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COMPLEXATION OF A FLUORESCENT ANTHOCYANIN WITH PURINES AND POLYPHENOLS

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Key Word Index—Anthocyanin complexation; purines; polyphenols; UV-visible, fluorescence and ^{1}H NMR spectroscopy.

Abstract—It is demonstrated that the synthetic anthocyanin 7-hydroxy-3,4′-dimethoxyflavylium chloride associates with copigments in a manner which mimics that of natural anthocyanins. When studying copigmentation, 7-hydroxy-3,4′-dimethoxyflavylium chloride offers several advantages over its natural homologues. Firstly, it is easy to prepare pure. Secondly, its unexpected strong fluorescence opens up a new field in copigmentation studies. Finally, being sufficiently water-soluble, its complexation can also be investigated by means of ^{1}H NMR spectroscopy. Copigments investigated are: chlorogenic acid, gallic acid, caffeine, adenosine 5′-monophosphate and adenosine 5′-triphosphate.

INTRODUCTION

Phytochemical aspects of copigmentation are closely related to plant pigmentation mechanisms due to the simultaneous presence of anthocyanins and their copigments [1]. However, on the basis of a recent work [2], it has been shown that the natural copigmentation phenomenon belongs to the much wider field of bio-organic molecule complexation, including polyphenol complexation [3]. Much of the work done on copigmentation has been performed using natural anthocyanins [4]. In order to scrutinize structural features necessary for an anthocyanin to associate strongly with copigments, we designed a synthetic, non-natural homologue, with the hope that it would behave like the good copigmenting natural anthocyanins. The anthocyanin investigated in the present work is 7-hydroxy-3,4′-dimethoxyflavylium chloride (**1**). With its hydroxyl group at C-7, this flavylium cation deprotonates to the corresponding quinonoidal base **1A**, a characteristic structural change of natural anthocyanins. The presence of the methoxy group at C-3 favours the nucleophilic attack of water on the C-2 site of the flavylium cation thus giving large amounts of colourless forms. This hydration process largely regulates the magnitude of the copigment effect [5]. We found that **1** not only mimics natural anthocyanins with regard to copigmentation, but also it turned out to be a highly fluorescent molecule. Moreover, being sufficiently soluble in heavy water–organic solvent mixtures, further investigation of its copigmentation could be conducted with the help of ^{1}H NMR spectroscopy.

In this paper, we report on the copigmentation of 7-hydroxy-3,4′-dimethoxyflavylium chloride (**1**). Measurements were made with either electronic spectroscopy—absorption and fluorescence—or ^{1}H NMR spectroscopy. The copigments selected are all naturally occurring substances: chlorogenic acid (**2**), gallic acid (**3**), caffeine (**4**), adenosine 5′-monophosphate or AMP (**5**) and adenosine 5′-triphosphate or ATP (**6**).

RESULTS AND DISCUSSION

A short description of the behaviour of the flavylium cation **1**, in aqueous solution, is given before we report on its association with the above copigments.

Compound **1** has been prepared previously [6], but its evolution, according to the solution acidity, has never been studied before. Using UV-visible and ^{1}H NMR spectroscopy, we were able to demonstrate the existence of four, out of five, of the species shown in Fig. 1 which are the flavylium cation $\mathbf{1AH^+}$, the quinonoidal base **1A**, the carbinol pseudobase **1B** and the retrochalcone $\mathbf{1C}_Z$; the retrochalcone $\mathbf{1C}_E$ could not be detected because of its low amount and/or because of its fast equilibrium with the carbinol pseudobase **1B** [7]. The flavylium cation is only stable in the more acidic solutions (pH lower than 1) and, as the pH increases, it transforms into a mixture of the neutral species **1A**, **1B**, $\mathbf{1C}_E$ and $\mathbf{1C}_Z$ according to the overall equilibrium: $\mathbf{1AH^+} \rightleftharpoons (\mathbf{1A}+\mathbf{1B}+\mathbf{1C}_E+\mathbf{1C}_Z) + H^+$. Since at equilibrium **1A** is a very minor species, the constant K_h for this equilibrium fully corresponds to the hydration process (Fig. 1). The analysis of the absorption data based on Sondheimer's method [8] gives $pK_h = 2.23$ at 20°. This value is close to those observed for the commonest natural anthocyanins [9], and it indicates that **1** should exhibit strong copigment effects.

