Kinetic and Thermodynamic Investigation of the Aluminium–Anthocyanin Complexation in Aqueous Solution

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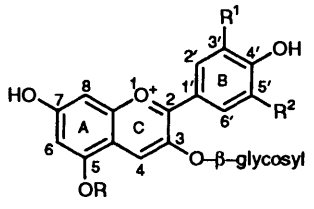
Complexation of the aluminium ion with a synthetic model of anthocyanin has been thoroughly investigated in aqueous solution. From UV–VIS spectroscopic data collected within the pH range 2–5, the presence of complexes involving not only the coloured forms but also the colourless forms of the pigment is demonstrated. A theoretical treatment is developed for the calculation of the corresponding stability constants, from which the percentage of the different pigment forms can be plotted as a function of pH. These results are important to plant pigmentation and, for instance, a narrow pH domain in which colour amplification due to complexation is at a maximum has been found. From relaxation kinetics measurements (pH-jump), it is made clear that the whole process of equilibration is governed by the kinetics of complexation between Al^{3+} and the minor anionic quinonoidal form of the anthocyanin. Finally, ^1H NMR analysis in CD_3OD in which complexation is much stronger than in water provides additional evidence of the anthocyanin passing from the red flavylium form to the deep-purple quinonoidal form upon coordination to Al^{3+}, a result that quantitatively demonstrates for the first time the large potential of aluminium–anthocyanin complexation in colour variation.

Anthocyanins are natural pigments from the flavonoid family (polyphenols) which are largely responsible for the red, purple and blue colours displayed in the plant world, in particular in flowers and fruits.[1-2] Their main coloured form is a 2-phenyl-1-benzopyrylium (flavylium) chromophore substituted in various ways by hydroxy, methoxy and *O*-β-glycosyl groups (Scheme 1). The natural medium of anthocyanins, *e.g.* the vacuoles of



Scheme 1 The flavylium form of common natural anthocyanins: R = H, β-glycosyl; R^1, R^2 = H, OH, OMe, *O*-β-glycosyl

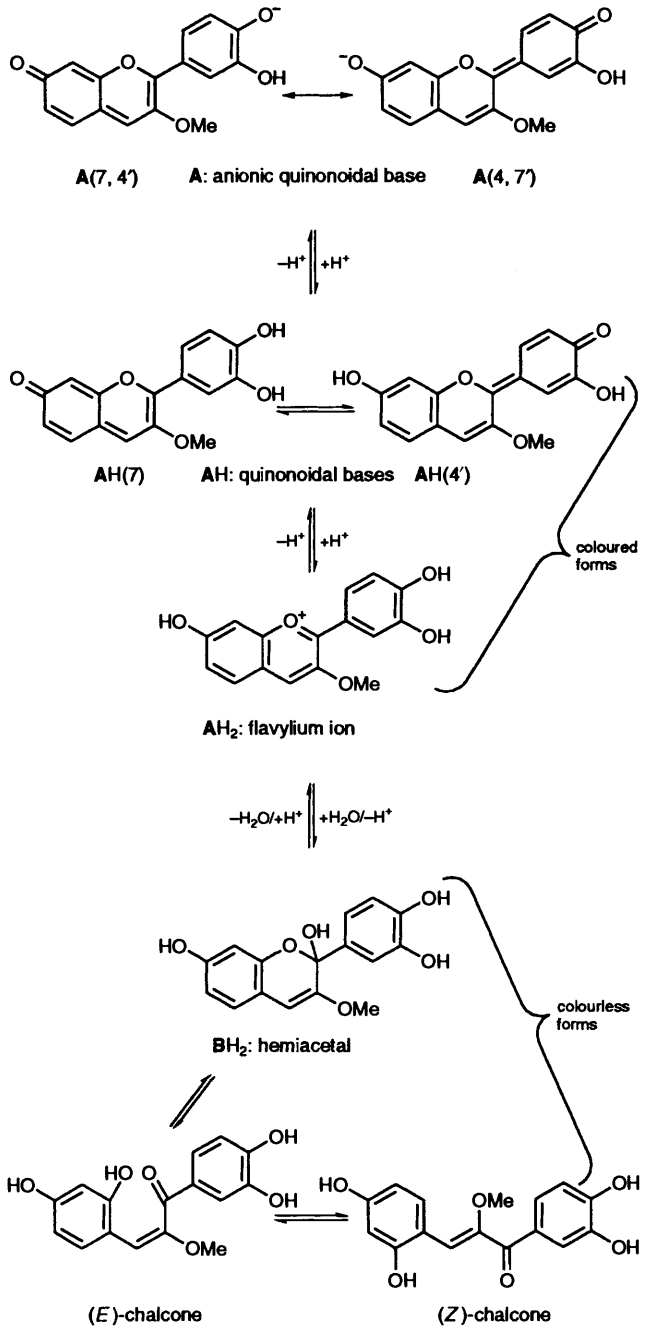
flower petal epidermal cells, is essentially aqueous, slightly acidic or neutral[3] and rich in inorganic ions and other (colourless) polyphenols.[4] *In vitro* studies have shown that most anthocyanins are paradoxically poorly coloured on their own because of the thermodynamic instability of the flavylium chromophore in water. Thus, except in strongly acidic solutions (pH < 2), the water molecule readily reacts at the 2-position of the flavylium ion with consecutive formation of dominant amounts of colourless hemiacetal according to a reversible hydration reaction (Scheme 2). Therefore, the percentage of coloured forms in a slightly acidic or neutral solution of anthocyanin is usually less than 10% of the overall pigment concentration at the thermodynamic equilibrium.[5] Clearly, *in vivo* colour stabilizing mechanisms are at work which involve other vacuolar components. It is now well known that colourless polyphenols (flavones, flavonols, cinnamic and benzoic acid esters, tannins) are able to form molecular (non-covalent) stacks with the large planar π-electron rich flavylium nucleus and thereby oppose the water's nucleophilic attack onto the pyrylium ring.[6-10] This process, called copigmentation, is able to restore colour to faded anthocyanin solutions by shifting the overall equilibrium between coloured and colourless forms towards the selectively complexed coloured forms. Copigmentation can also operate in an intramolecular way in sophisticated anthocyanins bearing cinnamic or benzoic acid residues on their glycosyl groups.[2,11,12]

