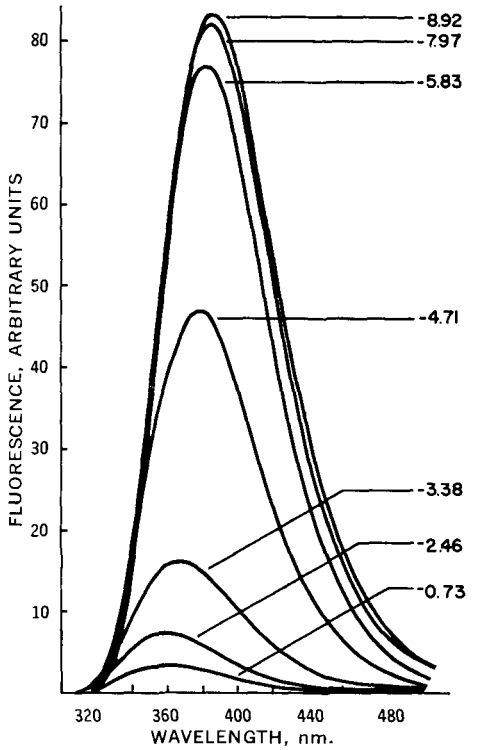


Figure 3—*Fluorescence spectra of ~10^{-4} M 4-hydroxycoumarin in sulfuric acid solutions. The curves are labeled as to the Hammett acidity value in which each spectrum was obtained.*

emission maximum shifting from 364 nm. at $H_0 = -2.46$ to 385 nm. at $H_0 = -8.92$.

3-(α-Acetonylbenzyl)-4-hydroxycoumarin (Warfarin)—The fluorescence of warfarin is unique among the coumarins studied due to the presence of an isoemissive point during part of the titration in the Hammett acidity region (Fig. 5). At the lower Hammett acidities, the emission maximum occurs at 354 nm. As the acidity is increased, the 354-nm. peak decreases and a corresponding increase in fluorescence at 470 nm. is observed. At $H_0 = -10.0$, a large (almost 10-fold) increase in fluorescence intensity occurs, with a maximum at 430 nm. Warfarin also fluoresces in the neutral pH region, having a maximum at 390 nm. The low solubility of the neutral warfarin molecule in aqueous solution prevented the determination of pKa, which occurs in the pH region around 4.

The absorption spectra of warfarin in the strong acid region are similar to those of the other 4-substituted derivatives studied (Fig. 6).

3-(α-Acetonylbenzyl)-4-methoxycoumarin (4-Methoxywarfarin)—The methoxy derivative of warfarin emits at 376 nm. ($H_0 = -0.73$) and at 416 nm. ($H_0 = -10.0$). The 416-nm. emission is much more intense than the emission observed in the lower acidities and is comparable to that seen with warfarin at $H_0 = -10.0$. The long wavelength emission and isoemissive point observed during the warfarin titration are absent in the titration of the methoxy derivative.

The absorption and emission spectral features of the 4-hydroxycoumarin derivatives studied are summarized in Table I.

DISCUSSION

The possible protolytic equilibria involving 4-hydroxycoumarin can be depicted as in Scheme I. Unlike the 7-isomer (5), 4-hydroxycoumarin does not appear to demonstrate phototautomerism in the lowest excited singlet state; the predominant uncharged species in the excited state, as well as the ground state, is N. In the neutral and alkaline pH range, the fluorescence emission maximum at 374 nm. is due to the anion, A. This maximum shifts to a shorter wavelength (357 nm.) as the neutral molecule, N, is formed. At higher acidities the cation, C, forms with a concomitant shift to a longer wavelength (374 nm.). The assignment of the path A → N → C is consistent with the fluorescence emission shifts expected to occur on successive protonation of the anion and then the neutral mole-

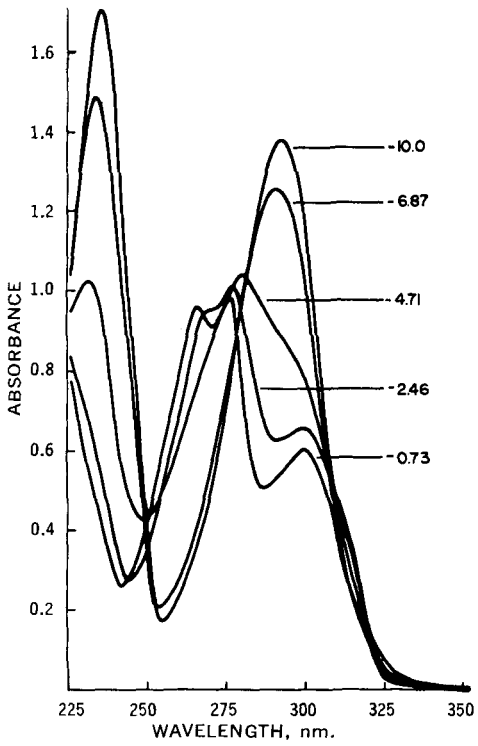


Figure 4—*Absorption spectra of ~10^{-4} M 4-hydroxycoumarin in sulfuric acid solutions. The curves are labeled as to the Hammett acidity value in which each spectrum was obtained.*