The ^{1}H NMR spectra of **1** were recorded at different pD values: 1.15, 2.4, 3.6 and 4.5. Compounds $\mathbf{1AH^+}$, **1B** (in fact two stereoisomers $\mathbf{B}_2$ and $\mathbf{B'}_2$) and $\mathbf{1C}_z$ were characterized by this method and the corresponding results are shown in Figs. 2 and 3 and also in Table 1. At pD 1.15, the only stable form is the flavylium cation $\mathbf{1AH^+}$ (Fig. 2). Peaks belonging to the carbinol bases ($\mathbf{B}_2$

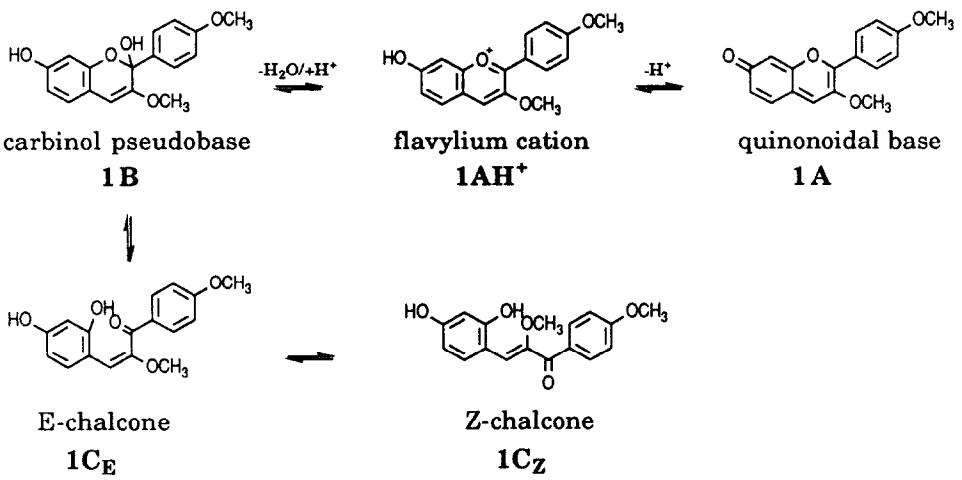
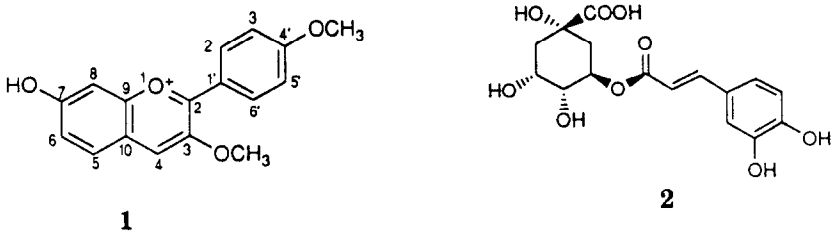


Fig. 1. Anthocyanin equilibria [7].



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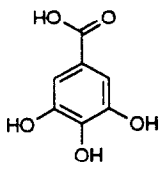

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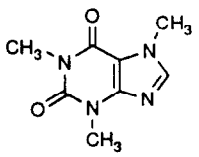

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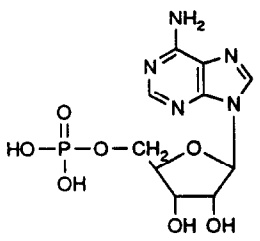

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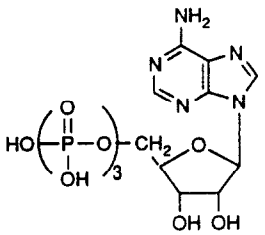

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and B'_2) and to the chalcone $1C_Z$ (Fig. 3) were identified in the following way. To a pD 4.5 solution a certain amount of DCl was added until the pD reached a value near 1. During the acidification process, all the neutral species rapidly revert to the flavylium cation, with the exception of the chalcone $1C_Z$ whose ^1H NMR spectrum could be recorded again after acidification [7]. It appears from these experiments that at pD 2.4 the dominant