Unlike polyphenolic copigments, metal ions which could be present in the anthocyanin natural medium seem to be rarely involved in colour stabilization and, so far, no quantitative investigation has been carried out on the complexes which could form between catechol-type anthocyanins (R^1 and/or R^2 = OH in Scheme 1) and small highly charged metal ions such as Al^{3+} and Mg^{2+}. However, those ions have been reported possibly to strengthen the pigment–copigment interaction, thus leading to enhanced hyperchromic and bathochromic shifts. In particular, it has been proposed that the blue colour displayed by some flowers is the result of pigment–copigment–metal ion assemblies.[13] The most remarkable achievement in this field is certainly the X-ray structure of the commelinin pigment elucidated by Goto and co-workers which shows up to six pigment and six copigment molecules packed around two magnesium ions in the crystal.[2,14]

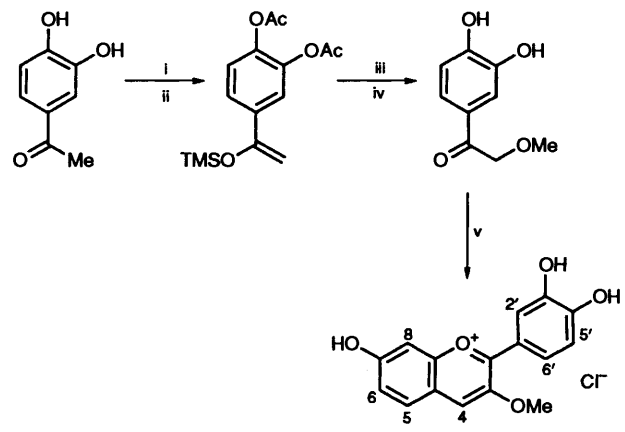
The present report is the first step in a programme aimed at studying quantitatively the pigment–copigment–metal ion complexes in aqueous solution and essentially deals with the anthocyanin–aluminium system. From UV–VIS spectroscopic measurements on equilibrated solutions at different pH values, the presence of complexes involving the different pigment forms is demonstrated. Relaxation kinetics (pH-jump) has allowed us to monitor the complexation kinetics, thus giving information on the mechanism. Anthocyanin–aluminium complexation has also been investigated in methanolic solution with the help of UV–VIS and ^1H NMR spectroscopy. As far as possible, data are analysed in the frame of theoretical treatments giving access to thermodynamic and kinetic parameters and interpreted in terms of colour stabilization and variation.

Experimental

Materials.—Aluminium chloride hexahydrate (Aldrich, 99%) was used as received. 3',4',7-Trihydroxy-3-methoxyflavylium chloride was synthesized according to Scheme 3. Diacetylation of 3,4-dihydroxyacetophenone required drastic conditions [AcCl, NaH, dimethylformamide (DMF), T −78 to 20 °C, yield



Scheme 2 The structural transformations of the 3',4',7-trihydroxy-3-methoxyflavylium ion in slightly acidic to neutral aqueous solutions



Scheme 3 Synthesis of 3',4',7-trihydroxy-3-methoxyflavylium chloride. *Reagents and conditions:* i, AcCl, NaH, DMF; ii, Me_3SiCl, NaI, NEt_3, DMF, 85 °C; iii, $PhI(OAc)_2$, MeOH, BF_3, Et_2O; iv, $NaHCO_3$, H_2O, EtOH; v, 2,4-$(HO)_2C_6H_3CHO$, AcOEt, HCl.

