

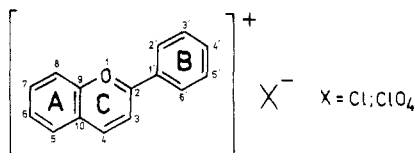
# Spectrophotometric Study of the Influence of Individual Substituted Positions on Flavylum Chromophore Stability

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In this paper the influence of C-7, C-4', and C-5 substituent positions and of the substituent type on structural transformations and on the stability of 4-substituted flavylum compounds was studied. Stability of the flavylum chromophoric system was highest in 7,4'-dihydroxy-4-methylflavylum chloride, out of four compounds studied, in a series of constant ionic strength buffer solutions (pH 2.00-11.00).

Flavylum (2-phenylbenzopyrylium) compounds, of the empirical formula  $(C_{15}H_{11-n}O(OH)_n)X$ , are the object of investigation of many authors, as reviewed by Brouillard (1988), because of their wide distribution in nature.



Structural transformations of flavylum compounds in aqueous solutions are well-known today (Brouillard, 1982): A complex equilibrium takes place between two colored forms (flavylum cation  $AH^+$  and anhydrobase A) and two leuco forms (pseudobase B and chalcone C). The sequence of transformations and the prevalence of individual transformational forms depends not only on the presence and type of the substituents in the A, B, and C rings of flavylum chromophore but also on positions which the substituents occupy.

The main approach in studying these compounds was the investigation of their stability and searching for optimum conditions in which it was reached. It has been recognized that positions C-7 and C-5 (A ring) and C-4' (B ring) (Timberlake and Bridle, 1967) have particular influence on the stability of the flavylum chromophore. In recent papers (Brouillard et al., 1982; Sweeny and Iacobucci, 1983; Mazza and Brouillard, 1987), position C-4 (C ring) was represented as having a more significant role. This is why the investigations in this paper refer to the spectroabsorptometric observation of transformational reactions of variably substituted flavylum compounds as a function of the pH value of the solution, as well as the determination of the optimum combination of substituents in the A, B, and C rings producing the highest stability of the flavylum chromophore.

## MATERIALS AND METHODS

**Samples.** The investigated flavylum compounds: 7,4'-dihydroxy-4-methylflavylum chloride (I), 7,4'-dihydroxy-4-phenylflavylum chloride (II), 7-hydroxy-4'-methoxy-4-phenylflavylum chloride (III), and 7-hydroxy-5-methyl-4-phenylflavylum chloride (IV) were synthesized according to the method of Timberlake (1968).

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**Table I. Parameter Values for the HMO Calculations**

element of group (X)	Coulomb integral ( $h_x$ )	resonance integral ( $K_{c-x}$ )
carbon (C)	0	1
pyrylium oxygen ( $O^+$ )	2	0.9
hydroxyl (OH)	1.2	0.8
methoxyl ( $OCH_3$ )	1.65	0.9
methyl ( $CH_3$ ) <sup>a</sup>	2	0.7

<sup>a</sup> Methyl group was treated on the basis of a heteroatom model.