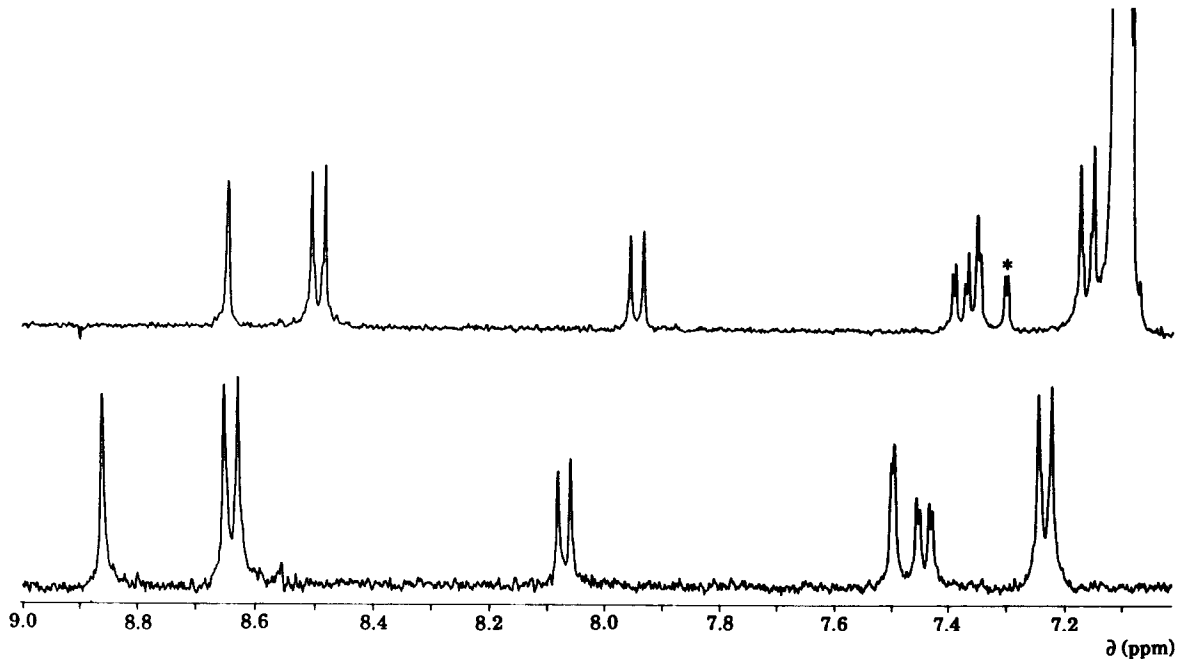

Fig. 2. ^{1}H NMR spectra (400 MHz) of 7-hydroxy-3,4′-dimethoxyflavylium chloride (**1**) (9.3×10^{-4} mol dm^{-3}) in D_2O–CD_3OD–DCl (66:33:1) at 27° and pD 1.15. Lower trace: **1** alone. Upper trace: **1** in the presence of gallic acid (3.72×10^{-2} mol dm^{-3}). Copigment to pigment molar ratio: 40. The star indicates satellites related to the strong gallic acid signal.

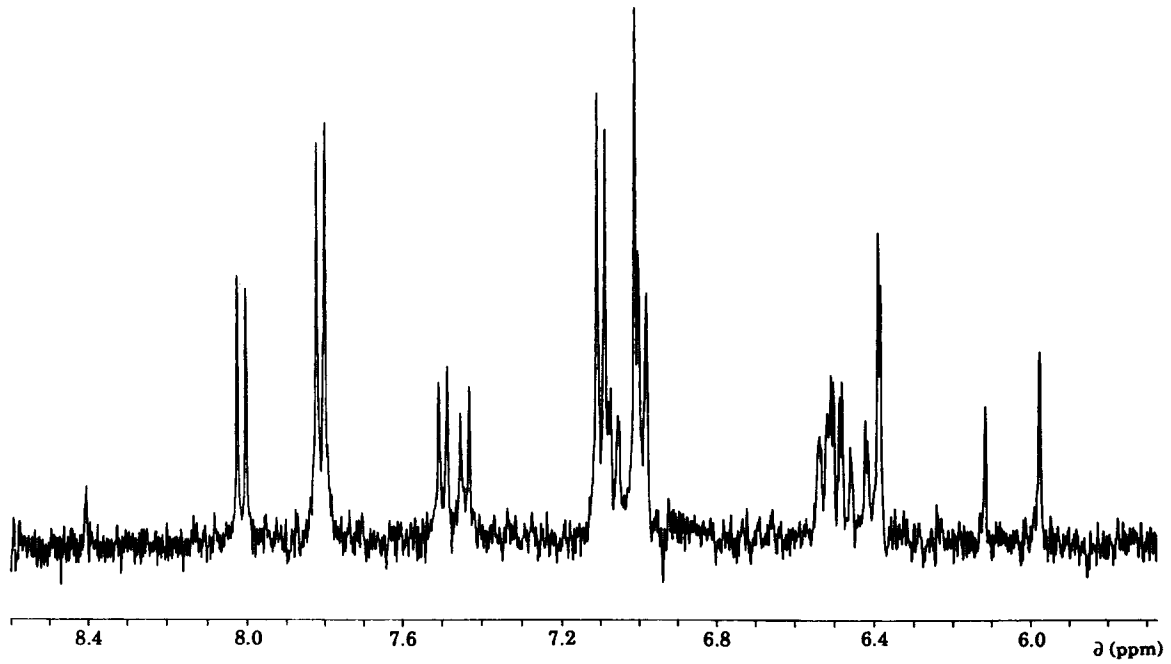

Fig. 3. ^{1}H NMR spectrum (400 MHz) of 7-hydroxy-3,4′-dimethoxyflavylium chloride (**1**) (9.3×10^{-4} mol dm^{-3}) in D_2O–CD_3OD–DCl (66:33:1). The free acidity, measured as pD, was adjusted to 4.5 by injecting into the solution of **1** a few microliters of concentrated NaOD. $T = 27°$.