70%). The corresponding enol ether was prepared in high yield (88%) owing to *in situ* formation of trimethylsilyl iodide.[15] Methoxylation of the trimethylsilyl enol ether of 3,4-diacetoxyacetophenone was adapted from a procedure developed by Moriaty *et al.*,[16] commercially available iodosobenzenediacetate replaced iodosobenzene. The yield was *ca.* 40% after chromatography on silica gel. In the final condensation step, gaseous hydrogen chloride was gently bubbled (3 h) into an equimolar solution of 2'-methoxy-3,4-dihydroxyacetophenone and 2,4-dihydroxybenzaldehyde in distilled ethyl acetate at 0 °C. Precipitation of deep-red crystals of 3',4',7-trihydroxy-3-methoxyflavylium chloride occurred and was completed by keeping the reaction mixture at −20 °C for 3 days. The crystals were then filtered, thoroughly washed with ethyl acetate and dried under vacuum (yield 65%). 3',4',7-Trihydroxy-3-methoxyflavylium chloride was characterized by electro-spray mass spectrometry (positive mode, m/z = 285); UV–VIS spectroscopy (λ_{max} = 270 and 500 nm in 0.2 mol dm^{-3} HCl); δ_H(400 MHz, [$^2H_6$]DMSO–CF_3CO_2D) 8.80 (s, 4-H), 8.12 (dd, 8.7 and 2.4 Hz, 6'-H), 8.03 (d, 2.4 Hz, 2'-H), 7.93 (d, 8.9 Hz, 5-H), 7.33 (d, 2.2 Hz, 8-H), 7.30 (dd, 8.9 and 2.2 Hz, 6-H), 6.99 (d, 8.7 Hz, 5'-H) and 4.07 (s, CH_3); δ_C(100 MHz, [$^2H_6$]DMSO–DCl) 165.56 (C-7), 163.33 (C-2), 155.46 (C-9), 155.11 (C-4'), 149.18 (C-3'), 146.71 (C-3), 134.38 (C-4), 131.51 (C-6'), 125.80 (C-5), 121.98 (C-1'), 120.41 (C-2'), 118.98 (C-10), 118.49 (C-5'), 117.57 (C-6), 102.48 (C-8) and 58.75 (CH_3) (tentative assignments from ref. 17). Its purity was checked by reversed-phase HPLC on a Merck C-8 column (5 μm, 125 × 4 mm) with a flow rate of 1 cm^3 min^{-1} and linear gradient elution for 30 min from 5 to 20% solvent A [5% HCO_2H in MeCN–H_2O (1 : 1)] in solvent B (5% HCO_2H in H_2O) followed by linear gradient elution for 30 min from 20% solvent A in solvent B to 100% solvent A. The chromatogram was recorded on a Spectra-Physics apparatus equipped with a Hewlett-Packard diode-array detector monitoring at 260 and 500 nm: R_t = 35.1 min at 20 °C. The only by-product that could be detected was the (*Z*)-chalcone (t_R = 37.3 min, only traces) in slow equilibrium with the flavylium ion.

Absorption Spectra.—Spectra were recorded with a Hewlett-Packard diode-array spectrometer fitted with a quartz cell (d = 1 cm) equipped with a stirring magnet. A constant temperature in the cell was obtained by use of a Lauda water-thermostatted bath. The temperature was measured in the spectrometer cell with a Comark thermocouple and was kept at 25 ± 0.1 °C throughout this work. The ionic strength was fixed by NaCl at 0.5 mol dm^{-3}. Water used in sample preparation was distilled, deionized, ultrafiltered to a resistance of about 18 MΩ using a Millipore Milli-Q apparatus. Methanol was spectroscopy grade (Merck).

pH *Measurements.*—The pHs of the solutions were recorded with a Metrohm model 654 pH meter fitted with a small combined glass electrode. The buffers used for calibration were pH 7 and pH 4 Aldrich standards.

Data Analysis.—The curve fittings were carried out on a Macintosh IIsi computer using the Kaleida Graph program. Standard deviations are reported.

Semi-empirical Quantum Mechanical Calculations.—Semi-empirical quantum mechanical calculations were performed on a Compaq ProLinea 4/50 PC using the HyperChem program (Autodesk, Sausalito, CA) in the AM1 parametrization. For a given quinonoidal form, the geometry optimization procedure was repeated with different sets of input data files with respect

Table 1 Relative energies (in kcal mol^{-1}) of the quinonoidal forms calculated *in vacuo* by the HyperChem program in the AM1 parametrization

AH(7)	AH(4')	A(7,4')–A(4',7)	A(7,3')	A(4',3')
0	1.4	0	12.7	19.1

to the torsion angles around the C(1')–C(2) and C(3)–OMe bonds. The relative energies corresponding to the most stable conformations of the different quinonoidal forms are reported in Table 1.

Thermodynamic Measurements.—Structural transformations of the 3',4',7-trihydroxy-3-methoxyflavylium ion. The value of the thermodynamic constant (K_h') of the overall hydration equilibrium connecting the flavylium ion (AH_2^+) and the mixture of colourless forms (hemiacetal BH_2 and chalcone forms) was gained by recording the visible absorbance at the wavelength of the flavylium absorption maximum (500 nm) on fully equilibrated solutions of pigment at different pH values. The value of the thermodynamic constant (K_{a1}) of the AH_2^+/AH proton-transfer equilibrium was obtained from pH-jump experiments and curve fitting of the plot of the apparent rate constant of hydration (first-order) *vs.* final pH [eqn. (4) and Fig. 7(*a*)]. Both procedures were published recently with details.[12] In order to estimate the value of the thermodynamic constant (K_{a2}) of the AH/A^- proton-transfer equilibrium, 2 cm^3 of an equilibrated solution of anthocyanin (5×10^{-5} mol dm^{-3}) in 0.05 mol dm^{-3} H_3PO_4 containing 0.5 mol dm^{-3} NaCl (pH adjusted to 2.5 after addition of concentrated NaOH) were magnetically stirred in the spectrometer cell. Small volumes (30 to 80 mm^3) of 1.5 mol dm^{-3} NaOH solution were added quickly to the cell and the visible absorbance at the wavelength of absorption maximum of A^- (586 nm) was recorded immediately. The final pH value was carefully measured and ranged from 5.9 to 8.7. In that way the initial amount of flavylium ion was quantitatively transformed into the corresponding quinonoidal bases (AH, A^- or a mixture of both depending on the final pH), the overall amounts of colourless forms being kept constant (hydration was slow in the pH range investigated). From the absorbance *vs.* pH plot the K_{a2} value was calculated.