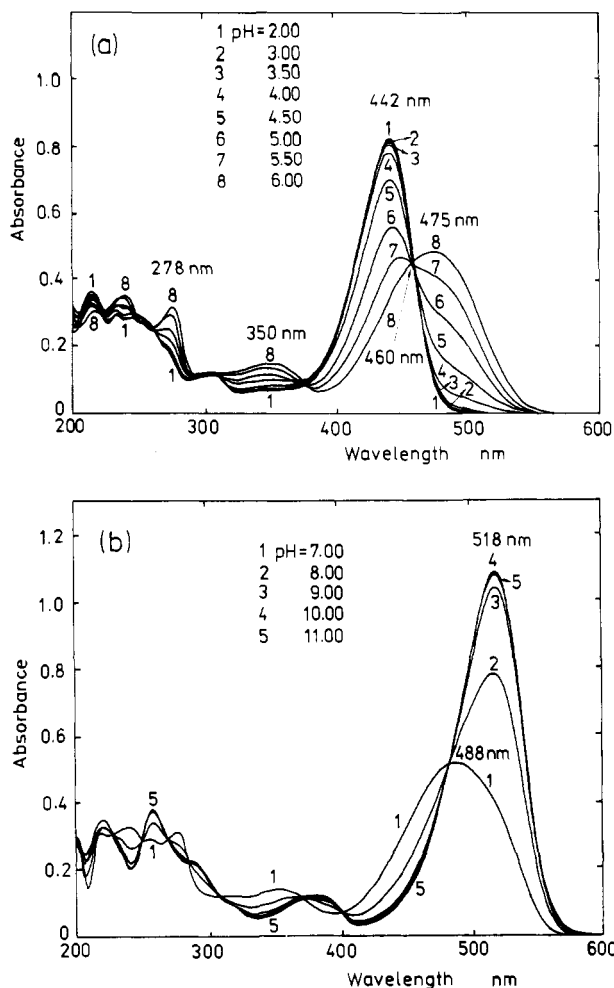
**Spectral Analysis.** Absorption spectra were recorded in a series of Britton-Robinson buffer solutions (pH 2.00-11.00) of constant ionic strength ( $I = 0.2 \text{ mol dm}^{-3}$ ). Solution concentrations were on the order of magnitude of  $10^{-5} \text{ mol dm}^{-3}$ . Spectra were recorded immediately after the solutions were made up at 20 °C. For that purpose a Pye Unicam UV-vis SP 8-100 recording spectrophotometer was used.

**Calculations.** HMO calculations were performed on an Atari 1040ST computer through a Pascal program (Amić et al., 1989). The parameters listed in Table I (Ohta et al., 1980; Trinajstić, 1987) were used in the calculations.

## RESULTS

Absorption spectra of compound I in buffer solutions (pH 2.00-6.00) are presented in Figure 1a. Characteristic absorption of the cationic form of this compound, which appears at  $\lambda = 442 \text{ nm}$ , is stable and almost unchanged up to pH 4.00. When the pH value of the solution is increased from 4.00 up to 6.00, a new absorption maximum is formed at  $\lambda = 475 \text{ nm}$ , while, at the same time, the absorption band of the cationic form disappears. The isosbestic point at  $\lambda = 460 \text{ nm}$  is evidence of the deprotonational equilibrium established between the cationic and the anhydrobase forms. In the UV region of the spectrum, the chalcone bands ( $\lambda = 350 \text{ nm}$ ) and the bands of flav-3-ene form of the pseudobase of this compound ( $\lambda = 278 \text{ nm}$ ) are indicated. In the neutral and alkaline solutions (pH 7.00-11.00), the presence of hydroxy groups in C-7 and C-4' positions causes further deprotonation. As can be seen from Figure 1b bathochromic and hyperchromic changes of the absorption band of the anhydrobase form are observable up to pH 10.00 (curve 4) where the anionic form of this chromophore is completely formed, with the absorption maximum at  $\lambda = 518 \text{ nm}$ .

In Figure 2a absorption spectra of compound II in acidic buffer solutions are presented. The cationic form of this