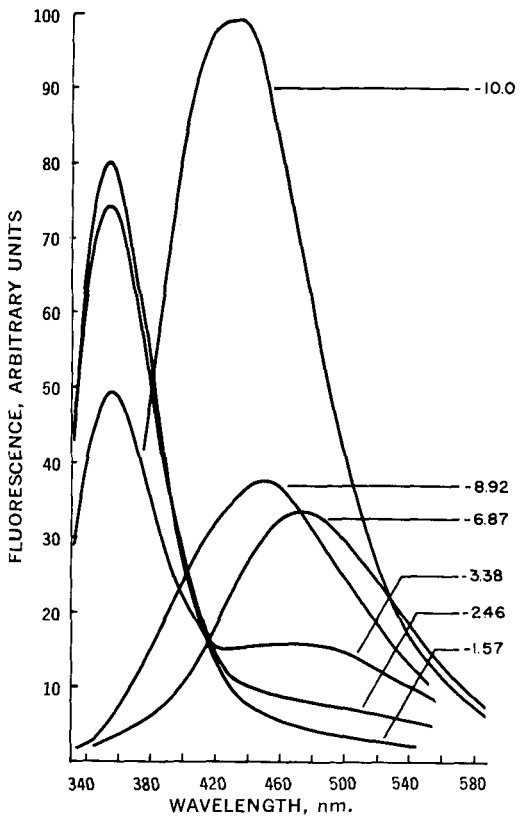


Figure 5—*Fluorescence spectra of ~10^{-4} M warfarin in sulfuric acid solutions. The curves are labeled as to the Hammett acidity value in which each spectrum was obtained. The curve at $H_0 = -10.0$ was obtained using a threefold attenuation of the fluorescence signal over that used for the other curves.*

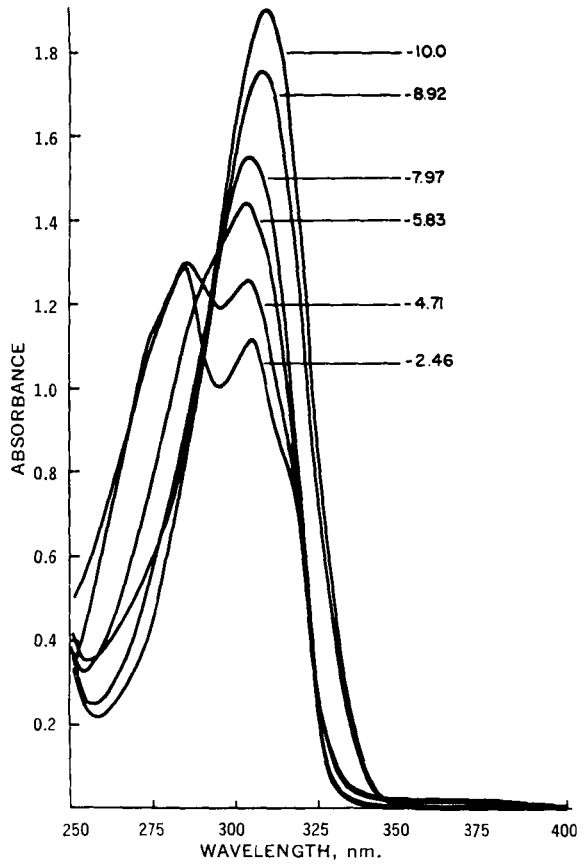
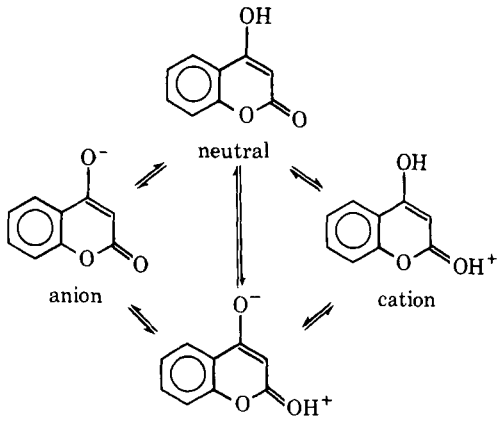