Complexation equilibria. A pH 3.0 HCO_2H–HCO_2^- buffer was prepared by mixing a 0.5 mol dm^{-3} HCO_2H solution containing 0.5 mol dm^{-3} NaCl with the required volume of 0.5 mol dm^{-3} NaOH solution (solution 1). A methanolic solution (2 cm^3) of 3',4',7-trihydroxy-3-methoxyflavylium chloride was increased in volume to 100 cm^3 by addition of solution 1 and mixed with $AlCl_3 \cdot 6H_2O$ (solution 2). Solution 2 was diluted twice either with solution 1 or solution 1 mixed with 0.5 mol dm^{-3} NaOH so that the final pH was 4.9. After dilution the pigment and aluminium concentrations were 7.5×10^{-5} and 3.75×10^{-3} mol dm^{-3}, respectively (metal : pigment ratio = 50). The solutions at pH 3.0 and pH 4.9 were then mixed so as to prepare solutions at intermediate pH values. All solutions had an ionic strength of *ca.* 0.5 mol dm^{-3} and were fully equilibrated for 1 h before the spectroscopic measurements were made. The values of the complexation constants were obtained from a curve fitting of the visible absorbance (at a given wavelength) *vs.* pH plot according to eqn. (2) [see Fig. 1(*a*)].

Kinetic Measurements.—Structural transformations of the 3',4',7-trihydroxy-3-methoxyflavylium ion. The values of the rate constants (k_1, k_2) of the hydration process were deduced from pH-jump experiments (quick addition of concentrated NaOH without significant dilution) on equilibrated pigment solutions.[12] The visible absorbance at the wavelength of flavylium absorption maximum (500 nm) was recorded immediately every 1 s for 30 s. Under those conditions, the hydration equilibrium was usually reached before the end of the runs for final pH values ranging from 3.3 to 5.0. The spectrometer software automatically computed the first-order apparent rate constant of hydration as well as the absorbance at equilibrium. The standard deviations were less than 2%. In the calculations, $[H^+]$ is approximated to 10^{-pH}.

Complexation equilibria. A methanolic solution (1 cm^3) of 3',4',7-trihydroxy-3-methoxyflavylium chloride was diluted to 50 cm^3 by addition of a 0.05 mol dm^{-3} HCO_2H solution containing 0.5 mol dm^{-3} NaCl as an ionic strength buffer. $AlCl_3 \cdot 6H_2O$ was then added, the pH brought to 2.5 by addition of a few drops concentrated NaOH and the solution equilibrated for 1 h. The pigment and aluminium concentrations were 10^{-4} and 5×10^{-3} mol dm^{-3}, respectively (metal : pigment ratio = 50). The solution (2 cm^3) was placed into the spectrometer cell and quickly mixed with a small volume (30–80 mm^3) of 1.5 mol dm^{-3} NaOH solution. The visible absorbance at the wavelength of the absorption maximum of the aluminium complex (544 nm) was immediately recorded every 1 s for 1 min. The final pH value was carefully measured and ranged from 3.8 to 4.6. The first-order apparent rate constant of the complexation reaction (computed by the spectrometer software) was then plotted as a function of the final pH and the corresponding curve was fitted against eqn. (13) [Fig. 7(*b*)].

Results

In order to simplify the notations, the charges in the pigment forms and their metal complexes will be largely omitted from now on.

Structural Transformations of the 3',4',7-Trihydroxy-3-methoxyflavylium Ion.—The combination of static and kinetic measurements allows one to estimate the values for the following parameters: the thermodynamic constants of the AH_2/AH and AH/A proton transfer reactions (K_{a1} and K_{a2}, respectively), the thermodynamic constant (K_h') of the overall hydration of AH_2 into the mixture of colourless forms (hemiacetal BH_2 and chalcone forms) and the rate constants of the hydration process (k_1 and k_2 for the forward and backward reactions, respectively) connecting AH_2 and hemiacetal BH_2 which in our kinetic experiments is indistinguishable from the (*E*)-chalcone because of fast cycle–chain tautomerism (Scheme 2).[18] The corresponding thermodynamic constant is noted K_h and equals k_1/k_2. K_h', K_{a1} and K_{a2} are defined as $a_H\{[BH_2] + [(E)\text{-chalcone}] + [(Z)\text{-chalcone}]\}/[AH_2]$, $a_H[AH]/[AH_2]$ and $a_H[A]/[AH]$, respectively, a_H being 10^{-pH}. K_h, which is obtained from the values of the visible absorbance at equilibrium in the kinetic runs, does not take into account the slow forming (*Z*)-chalcone [about 1 h is needed for (*Z*)–(*E*) isomerism to reach equilbrium]. K_h is thus expressed as $a_H\{[BH_2] + [(E)\text{-chalcone}]\}/[AH_2]$. The thermodynamic constant of the (*Z*)–(*E*) isomerism (K_i) can be estimated from the relationship, $K_h' = K_h(1 + K_i)$. The values for the above-defined parameters are reported in Table 2. The pK_h' value lies within the domain of the values found for common natural anthocyanins[5c,19] (pK_h' typically ranging from 2 to 3). This makes 3',4',7-trihydroxy-3-methoxyflavylium chloride a valuable model for the study of the role of anthocyanins, having a catechol B-ring, in colour expression. At pH higher than 3, the colourless forms are largely predominant and any process tending to restore the initial colour (selective complexation of the minor coloured forms) should be detected with high sensitivity. As can be seen from the low K_i value, the (*Z*)-chalcone represents only one fifth to one quarter of the overall concentration of colourless forms. From fast kinetics and ^1H NMR investigations of natural and synthetic flavylium ions, it has been made clear that the (*E*)-chalcone too is a minor component.[18] Therefore, we may