species are the flavylium cation, the *Z*-chalcone and the carbinol bases. At higher pD (3.6 and 4.5), the flavylium cation could no longer be observed in this way.

Unexpectedly, we found out that **1** fluoresces in daylight. Anthocyanin fluorescence is usually not so strong although it has been used to distinguish between different anthocyanin categories [10]. Excitation and emission spectra of **1** are shown in Fig. 4. The excitation spectrum closely resembles the flavylium cation absorption spectrum with, in particular, the same maximum at 486 nm.

Table 1. ^{1}H NMR chemical shifts (ppm) and coupling constants (400 MHz) of the AH^+, B_2, B'_2 and C_Z forms of compound **1** (in D_2O–CD_3OD–DCl, 66:33:1 at 27°)

H	AH^+	B_2 and B'_2	C_Z
4 *s*	8.86	5.97; 6.11	6.99
5 *d* (8 Hz)	8.07	7.05; 7.06	7.98
6 *dd* (2 and 8 Hz)	7.44	6.46; 6.52	6.50
8 *d* (2 Hz)	7.50	6.37; 6.45	6.41
2′ and 6′ *d* (8 Hz)	8.64	7.45; 7.48	7.79
3′ and 5′ *d* (8 Hz)	7.24	6.99; 6.99	7.09
3 or 4′-OMe *s*	4.21*	3.83; 3.84	3.90
3 or 4′-OMe *s*	3.99	3.61; 3.63	3.70

*Assigned to OMe-3 by NOE.

B_2 and B'_2 are stereoisomers differing only in the configuration at C–2. No attempt was made to assign signals separately to each of these structures.

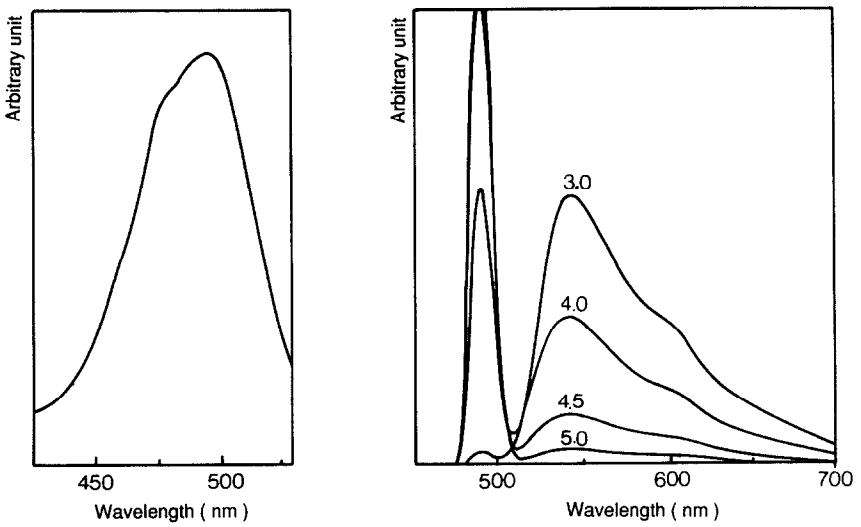

Fig. 4. Left: fluorescence excitation spectrum recorded at 543.6 nm of 7-hydroxy-3,4′-dimethoxyflavylium chloride (**1**) (10^{-4} mol dm^{-3}). pH 3.50; T=20°C; optical path length: 1 cm; solvent: aqueous citric acid–sodium hydrogen phosphate buffer. Right: fluorescence emission spectra of **1** (3×10^{-4} mol dm^{-3}) at pH 3.0, 4.0, 4.5 and 5.0. T=20°; optical path length: 1 cm; solvents: aqueous citric acid–sodium hydrogen phosphate buffers. In these experiments, Rayleigh scattering could not be completely eliminated. Reduction in the intensity of light scattered, as a function of pH, is explained by the solution self-absorption.