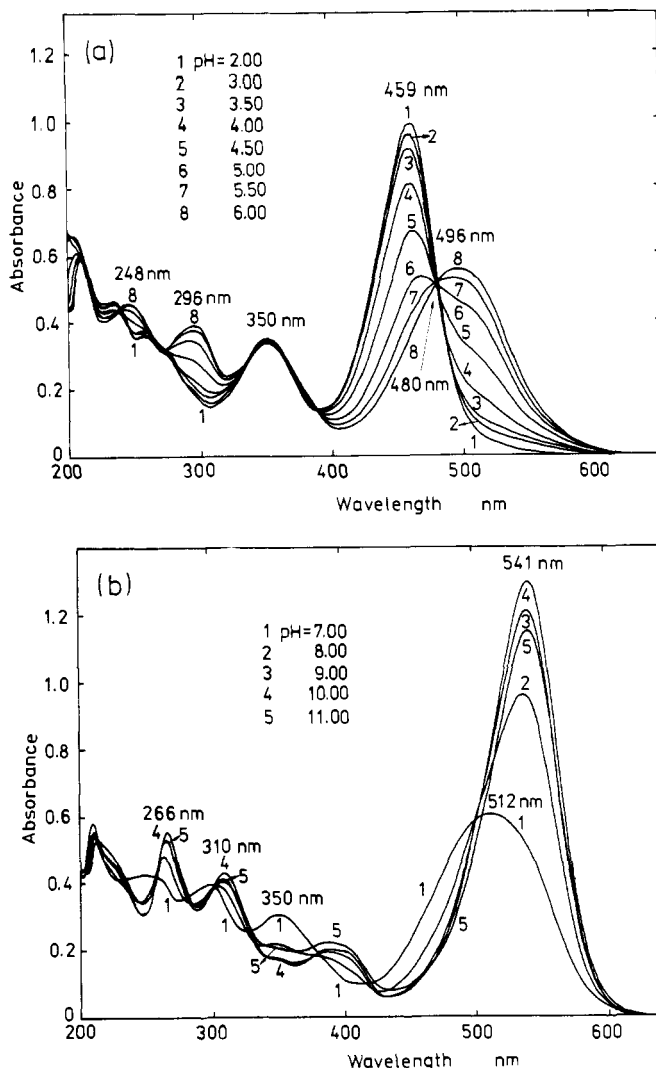


**Figure 1.** Absorption spectra of compound I in buffered solutions: (a) acidic; (b) neutral and alkaline.

compound at  $\lambda = 459$  nm is stable in acidic solutions (pH 2.00–3.50), being converted above that point by the process of deprotonation into the anhydrobase form ( $\lambda = 496$  nm). The isosbestic point appears here at  $\lambda = 480$  nm. In this range of pH values the chalcone bands at  $\lambda \approx 300$  nm were also observed. With this compound, hydroxy groups in positions C-7 and C-4' cause formation of the anionic form of the molecule in neutral and alkaline solutions. The absorption band of the anhydrobase form at  $\lambda = 496$  nm formed at pH 6.00 (Figure 2a, curve 8) shifts bathochromically and hyperchromically when the pH is raised. At pH 10.00 and  $\lambda = 541$  nm, the band of the anionic form of flavylium chromophore is formed (Figure 2b, curve 4).

The cationic form of compound III (Figure 3a) at  $\lambda = 458$  nm is stable in acidic solutions (pH 2.00–3.00). By raising the pH value and deprotonation of the 7-OH group, it is converted to the anhydrobase form ( $\lambda = 492$  nm) and the isosbestic point appears at  $\lambda = 476$  nm. The anhydrobase form, produced at pH 7.00 is stable in alkaline solutions up to pH 10.00 (Figure 3b). In alkaline solutions compound III cannot produce the ionized form of the anhydrobase, because the C-4' position is substituted by an  $\text{OCH}_3$  group. At pH 11.00, as can be seen in Figure 3b, curve 5, the anhydrobase form of this compound is converted into the ionized chalcone form ( $\lambda = 478$  nm).

Absorption spectra of compound IV in buffer solutions (pH 2.00–4.50) are presented in Figure 4a. The cationic form of this compound ( $\lambda = 432$  nm) is stable only in buffer solutions at pH 2.00 and 2.50. The lowering of