Table 2 Main thermodynamic constants of the structural transformations of the 3',4',7-trihydroxy-3-methoxyflavylium ion in aqueous solution[a]

pK'_h	pK_{a1}	pK_{a2}	pK_h	K_i
2.42 ± 0.02	4.40 ± 0.06	7.62 ± 0.07	2.52 ± 0.02	0.28 ± 0.05

[a] At 25 °C and 0.5 mol dm^{-3} ionic strength (see the text for definitions). From the kinetic runs, $k_1 = (23.2 \pm 1.5)$ min^{-1}; $k_2 = (7.7 \pm 0.7) \times 10^3$ dm^3 mol^{-1} min^{-1} under the same conditions.

Table 3 Thermodynamic constants[a] of complexation of the 3',4',7-trihydroxy-3-methoxyflavylium ion in aqueous solution and absorption maxima[b]

$\log K_{AM}$	$\log K'_{AM}$	$\log K''_{AM}$	$\log K_{BM}$
−4.04 ± 0.06	0.36 ± 0.12	7.98 ± 0.19	−6.39 ± 0.05
λ_{max} AH_2	λ_{max} AH	λ_{max} A	λ_{max} AM
500	528	586	544

[a] At 25 °C and 0.5 mol dm^{-3} ionic strength (see the text for definitions). [b] Of the coloured forms in aqueous solution; in nm.

assume the hemiacetal to be by far the most abundant pigment form in slightly acidic to neutral aqueous solutions at equilibrium.

In methanolic solution, the 3',4',7-trihydroxy-3-methoxyflavylium ion undergoes the nucleophilic attack of methanol with simultaneous fading. The colourless product is probably the methylacetal [m/z = 315 in electrospray MS (negative mode) after deprotonation of a phenolic group]. From spectra collected in methanol at different HCl concentrations, the corresponding pK value could be estimated as 3.53 ±0.01, K being defined as $[H^+]$[methylacetal]/$[AH_2]$. The water content was kept constant (about 6%) in all samples. No trace of hemiacetal could be detected by MS.

Complexation Equilibria.—Fig. 1(*a*) reports the variations of visible absorbance at a fixed wavelength when the pH is varied in equilibrated solutions of 3',4',7-trihydroxy-3-methoxyflavylium ion in the presence of Al^{3+}. The corresponding solutions without aluminium are almost colourless in the pH range investigated (pH 3–5). The experimental curve on Fig. 1(*a*) is best fitted by a theoretical curve postulating the formation of two metal complexes of 1:1 stoichiometry, the one (**AM**) involving the coloured forms of the pigment and the other (**BM**) involving the colourless forms taken as a whole. When complex formation arises from the protonated ligands, the corresponding thermodynamic constants K_{AM} and K_{BM} are expressed as $a_H{}^2[AM]/([AH_2][M])$ and $a_H{}^2[BM]/([BH_2][M])$, **M** being Al^{3+}. Formation of complex **AM** from the **AH** and **A** forms can be characterized by the constants K'_{AM} and K''_{AM}, respectively. We can write: $K'_{AM} = a_H[AM]/([AH][M]) = K_{AM}/K_{a1}$; $K''_{AM} = [AM]/([A][M]) = K_{AM}/(K_{a1}K_{a2})$. The values for these parameters are reported in Table 3. From the pK_{AM} and pK_{BM} values it is clear that the affinity of Al^{3+} for the coloured forms is much stronger than for the colourless forms. As a consequence, at a metal: pigment molar ratio of 50, large concentrations of coloured forms are retained in the pH domain from 3 to 5 (see distribution diagrams displayed in Fig. 4). The values for the wavelength of visible absorption maximum of the different coloured forms in their free and complexed forms are reported in Table 3. The strong bathochromic shifts accompanying complexation (44 nm relative to free AH_2, 16 nm relative to free AH) point to the pigment having the **A** form in the complex.