The latter value was, therefore, selected throughout this work as the excitation wavelength. Emission spectra of **1** have been recorded at various acidities (Fig. 4). It turns out that the fluorescence intensity decreases as the acidity decreases, following the disappearance of **1** coloured forms. A maximum is seen close to 540 nm and a shoulder consistently appears near 600 nm. The emission spectrum is not symmetrical with the excitation spectrum. This could come from different origins. Firstly, it may represent a vibrational feature belonging to the flavylium cation electronic ground state. Secondly, in the excited singlet state the acidity of the hydroxyl at C-7 may well be sufficient as to permit the excited flavylium cation **1** to deprotonate to the excited quinonoidal base **1A**, whose fluorescence is then responsible for the 600 nm shoulder. Effectively, it has been demonstrated that, in the case of phenolics, the acidity constant of an hydroxyl group drastically increases, when the molecule is in the excited state, as compared to its value in the ground state [11]. A third plausible situation corresponds to the instantaneous formation of an excimer (self-association between molecules in their ground and excited electronic states). Fluorescence emission of an excimer usually occurs at a longer wavelength than the monomer emission. More work is needed for a safe attribution of the spectral features associated with the fluorescence of **1**. Nevertheless, this spectroscopic property can be used to monitor the copigment effect as is shown below.

The absorption spectra of **1** are represented in Fig. 5, in the absence and in the presence of different copigments

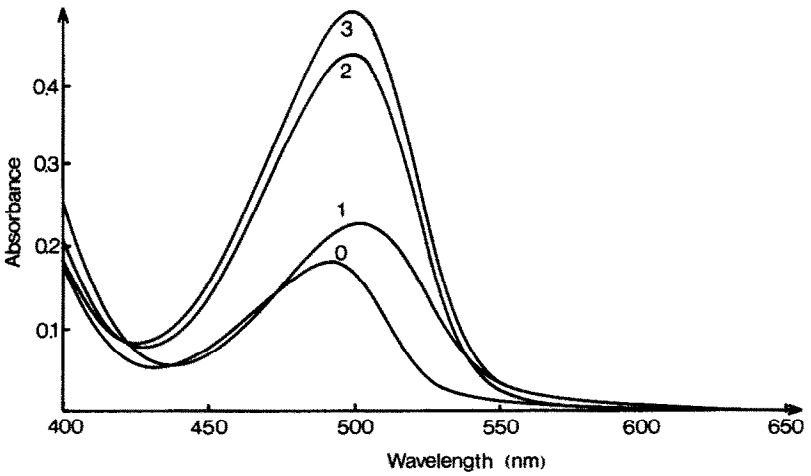

Fig. 5. Visible absorption spectra of 7-hydroxy-3,4'-dimethoxyflavylium chloride (**1**) (10^{-3} mol dm^{-3}) without copigment (0) and with either caffeine (1), ATP (2) or AMP (3). Optical path length: 0.1 cm; T = 20°; pH 3.5. Copigment to pigment molar ratio: 50. Solvent: aqueous citric acid–sodium hydrogen phosphate buffer.

caffeine, **4**; adenosine 5'-monophosphate, **5** or adenosine 5'-triphosphate **6**. It is seen that **5** and **6** behave similarly, while caffeine seems to associate only weakly with **1**. The influence of caffeine on the absorption of **1** is intriguing: there is a large bathochromic shift with only a small increase in the absorption band. In particular, the poor correlation usually observed between the bathochromic and hyperchromic effects completely fails in this case [12]. To derive from the UV data the stability constants for complex formation of both the flavylium cation **1** and the quinonoidal base **1A**, we used a previously established relationship [13]. K_1 and K_2 stand for the flavylium cation complexation constant and the quinonoidal base complexation constant, respectively. With chlorogenic acid (**2**) as copigment, K_1 is 250 $mol^{-1}dm^3$ and K_2 180 $mol^{-1}dm^3$. Such values are close to those reported in the case of the malvin to chlorogenic acid complex [13]. For AMP (**5**) and ATP (**6**) almost identical low values were measured: $K_1 = 40$ and $K_2 = 30$ $mol^{-1}dm^3$ (AMP) and $K_1 = K_2 = 30$ $mol^{-1}dm^3$ (ATP). Gallic acid (**3**) has a K_1 value equal to 100 $mol^{-1}dm^3$. Caffeine (**4**) deserves special attention since when using the copigmentation general equation [13], for estimating its complexation constants only fluctuating weak values could be obtained and they spread within the range 0–4 $mol^{-1}dm^3$, depending on the wavelength of observation. Note that when caffeine is mixed with malvin it behaves in a normal manner and significantly precise values for the strength of its association with malvin can be measured [3, 13]. This may point to the determining influence of the anthocyanin glycosyl residues in the copigmentation of malvin by caffeine (see concluding remarks). In the present work, complexation constants K_1 and K_2 derived from absorption data are given for a temperature of 20°.