Figure 6—*Absorption spectra of ~10^{-4} M warfarin in sulfuric acid solutions. The curves are labeled as to the Hammett acidity value in which each spectrum was obtained.*

cule. The formation of the zwitterion, Z, from the anion, would be accompanied by a shift to a longer wavelength (5). Protonation of the zwitterion to form the cation would result in a shift of the emission maximum to a shorter wavelength. In each case, the opposite shift is encountered for the 4-isomer, indicating that the zwitterionic form does not contribute to the fluorescence of 4-hydroxycoumarin. The fact that 4-methoxycoumarin, from which the zwitterion cannot be formed, shows fluorescence in the same wavelength region as that attributed to the neutral 4-hydroxycoumarin confirms the absence of any contribution from the zwitterion. The absorption spectra of both the 4-hydroxy and 4-methoxy derivatives behave almost identically in concentrated sulfuric acid, indicating that the only functional group undergoing

Table I—Features of the Absorption and Fluorescence Spectra of Some 4-Substituted Coumarin Derivatives in Sulfuric Acid Solution

Compound	Absorption Maxima, λ, nm.		Fluorescence Maxima, λ, nm.	
	H_0 = −0.73	H_0 = −7.97	H_0 = −0.73	H_0 = −7.97
4-Hydroxycoumarin	316 (shoulder)	293	357	387
	303	233		
	280			
	271			
4-Methoxycoumarin	315 (shoulder)	291	364	385
	300	235		
	276			
	266			
Warfarin	315 (shoulder)	306	354	470
	305	234		
	283			
	275			



Scheme I

protonation in this acidity region is the oxygen of the carbonyl group.

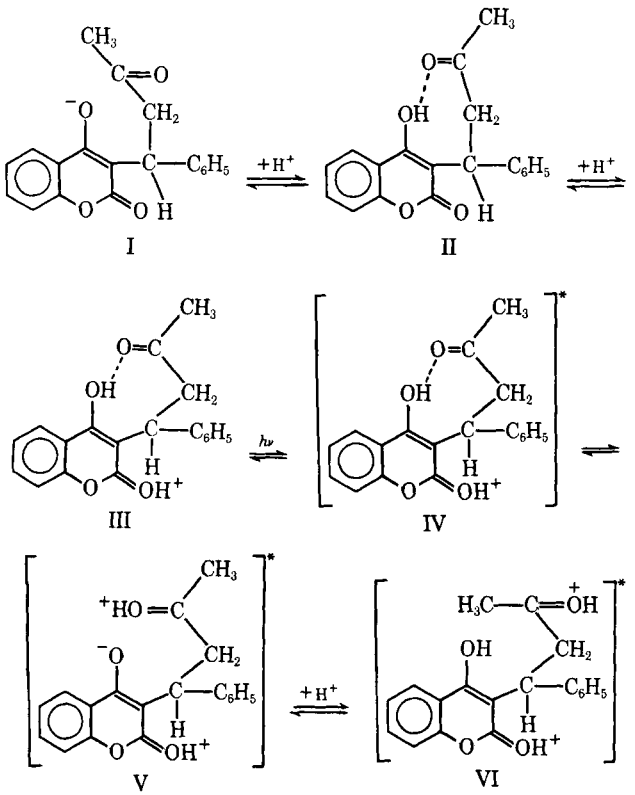
In a study on the metabolism of hydroxycoumarins, Mead *et al.* (10) reported a pKa for 4-hydroxycoumarin of 5.8. The authors did not state how this value was obtained or even whether they themselves had measured it. The present data (Table II) show that a pKa value of 4.1 was obtained by both fluorescence and absorption spectrophotometry. The difference in the ground state dissociation constants for ionization of the hydroxyl group in the 4- and 7-hydroxycoumarins is considerable and likely reflects the fact that these hydroxyl groups are different, the 7-hydroxyl being phenolic in nature and the 4-hydroxyl being vinylic.

The fluorescence shifts observed for the equilibrium between the neutral and anionic forms of 4-hydroxycoumarin indicate that the molecule should become more acidic in the excited state by approximately 3 units (11). Similarly, the shifts observed for the equilibrium between the neutral molecule and the cation indicate that the latter should become less acidic in the excited state by almost 5 units (11). In both cases, no variations of the fluorescence were observed in the acidity regions where excited state prototropism might occur. In fact, the pKa values obtained (Table II) were identical whether fluorescence or absorption measurements were used. Thus, dissociation of a proton from the cation or from the neutral species in the excited state must take place much more slowly than fluorescence can occur, and the thermodynamics of the ground state prototropic reactions determine which species fluoresce (11).