Complexation has been investigated in methanol as a function of the metal concentration, the pigment concentration being held constant (5×10^{-5} mol dm^{-3}). Large colour increases reaching saturation at a metal: pigment molar ratio of about 2 have been observed (Fig. 6). The intense blue–purple colour of the metal containing solutions is in sharp contrast to the total absence of colour of the solution without aluminium due to complete conversion (in dilute pigment solution) of the flavylium ion into a colourless methylacetal. The absorption wavelength of the aluminium complex is 564 nm, *i.e.* 46 nm higher than that of the free flavylium ion in methanol (518 nm). The much higher solubility of 3',4',7-trihydroxy-3-methoxyflavylium chloride in methanol (*vs.* water) and the apparent much higher stability of its aluminium complex allow one to study the complexation by ^1H NMR spectroscopy. In Fig. 5 are displayed the spectra of 3',4',7-trihydroxy-3-methoxyflavylium chloride in CD_3OD in the presence and in the absence of Al^{3+}. Owing to the acidic properties of the pigment (pK = 3.53 ± 0.01 in methanol) and its relatively high concentration in the samples (4×10^{-3} mol dm^{-3}), no acidification is needed to convert the pigment mainly into the flavylium form in the absence of metal. However, small amounts of colourless form are present. Addition of Al^{3+} causes large diamagnetic shifts of the NMR signals for all protons with the exception of 6'-H whose signal moves to a higher δ value.

Quick addition of concentrated NaOH (without dilution) to a fairly acidic (pH about 2.5) equilibrated aqueous solution of 3',4',7-trihydroxy-3-methoxyflavylium chloride in the absence of aluminium results in an exponential decay of the visible absorbance featuring the relaxation of the pH-dependent hydration equilibrium according to apparent first-order kinetics. The plot of the corresponding apparent rate constant *vs.* pH shows acid catalysis [Fig. 7(*a*)]. In the presence of aluminium, the situation is reversed: the pH-jump results in an exponential increase of the visible absorbance with a corresponding first-order apparent rate constant increasing with the pH [Fig. 7(*b*)]. Clearly, aluminium complexation is now the rate determining step in the overall process of equilibration.

Discussion

Anthocyanins having a catechol group in their structure are widespread in flowers. They are glycosylated derivatives of the cyanidin (see Scheme 1 with R^1 = OH, R^2 = H), delphinidin (R^1 = R^2 = OH) and petunidin (R^1 = OH, R^2 = OMe) chromophores. Their complexation with Al^{3+} has been known for a long time and constitutes the basis of a popular qualitative test for their presence in plant extracts.[20] Moreover, aluminium being relatively abundant in plants, its complexation with anthocyanin could be of biological relevance in the expression of the blue colour in flowers. At the end of the 1960s, works were published which described the spectral changes occurring in aluminium containing solutions of cyanidin-3-glucoside and cyanidin-3,5-diglucoside (cyanin) when the pH was varied.[21] Those observations on natural pigments parallel ours on 3',4',7-trihydroxy-3-methoxyflavylium chloride and, in particular, mention was made of a pH domain in which hyperchromism due to complexation was at a maximum. However, the lack of kinetic and thermodynamic information on the structural transformations of anthocyanins in aqueous solution did not permit one to go further in the interpretation of the data. More recently, Takeda and co-workers[13] have demonstrated that Al^{3+} is highly effective at producing blue colour in slightly acidic (pH 3.7) solutions of delphinidin-3-glucoside in the

presence of copigments such as 3-*p*-coumaroyl-and 3-caffeylquinic acids, themselves liable to complex Al^{3+} through their α-hydroxycarboxy group. Both aluminium and copigment were required to produce a blue colour, a result which led the authors to conclude that the blue colour probably developed through the formation of ternary complexes involving pigment, copigment and aluminium. In such assemblies, Al^{3+} would have the key-function of building a covalent link between pigment and copigment that would bring their respective aromatic moieties close enough to interact in an efficient intramolecular way.

Thermodynamic Investigation in Water.—In a physicochemical investigation of aluminium complexes in aqueous solution two main difficulties are expected. (*i*) Al^{3+} is an octahedrally hexacoordinated ion liable to accommodate one, two or three molecules of bidentate ligand with consecutive formation of 1:1, 1:2 or 1:3 complexes. The simultaneous occurrence of those complexes greatly complicates any theoretical treatment. In slightly acidic aqueous solution, aluminium binds moderately to the synthetic anthocyanin so that large aluminium:pigment molar ratios are required for a sensitive investigation of complexation. In such conditions 1:2 and 1:3 complexes must be very minor species and can be neglected. This is no longer true in methanol because of much stronger complexation reaching saturation at low aluminium:pigment molar ratios. (*ii*) Al^{3+} is a very small highly charged metal ion, one of the hardest chemical species after the proton. As a consequence, its hydrolytic properties are complex and may result in the formation of numerous hydroxo species, in particular aluminium hydroxide which precipitates.[22] However, these species form significantly at pH higher than 5 (for a total aluminium concentration of the order of 10^{-3} mol dm^{-3})[23] which is the upper limit of the pH domain investigated in this work. Therefore we will start our quantitative spectroscopic investigation of the anthocyanin–aluminium complexation in equilibrated aqueous solution with the simple model described on Scheme 4 which, in addition to the anthocyanin structural

$$\mathbf{AH_2}^+ + H_2O \rightleftharpoons \mathbf{BH_2} + H^+ \qquad K'_h$$

$$\mathbf{AH_2}^+ \rightleftharpoons \mathbf{AH} + H^+ \qquad K_{a1}$$

$$\mathbf{AH_2}^+ + \mathbf{M}^{3+} \rightleftharpoons \mathbf{AM}^{2+} + 2H^+ \qquad K_{AM}$$

$$\mathbf{BH_2} + \mathbf{M}^{3+} \rightleftharpoons \mathbf{BM}^{+} + 2H^+ \qquad K_{BM}$$