Good fluorescence spectra could be recorded from more or less acidic solutions of **1** mixed with either caffeine (**4**), AMP (**5**) or ATP (**6**) (Fig. 6). The pH range investigated lies between 3 and 5 and temperature was maintained at 20°. All copigments studied show a pronounced effect on the emission spectra of **1** at any pH. For instance, at pH 3, AMP and even more caffeine, induce a decrease in the fluorescence intensity, whereas ATP produces a slight increase in fluorescence. In each case, small bathochromic shifts are observed. At pH 5, each copigment exhibits a distinct behaviour in the reshaping of **1** initial emission spectrum. Emission intensity at 540 nm is favoured by both AMP and ATP with the 600 nm shoulder now appearing as a separate band. For AMP, fluorescence intensity at 600 nm is even stronger than that at 540 nm. Caffeine also induces characteristic spectral features; while the 540 nm emission band is significantly weakened, the 600 nm shoulder here too transforms into a strong fluorescence band with a large bathochromic shift. Thus, depending on pH and nature of a given copigment, fluorescence quenching or fluorescence enhancement can be observed. This contrasts with the visible absorption experiments where an hyperchromic effect is always observed on copigment addition [12]. With some caution, it may be concluded from the fluorescence spectra, that caffeine and, to a lesser extent, AMP have more affinity for the quinonoidal base **1A** whereas ATP associates preferentially with **1** in the flavylium form AH^+. Note also that fluorescence spectrometry is more appropriate to the study of caffeine complexation than is absorption spectrometry.

It has been demonstrated by Haslam [14] that ^1H NMR spectroscopy is a good tool in the quantitative evaluation of the association between caffeine and a wide range of structurally different phenolic molecules. Haslam and his colleagues [3] also applied this technique to investigating complex formation between malvin and either β-1,2,3,4,6-pentagalloyl-D-glucose or caffeine. We, too, used the procedure of Haslam [3] to study the association of **1** with the simplest possible copigment, seen from the ^1H NMR point of view, that is to say gallic acid (**3**). Effectively, in the ^1H NMR experiments gallic acid is characterized by a unique singlet which does not interfere in the spectrum of **1** (Fig. 2). In the presence of gallic acid all anthocyanin protons are shifted upfield (Fig. 2 and Table 2). Such a result is in good agreement with data reported by Haslam [3] in the case of malvin-β-1,2,3,4,6-pentagalloyl-D-glucose and malvin-caffeine complexes. In the case of the present system, the proton

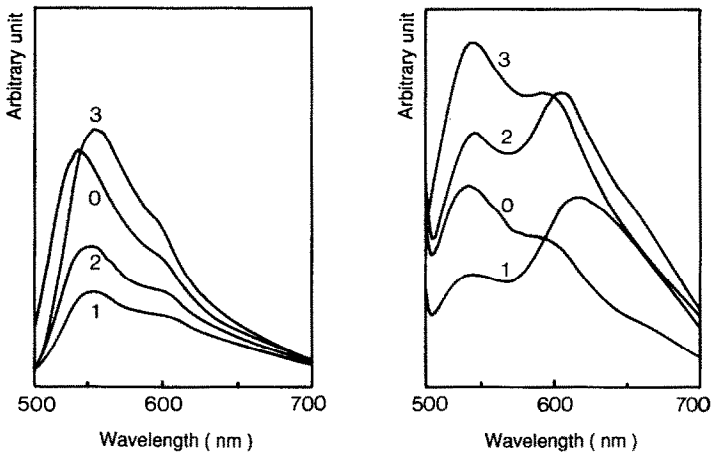

Fig. 6. Fluorescence emission spectra of 7-hydroxy-3,4'-dimethoxyflavylium chloride (**1**) (3×10^{-4} mol dm^{-3}) without copigment (0) and with either caffeine (1), AMP (2) or ATP (3). Optical path length: 1 cm; T = 20°; solvent: aqueous citric acid–sodium hydrogen phosphate buffer. Copigment to pigment molar ratio: 100. Left: pH 3.0. Right: pH 5.0.