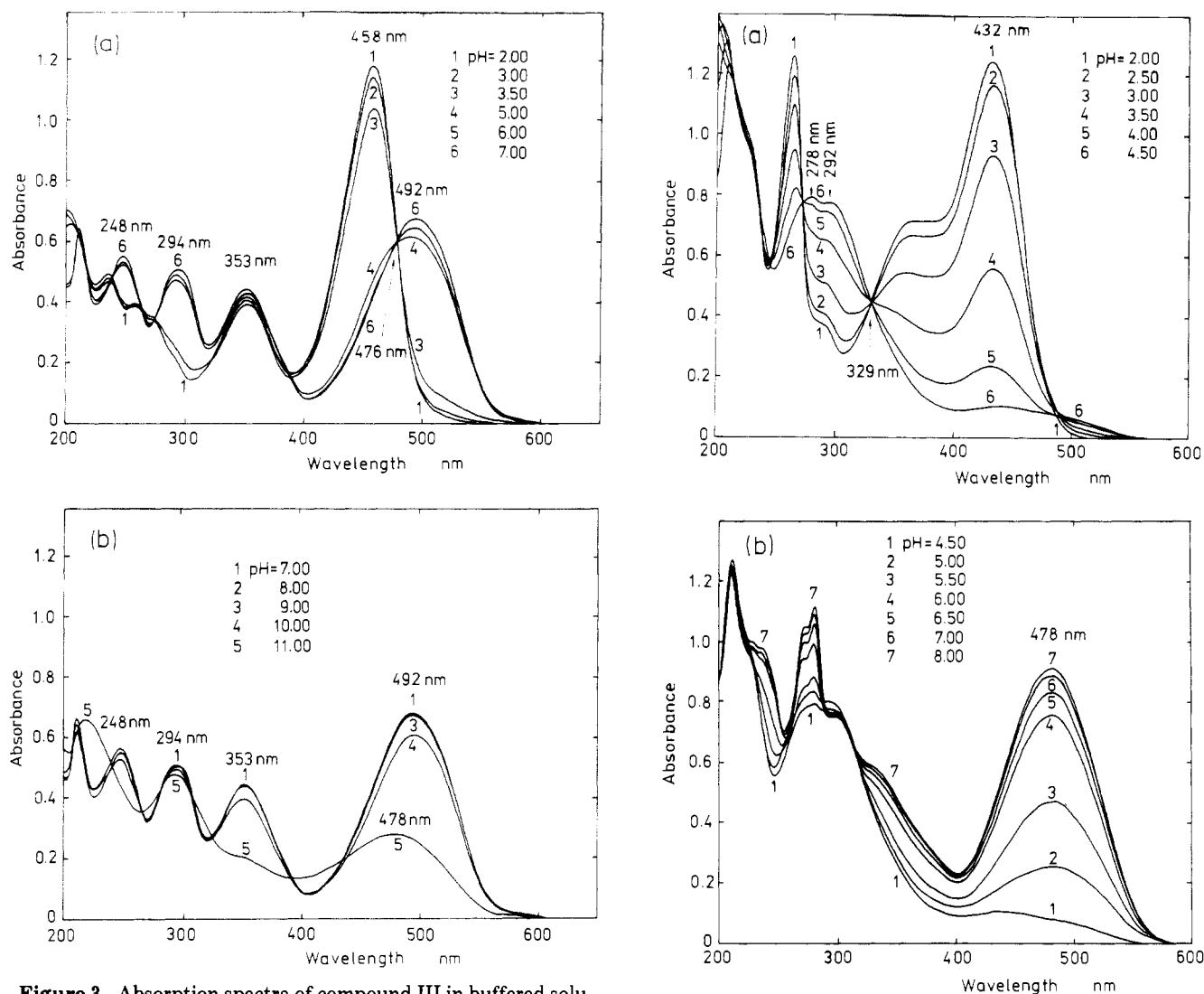


**Figure 2.** Absorption spectra of compound II in buffered solutions: (a) acidic; (b) neutral and alkaline.

solution acidity is followed by a pronounced hypochromic effect, while, simultaneously, a wide double band is formed with two maxima:  $\lambda = 278$  and  $292$  nm. This is why buffer solution at pH 4.50 (Figure 4a, curve 6) is practically colorless. In weakly acidic and neutral solutions the absorption band of the anhydrobase form appears. In Figure 4b, curve 7, it can be seen that the anhydrobase form of compound IV is completely formed at pH 8.00 and  $\lambda = 478$  nm. Stability of the anhydrobase form is pronounced at pH 9.00 (Figure 4c, curve 2), while at pH 10.00 a hypochromic effect on the fundamental band can be observed (curve 3). In buffer solution of pH 11.00 the anhydrobase disappears, probably followed by chalcone formation (curve 4).