Scheme 4

transformations, assumes 1:1 complexation between the free aluminium ion and the coloured and colourless forms of the pigment. The weak binding ability of the formate ions (buffer) is neglected. The complexation equilibria are written from the completely protonated ligands $\mathbf{AH_2}$ and $\mathbf{BH_2}$. The $\mathbf{BH_2}$ notation relates to the mixture of colourless forms (hemiacetal and chalcones) at equilibrium, **BM** representing the corresponding mixture of aluminium complexes. Our model does not take into consideration flavylium ion self-association which has turned out to be significant only in strongly acidic solutions in which the pigment is in a pure flavylium form. Thus, in 0.5 mol dm^{-3} HCl, the visible absorbance (at 500 nm) *vs.* pigment concentration plot departs steadily from linearity (Beer's law) when the concentration is raised above 10^{-5} mol dm^{-3}. The experimental plot (23 points collected at concentrations ranging from 10^{-6} to 6.4×10^{-5} mol dm^{-3}) can be fitted against eqn. (1) with the following notations: c is the pigment concentration and ε and ε' the molar absorption coefficients of the flavylium chromophore in the monomer and in the dimer, respectively (in other words, ε' equals half the dimer molar absorption coefficient). Finally, K_d represents the dimer stability constant. The optical pathlength (taken equal to 1) has been omitted. In the curve fitting ε' and K_d are the parameters to optimize, ε being determined in the most dilute solutions in which the flavylium ion is a pure monomer form. Thereby, a K_d value of $(2.6 \pm 0.7) \times 10^4$ dm^3 mol^{-1} has been obtained at 25 °C and 0.5 mol dm^{-3} ionic strength (ε = 43 000, ε' = 27 400 dm^3 mol^{-1} cm^{-1}, correlation coefficient = 0.9998). The K_d value is much higher than those determined for natural glycosylated flavylium ions which are typically lower than 10^3 dm^3 mol^{-1}.[24] However, in the weakly acidic conditions (pH 3 to 5) used for the investigation of metal complexation the flavylium concentration usually drops to less than 10^{-5} mol dm^{-3} because of the hydration process. In this concentration range the relatively strong non-covalent dimerization of the flavylium ion does not operate significantly. This is true equally for the neutral quinoidal bases which are expected to self-associate even more strongly than the flavylium ion, but never represent more than a few percent of the total pigment concentration in equilibrated solutions. At a fixed wavelength of the visible range, the absorbance is expressed as: $D = \varepsilon_{AH_2}[\mathbf{AH_2}] + \varepsilon_{AH}[\mathbf{AH}] + \varepsilon_{AM}[\mathbf{AM}]$, ε being the molar absorption coefficients. At pH lower than 5, the anionic quinonoidal base **A** is a very minor species and has been neglected. The total concentration of pigment can be written as: $c = [\mathbf{AH_2}] + [\mathbf{AH}] + [\mathbf{AM}] + [\mathbf{BH_2}] + [\mathbf{BM}]$. These relations are combined with the thermodynamic constants K'_h, K_{a1}, K_{AM} and K_{BM} (see the Results section for their definitions) to give eqn. (2) (simplification occurs from $K'_h \gg K_{a1}$). In eqn. (2), M_t is the total metal concentration and can be approximated to the free metal concentration because of the large metal:pigment molar ratio used in aqueous solution. Finally, D_0, D_1 and D_2 are $\varepsilon_{AH_2}c$, $\varepsilon_{AH}c$ and $\varepsilon_{AM}c$, respectively. Eqn. (2) can be checked either by

$$D = \frac{D_0 + D_1K_{a1}10^{pH} + D_2M_tK_{AM}10^{2pH}}{1 + K'_h10^{pH} + M_tK_{AM}10^{2pH} + M_tK'_hK_{BM}10^{3pH}} \qquad (2)$$

varying the pH in aqueous solutions of pigment and aluminium at fixed concentrations [Fig. 1(*a*)] or by varying the metal concentration in aqueous solutions of pigment at fixed concentration, the pH being held constant (Fig. 2). In the first case, a curve fitting yields the best values for K_{AM} and K_{BM}, D_1 and D_2 being additional floating parameters. D_0 is determined in a strongly acidified solution in which neither hydration nor complexation occurs (100% free flavylium). In the second case, the pH selected is 3.75 so that both **AH** and **BM** can be neglected. Under those conditions, eqn. (2) can be rearranged into eqn. (3) (with additional simplification due to $K'_h10^{pH} \gg 1$)

$$\frac{1}{D} = \frac{1}{D_2} + \frac{K'_h}{D_2K_{AM}10^{pH}}\frac{1}{M_t} \qquad (3)$$

and a D *vs.* M_t double reciprocal plot gives a straight line (Fig. 3) from which K_{AM} can be estimated readily. The corresponding pK_{AM} value of 4.03 ± 0.05 is in very good agreement with that determined by the first method (4.04 ± 0.06). When the absorbance at 358 nm of an equilibrated aluminium-containing solution of pigment is plotted as a function of pH, a monotonous increase is observed throughout the pH range investigated [Fig. 1(*b*)]. At 358 nm, the absorbing species are the free and complexed coloured forms ($\mathbf{AH_2}$, **AH** and **AM**) and the free and complexed (*Z*)-chalcones ($\mathbf{CH_2}$ and **CM**) whose absorption reaches a maximum at that wavelength. Note that *cis*-2-hydroxychalcones [the (*E*)-chalcone in this work]

$$D = \frac{\varepsilon - \varepsilon'}{4K_d}[\sqrt{(1 + 8K_dc - 1)}] \qquad (1)$$