Table 2. ^{1}H NMR chemical shifts (ppm) of the AH^+ form of compound **1** at different copigment to pigment molar ratios

R	0	10	25	40	60	80	100
H-4	8.86	8.78	8.71	8.64	8.60	8.55	8.53
H-5	8.07	8.02	7.98	7.94	7.91	7.89	7.88
H-6	7.44	7.42	7.40	7.38	7.36	7.35	7.34
H-8	7.50	7.44	7.39	7.35	7.31	7.29	7.29
H-2' and H-6'	8.64	8.59	8.54	8.49	8.46	8.43	8.42
H-3' and H-5'	7.23	7.21	7.19	7.16	*	*	*

*Not measured.
R = [gallic acid]/[**1**] in D_2O–CD_3OD–DCl, 66:33:1 at 27°.

with the greatest shift is H-4 (Table 2). For a copigment to pigment molar ratio of 10, chemical shift differences for the protons at C-4 and C-3' in the free molecule and in the complex, are 32 and 8 Hz, respectively. From data in Table 2 we derived the value of the complexation constant of gallic acid with **1** using a Benesi—Hildebrand type plot. Formation of a 1:1 complex between **1** and **3** occurs and the stability constant K_1 is 18 $mol^{-1}dm^{-3}$ at 27° in a D_2O–CD_3OD–DCl (66:33:1) mixture. The latter value is significantly lower than that measured at 20° in water (100 $mol^{-1}dm^{-3}$, see above). Raising the temperature reduces the copigment effect [2] and replacing water by any other solvent has the same effect [13]. Finally, it is not known if the solvent isotope effect (D_2O instead of H_2O) produces a change in copigmentation strength but if it does, this can only be a small effect. It has been reported that anthocyanins self-associate [15 and refs therein]. On the basis of a concentration effect and also a temperature change, both experiments being performed in the absence of any copigment, we were able to demonstrate that, in the case of **1**, self-association does not take place to a measurable extent under our experimental conditions. We also investigated, by the NMR method and at 27°, the effect produced by caffeine on the ^{1}H NMR spectrum of **1**. We were surprised to discover that the chemical shifts of H-4 and H-6 remained unchanged while the other anthocyanin protons were only weakly upfield shifted in the presence of caffeine (at most 0.02 ppm or 8 Hz for protons H-2' and H-6', with a copigment to pigment molar ratio of 13.7). It may be concluded that the caffeine molecule associates with 7-hydroxy-3,4'-dimethoxyflavylium chloride (**1**) according to a mechanism different from that featuring other copigments such as, in particular, chlorogenic acid (**2**) and gallic acid. Caffeine is known to be an excellent hydrogen bond acceptor [14]. The caffeine-**1** complex could result from the formation of a hydrogen bond between the hydroxyl group at C-7 and one of the caffeine carbonyl groups, for instance. Unlike vertical stacking, this type of complexation would only provide a weak protection of the flavylium cation from hydration thus giving a very small gain of colour. Now, let us turn to the results obtained from the binding of caffeine to malvin chloride, a common natural anthocyanin. Haslam and his collaborators [3] and our group [13] have observed that caffeine stacks to malvin in the more usual manner (hydrophobic interaction with a good protection against hydration of the malvin pyrylium ring). Nevertheless, it is seen from the absorption experiments [3, 13] that caffeine produces on the malvin visible band a large bluing

effect comparable to the caffeine bathochromic shift shown on Fig. 5. In our opinion, for caffeine two types of association with anthocyanins are possible depending on the anthocyanin structure and also on medium factors. One type of complex forms by hydrogen bonding between the caffeine molecule and the pigment and the other type corresponds to the stacking of the large planar caffeine surface with the anthocyanin. In the case of malvin, the two types certainly coexist (vertical stacking enforced by hydrogen bonding) while caffeine complexation with **1** is only by the hydrogen bonding type. Molecular structures of caffeine complexes have been extensively analysed by Haslam [16]. In conclusion: the case of caffeine, if not unique, is probably rare since in water—copigmentation does not occur out of the presence of water—competition between hydrogen bond acceptors highly favours water itself so that complex formation by hydrogen bonding is quite unlikely. For the rest of the large copigment family, where most of the molecules are hydrogen bond donors and acceptors, the stacking association, with its good pyrylium ring protection against nucleophilic attack, is the rule. Finally, differences in the types of the complexes formed between caffeine and either malvin or the synthetic flavylium cation **1** points to a role, in the association of malvin with caffeine, to the anthocyanin sugar residues. It is assumed that malvin's sugar (β-D-glucosyl groups) can adopt a configuration satisfying the need for hydrogen bonding of the caffeine molecule; this would favour the stacking of caffeine on the malvin chromophore. A comparable cooperative binding has been described previously by Haslam in the case of the caffeine-chlorogenate complex [17] and also in the case of the interaction between proteins and polyphenols [18]. Being devoid of a sugar group, the structurally simple anthocyanin **1** cannot associate with caffeine in this way.