## DISCUSSION

It is known that the majority of flavylium compounds are unstable in weakly acidic solutions due to formation of leuco pseudobase forms (if they have an OH group in position C-3) or chalcone forms (if they do not have a substituent in the C ring). Introducing a substituent in position C-4 of the flavylium chromophore, as can be seen from the present results, leads to establishing of deprotonational equilibrium and to significant stabilization of the cationic and anhydrobase form, by defavoring hydration reactions (Figures 1a, 2a, and 3a). Positions C-7 and C-4', as well as the combination of their substituents, also

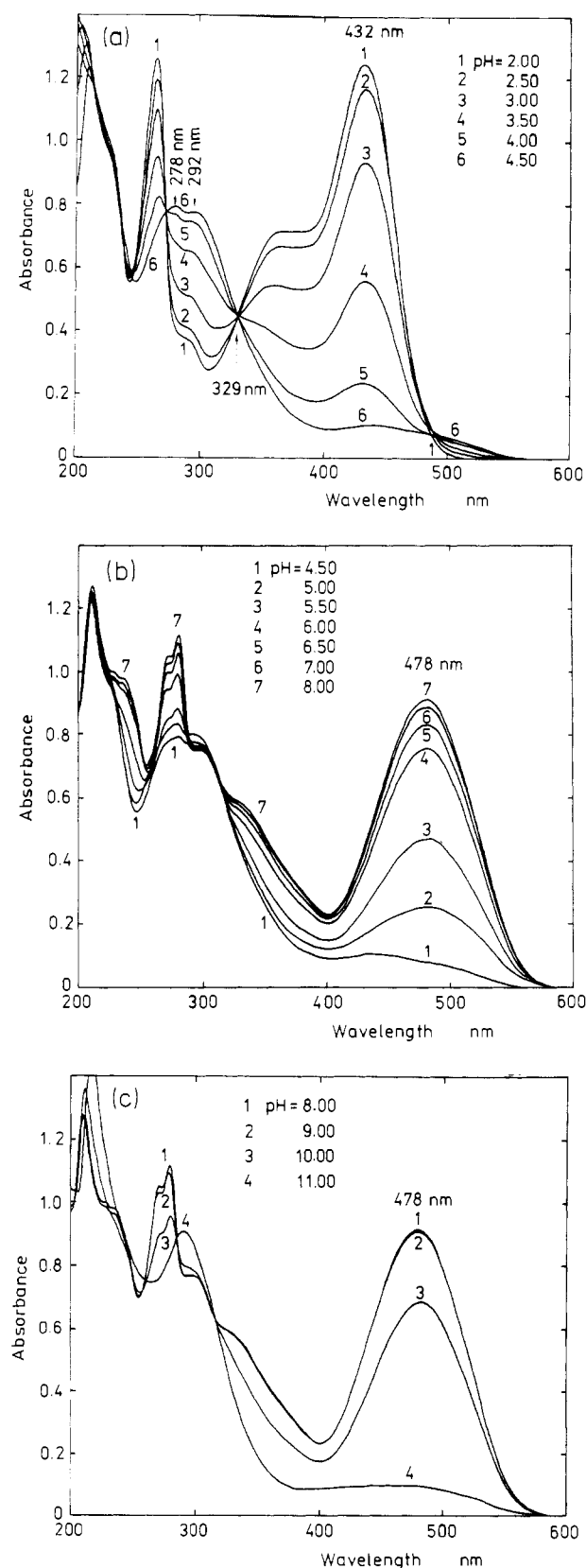


**Figure 3.** Absorption spectra of compound III in buffered solutions: (a) acidic; (b) neutral and alkaline.

have a significant influence on stability and possible further structural transformations of the anhydrobase form of thus substituted flavylum compounds in nearly neutral and alkaline solutions. If OH groups occupy those positions, tautomeric forms of anhydrobase are formed and further increase in alkalinity leading to deprotonation of the second hydroxy group and to formation of anionic forms (Figures 1b and 2b). Flavylum compounds having an OH group substituted only in position C-7, having OCH<sub>3</sub> in position C-4', can form neither tautomeric forms of anhydrobases nor anionic forms when alkalinity is raised, but they form ionized chalcone forms in strongly alkaline solutions (Figure 3b).