Although it is a derivative of 4-hydroxycoumarin, warfarin exhibits remarkably different fluorescence behavior in the Hammett acidity region. The warfarin anion fluorescence (λ = 390 nm.) shifts to a shorter wavelength on protonation in dilute acid (λ = 354 nm.). This is consistent with protonation of the enolate anion and with the similar behavior of 4-hydroxycoumarin. As the acidity is increased, a red shift consistent with protonation at the 2-carbonyl oxygen atom would be expected, as was observed with 4-hydroxycoumarin. The red shift does occur but is of a much greater magnitude than that observed for the parent compound. Above a Hammett acidity of −8.0, warfarin demonstrates a blue shift in fluorescence which is not observed with 4-hydroxycoumarin. This strongly indicates that the 3-acetonylbenzyl side chain plays a significant role in the complex emission behavior of warfarin. A

Table II—Dissociation Constants for Some 4-Substituted Coumarins Determined by Fluorescence and UV Spectra Titration Methods

Compound	pKa (Fluorometry)	pKa (Absorptiometry)
4-Hydroxycoumarin	−4.6	−4.6
	4.1	4.1
4-Methoxycoumarin	−4.5	−4.5
Warfarin	−4.0	~−5
	−9.2	



Scheme II

reasonable explanation of the fluorescence behavior of warfarin can be depicted as in Scheme II.

Protonation of the anion (I) in the ground state to form the neutral species (II) occurs in the region near pH 4. That protonation of the excited anion is too slow to compete with fluorescence is demonstrated by the coincidence of the pH regions for both absorptiometric and fluorometric titrations of the anion. The shift in fluorescence of the anion as it is converted to the neutral species is of such a magnitude that if prototropic equilibrium actually occurred in the lowest excited singlet state for these species, the fluorometric titration would occur in the region near $H_0 = -1.5$ (9).

Protonation of the neutral species in the ground state to form the cation (III) (protonated at the 2-carbonyl group) is demonstrated in the appropriate absorptiometric titration and occurs with pKa −5. However, after excitation, the excited cation (IV) transfers a proton from the 4-hydroxy group to the acetonyl oxygen atom of the side chain to form the excited charged zwitterionic species (V). This process obviously occurs within the lifetime of the excited state, since the absorption spectra do not reflect titration behavior appreciably different from 4-hydroxycoumarin or 4-methoxycoumarin while the fluorescence spectra do. In support of the process of intramolecular proton transfer in the excited state, the sum of the shifts to lower frequencies of 4-hydroxycoumarin fluorescence resulting from dissociation of the hydroxyl group and from protonation of the 2-carbonyl oxygen is almost identical with the shift to a lower frequency of the fluorescence of the neutral warfarin molecule produced by protonation. Moreover, the 4-methoxy derivative of warfarin, which has no hydroxy proton capable of being transferred, does not show the anomalous fluorescence behavior of warfarin in acidic media. Rather, protonation of the 4-methoxy derivative shows a red shift of fluorescence whose magnitude is consistent with simple protonation of the 2-carbonyl group. Furthermore, it is in the Hammett acidity region where the zwitterion forms that the acetonyl group [pKa −7.2 (12)] becomes capable of accepting a proton (the acetonyl group, being isolated from the aromatic system and transparent in the wavelength region of excitation, is thought to exhibit its ground state prototropic properties). That the phototautomerism of the excited warfarin cation is mediated by an intramolecular hydrogen bond between the 4-hydroxyl group and the acetonyl oxygen is supported by the failure of 4-hydroxycoumarin to exhibit phototautomerism. If a two-proton tautomerism (*i.e.*, transfer of a proton from the hydroxyl group to the solvent and transfer of a proton from the solvent to the acetonyl group) was responsible for the anomalous fluorescence behavior of warfarin, phototautomerism should also be exhibited by 4-hydroxycoumarin. That is, protonation of 4-hydroxycoumarin should result in excited state dissociation of the hydroxyl group, with an accompanying large red shift of fluorescence. Since this is not observed, it is concluded that the phototautomerism of the warfarin cation proceeds by reorientation of the intramolecularly hydrogen-bonded proton of the hydroxylic group in the excited state.

Protonation of the excited zwitterion in the most concentrated acid solutions, at the hydroxyl group, results in formation of the excited doubly protonated cation (VI) and causes the blue shift of the zwitterion fluorescence, which results in an emission similar to that of protonated 4-hydroxycoumarin. The pKa* for the latter excited state prototropic equilibrium is −9.2.

That warfarin fluoresces at much longer wavelengths in moderately concentrated sulfuric acid than do any of the other 4-hydroxycoumarin derivatives is analytically useful because it permits the selective fluorometric determination of warfarin in the presence of closely related structures.

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