EXPERIMENTAL

Materials. 2-Bromo-4'-methoxyacetophenone and 2,4-dihydroxybenzaldehyde, provided by Lancaster Synthesis and sodium methoxide (30% wt soln in methanol) purchased from Janssen Chimica, were used as such. Chlorogenic acid **(2)**, gallic acid **(3)**, caffeine **(4)**, AMP **(5)** and ATP **(6)** were provided either by Roth or by Sigma. NMR solvents were obtained from Merck (deuterium oxide, methanol-d_4 and trifluoroacetic acid-d_1) and from Aldrich (deuterium chloride and sodium deuteroxide).

7-Hydroxy-3,4'-dimethoxyflavylium chloride **(1)** [6]. 2,4'-Dimethoxyacetophenone was prepared by treatment of 2.5 g of 2-bromo-4'-methoxyacetophenone with an equivalent quantity of Na methylate in 40 ml dilute MeOH soln for 2 hr. The product obtained in 35% yield was sepd from a range of side products by chromatography on a silica gel column with CH_2Cl_2 as solvent. R_f (CH_2Cl_2) 0.16. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.93 (2H, *m*, H-2' and H-6'), 6.94 (2H, *m*, H-3' and H-5'), 4.65 (2H, *s*, H-2), 3.87 (3H, *s*, -OMe), 3.50 (3H, *s*, -OMe). 7-Hydroxy-3,4'-dimethoxyflavylium chloride **(1)** was prepared by mixing, in 100 ml of Et_2O with dry HCl at 0° and for 30 min, 590 mg of 2,4'-dimethoxyacetophenone with an equivalent amount of 2,4-dihydroxybenzaldehyde, **1** ppt. was repeatedly washed with Et_2O. Yield: 85%. UV-visible $\lambda_{max}^{H_2O-HCl}$ nm 265 (4.08), 486 (4.58).

UV-visible spectrometry. Visible absorption spectra of equilibrated soln of **1**, without and with copigment, were recorded using a Hewlett Packard diode-array spectrometer fitted with a thermostated quartz cell (d = 1 cm). Absorbance values were measured at the pigment visible λ_{max} (486 nm) and for a temperature of 20°. Solutions were left to equilibrate for several hr and kept at 20°. To determine the hydration constant (pK_h) of **1**, the following procedure was used: equally concd solns of **1** (3.45×10^{-4} mol dm^{-3}) were prepared at different pH values using citric acid–sodium hydrogen phosphate buffers and their absorbances at 486 nm recorded. In the copigmentation experiments the pH was set to 3.5 and 5.0 using the same buffer components as above. For copigmentation with chlorogenic acid **(2)**, 7-hydroxy-3,4'-dimethoxyflavylium chloride (6×10^{-4} mol dm^{-3}) was dissolved in the buffer. Chlorogenic acid (4.2×10^{-3} mol dm^{-3}) was then added to the solution. For copigmentation with caffeine AMP and ATP, **1** (10^{-3} mol dm^{-3}) was dissolved in a buffer containing 10% of ethylene glycol. The copigments were then added to the solutions at a concn of 5×10^{-2} mol dm^{-3}.

Spectrofluorometry [19]. Fluorescence spectra of equilibrated solutions of pigment **1**, without and with copigment, were recorded on a Shimadzu RF-540 spectrofluorometer. Solutions were prepared from 0.05 mol dm^{-3} citric acid–sodium hydrogen phosphate buffers at different pH values. The concentration of the pigment was 3×10^{-4} mol dm^{-3} and that of the copigment (caffeine, AMP or ATP) 100 times larger. Emission spectra were obtained for an excitation wavelength of 486 nm.

^{1}H NMR spectroscopy. ^{1}H NMR spectroscopic measurements were carried out using a Bruker AM-400 spectrometer at a constant temperature of 27°. Spectra of **1** (9.3×10^{-4} mol dm^{-3}), with and without gallic acid, were taken in D_2O–CD_3OD–DCl (66:33:1), sodium deuteroxide being added, if necessary. Spectra of **1** (9.3×10^{-4} mol dm^{-3}) in the presence of caffeine (1.28×10^{-2} mol dm^{-3}) were recorded from D_2O–CD_3OD–TFA (65:30:5).

Mass spectral data. The structure of **1** was confirmed by FAB mass spectrometry. Measurements gave a molecular ion $[M]^+$ at 282.9 *m/z* indicating the mass calculated for $C_{17}H_{15}O_4^+$.

pH measurements. pH values were recorded by means of a Metrohm model 654 pH meter fitted with a small combined glass electrode (Metrohm 6.0204). Buffers used to calibrate the pH meter were pH 4 and 7 NBS standards. In the case of solutions made of deuterated water, the reported pH values correspond to the values read from the pH meter to which a constant 0.40 value has been added. Such a correction gives pD values.

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