It has been recognized (Timberlake and Bridle, 1967; Ohta et al., 1980) that introducing the second substituent in position C-5 of the A ring of 4-nonsubstituted flavylum compounds raises the stability of flavylum chromophore. However, with 4-substituted flavylum compounds, according to our results (compound IV, Figure 4a) introducing the second substituent in the A ring of the flavylum chromophore significantly lowers the stability. According to Brouillard et al. (1982) a similar effect was observed with 5,7-dihydroxy-4'-methoxy-4-methylflavylum chloride.

**pK Values.** Stability of each of the investigated 4-substituted flavylum compounds was quantitatively defined by determining the equilibrium constant, namely the pK value. In Table II, besides our results, other pub-



**Figure 4.** Absorption spectra of compound IV in buffered solutions: (a) acidic; (b) approximately neutral; (c) alkaline.

lished pK values for some flavylum compounds are given as well.

Some pK values particularly indicate the raising of stability of the molecule when the substituent is introduced in position C-4 of the flavylum chromophore system. In this respect the methyl substituent in position C-4 has a greater effect than the phenyl one (Table II).

**Table II. Equilibrium Constants and pK Values of Some 4-Nonsubstituted and 4-Substituted Flavylium Compounds at 293 K and  $I = 0.2 \text{ mol dm}^{-3}$** 

compound <sup>b</sup>	$K, \text{ mol dm}^{-3}$	pK
4-H		
7-OH-fc <sup>c</sup>	$2.04 (\pm 0.2) \times 10^{-3}$	$2.69 \pm 0.04$
7,4'-(OH) <sub>2</sub> -fp	$3.55 (\pm 1.4) \times 10^{-4}$	$3.45 \pm 0.17$
7-OH-4'-OCH <sub>3</sub> -fp	$5.75 (\pm 1.9) \times 10^{-4}$	$3.24 \pm 0.14$
4-Ph		
7-OH-4-Ph-fc <sup>d</sup>	$5.37 (\pm 0.7) \times 10^{-5}$	$4.27 \pm 0.06$
7,4'-(OH) <sub>2</sub> -4-Ph-fc	$3.24 (\pm 0.2) \times 10^{-5}$	$4.49 \pm 0.03$
7-OH-4'-OCH <sub>3</sub> -4-Ph-fc	$3.63 (\pm 0.8) \times 10^{-5}$	$4.44 \pm 0.09$
7-OH-5-CH <sub>3</sub> -4-Ph-fc	$4.79 (\pm 1.9) \times 10^{-4}$	$3.32 \pm 0.17$
4-CH <sub>3</sub>		
7-OH-4-CH <sub>3</sub> -fc <sup>d</sup>	$3.80 (\pm 0.3) \times 10^{-5}$	$4.44 \pm 0.03$
7,4'-(OH) <sub>2</sub> -4-CH <sub>3</sub> -fc	$1.45 (\pm 0.1) \times 10^{-5}$	$4.84 \pm 0.03$
7-OH-4'-OCH <sub>3</sub> -4-CH <sub>3</sub> -fc <sup>e</sup>	$1.60 (\pm 0.1) \times 10^{-5}$	$4.80 \pm 0.03$
5,7-(OH) <sub>2</sub> -4'-OCH <sub>3</sub> -4-CH <sub>3</sub> -fc <sup>e</sup>	$7.90 (\pm 0.5) \times 10^{-5}$	$4.10 \pm 0.03$

<sup>a</sup> Refers to our measurements. <sup>b</sup> Key: fc = flavylium chloride; fp = flavylium perchlorate. <sup>c</sup> From Timberlake and Bridle (1967). <sup>d</sup> From Baranac and Amić (1985). <sup>e</sup> From Brouillard et al. (1982).

**Table III. Percentages of the Cationic Form in Aqueous Solutions at pH 4.50**

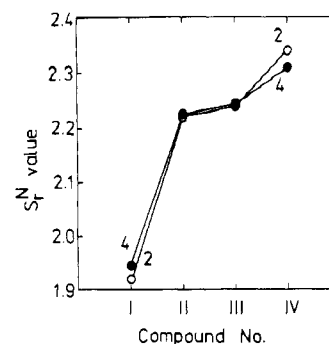
compound	AH <sup>+</sup> , %
7-OH-fc	2
7-OH-4'-OCH <sub>3</sub> -fp	5
7-OH-5-CH <sub>3</sub> -4-Ph-fc	6
7,4'-(OH) <sub>2</sub> -fp	8
5,7-(OH) <sub>2</sub> -4'-OCH <sub>3</sub> -4-CH <sub>3</sub> -fc	28
7-OH-4-Ph-fc	37
7-OH-4-CH <sub>3</sub> -fc	45
7-OH-4'-OCH <sub>3</sub> -4-Ph-fc	47
7,4'-(OH) <sub>2</sub> -4-Ph-fc	49
7-OH-4'-OCH <sub>3</sub> -4-CH <sub>3</sub> -fc	67
7,4'-(OH) <sub>2</sub> -4-CH <sub>3</sub> -fc	69

If a comparison is drawn to flavylium compounds with no substituents in position C-4, the effect is particularly pronounced. Among presently investigated compounds, the most efficient combination of substituents is achieved in 7,4'-disubstituted compounds where position C-4 is substituted. A methyl substituent in position C-4, with OH groups in C-7 and C-4', produces the highest stability of the flavylium chromophoric system ( $pK = 4.84$ ), which is even higher than with a phenyl substituent in the same position ( $pK = 4.49$ ). Further on, substituent OCH<sub>3</sub> (taking place of an OH group) in position C-4' lowers the stability of this chromophore.

Quantitative proof of the existing equilibrium and the equilibrium constants determined, namely the pK values, made possible the present amount of cationic (colored) form to be expressed as a percentage value. Thus, for example, the cationic form of investigated compounds, at pH 4.50, in our buffer solutions was present in percentages listed in Table III.

Favorable combination of substituents in A and B rings, together with substituted position C-4, produces not only maximum stability but also the highest percentage of the cationic form of this molecules and, thus, the highest coloration of the solution.

**$S_r^N$  Indices.** The effects of various substituents in different positions on the stability of flavylium compounds were followed through HMO reactivity parameters (Bendz et al., 1967; Kurtin and Song, 1968). For purposes of comparison of the reactivity of different positions in the  $\pi$ -electronic system of related molecules, the property of superdelocalizability index was introduced. For the nucleophilic attack superdelocalizability index is highest in positions C-2 and C-4 of flavylium chro-

**Figure 5. Effect of substituents on  $S_r^N$  values in positions C-2 (○) and C-4 (●) of the flavylium chromophore.  $S_r^N$  = superdelocalizability index for the nucleophilic attack.**

mophore, so that these positions show the highest reactivity toward nucleophilic reagents, like H<sub>2</sub>O. In our case that results in pseudobase formation and distortion of the flavylium chromophore. Superdelocalizability indices calculated for our investigations of compounds I-IV are presented in Figure 5. As can be seen, the lowest values were derived for compound I ( $S_2^N = 1.918$  and  $S_4^N = 1.944$ ; values previously published by Ohta et al. (1980) agree with ours).

Comparison with, for example, 7-hydroxyflavylium chloride ( $S_2^N = 2.411$  and  $S_4^N = 2.483$ ) shows a pronounced influence of the substituent in position C-4 on the stability. The effect is greater with the methyl substituent than with the phenyl one, as could have been assumed on the basis of the absorption spectra and the pK values. The hydroxy group in position C-4' (Figure 5, compounds II and III) shows a somewhat greater influence on the stability than the methoxy group. The greatest values of this index being derived for compound IV ( $S_2^N = 2.341$  and  $S_4^N = 2.312$ ) show that the stability of 4-substituted flavylium chromophore is, to a greater extent, disturbed by the substitution in position C-5. So, the  $S_r^N$  indices calculated for compounds I-IV are in agreement with experimental results derived through investigating the influence of positions C-7, C-5, C-4', and C-4, and the type of substituents, on the stability of flavylium chromophoric system.

## CONCLUSIONS

From the presented results a conclusion can be drawn that a substituent in position C-4 contributes to the stability of flavylium compounds. In this respect, the methyl group shows a greater effect than the phenyl one. The best combination of substituents was found with compound I, namely, 7,4'-dihydroxy-4-methylflavylium chloride, which shows maximum stability, both through pK values and through calculated superdelocalizability indices. It also has the highest percentage of the cationic form of this chromophore, which produces the highest coloration of the solution. These facts can be of particular applicative interest if flavylium compounds are discussed as possible dye additives in the food industry.

## ACKNOWLEDGMENT

We express our sincere thanks to Dr. C. F. Timberlake, University of Bristol, for valuable advice for preparation of flavylium compounds and for gifts of authentic samples of 7,4'-dihydroxy-4-methyl-, 7,4'-dihydroxy-4-phenyl-, and 7-hydroxy-4'-methoxy-4-phenylflavylium chlorides.

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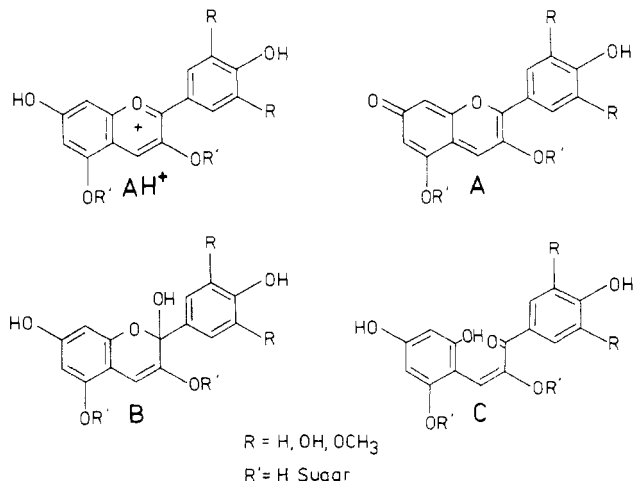
## Reactivity of Some Flavylium Cations and Corresponding Anhydrobases

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The indices of superdelocalizability were calculated for the nucleophilic attack in positions C-2 and C-4 of the cationic and anhydrobase forms of 10 synthetic flavylium salts. Compounds 1-5 have no substituent at position C-4, while compounds 6-10 are methylated at this position. The inductive model was shown to be the most appropriate for treating the methyl group. The obtained results indicate that decolorization of weakly acidic solutions of flavylium salts was caused by hydration reactions taking place at the C-2 position of the anhydrobase form of flavylium chromophore. UV-vis spectra of compounds 2 and 5 confirm the obtained results.

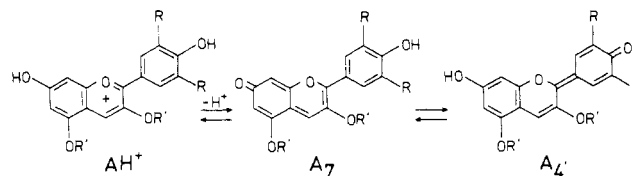
Flavylium (2-phenylbenzopyrylium) salts in acidic aqueous solutions attain an equilibrium between two colored forms, cationic AH<sup>+</sup> and anhydrobase A, and two colorless forms, pseudobase B and chalcone C (Brouillard, 1982):



Flavylium salt glycosides—anthocyanins—are associ-

ated with the largest number of red and blue hues of flowers, fruits, and vegetables. In vivo mechanisms such as intermolecular and intramolecular pigmentation stabilize the colored forms of flavylium chromophore (Brouillard, 1983). When separated from cell fluids, most of anthocyanins (as well as their aglycons anthocyanidins) are virtually colorless within the range pH  $\approx$  4-6 (Asen, 1976). The increased interest for the application of flavylium compounds as food colorants induced not only isolation of some stable natural acylated anthocyanins (Brouillard, 1981) but also the syntheses of a number of 4-substituted flavylium salts, which keep stability over a wide range of pH values (Timberlake, 1968).

Flavylium salts are stable only in strongly acidic solutions. Reduction of acidity causes deprotonation and transformation of cationic form into the anhydrobase form. If the flavylium core is substituted with OH groups in several positions, a number of prototropic tautomers is formed, for example:



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