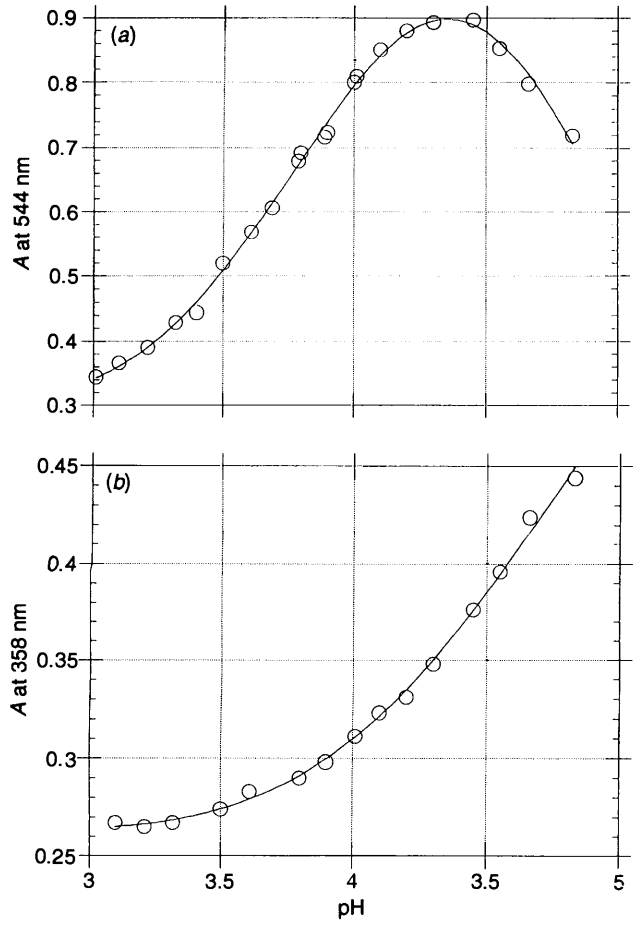


Fig. 1 Absorbance *vs.* pH plot for equilibrated solutions of 3′,4′,7-trihydroxy-3-methoxyflavylium chloride and Al^{3+} in 0.5 mol dm^{-3} formate buffers (T = 25 °C, I = 0.5 mol dm^{-3}). Pigment concentration: 7.5 × 10^{-5} mol dm^{-3}. Al^{3+}:pigment molar ratio: 50. (*a*) λ = 544 nm; the solid line is the result of the curve fitting according to eqn. (2) (r = 0.9990). (*b*) λ = 358 nm; the solid line is the result of the curve fitting according to modified eqn. (2) (see the text, r = 0.9990).

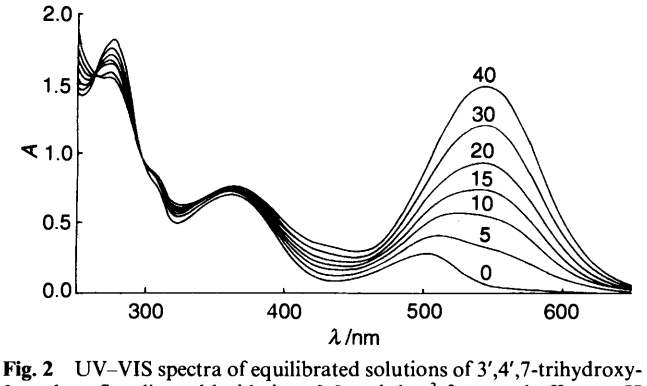


Fig. 2 UV–VIS spectra of equilibrated solutions of 3′,4′,7-trihydroxy-3-methoxyflavylium chloride in a 0.5 mol dm^{-3} formate buffer at pH 3.75 at different Al^{3+} concentrations (T = 25 °C, I = 0.5 mol dm^{-3}). Pigment concentration: 1.5 × 10^{-4} mol dm^{-3}. Al^{3+}:pigment molar ratios: 0, 5, 10, 15, 20, 30 and 40.

typically absorb at much lower wavelengths than the corresponding *trans*-isomers, probably because steric repulsions enforce a non-planar conformation in the *cis*-isomers [the (*Z*)-chalcone in this work].[25] For instance, the (*E*)-chalcone is probably responsible for an inflexion point around 300 nm in the UV–VIS spectrum of 3′,4′,7-trihydroxy-3-methoxyflavylium chloride in weakly acidic solution (Fig. 2). If complexation of the hemiacetal was mainly responsible for the colour loss (at 544 nm) when the pH was raised above 4.4,

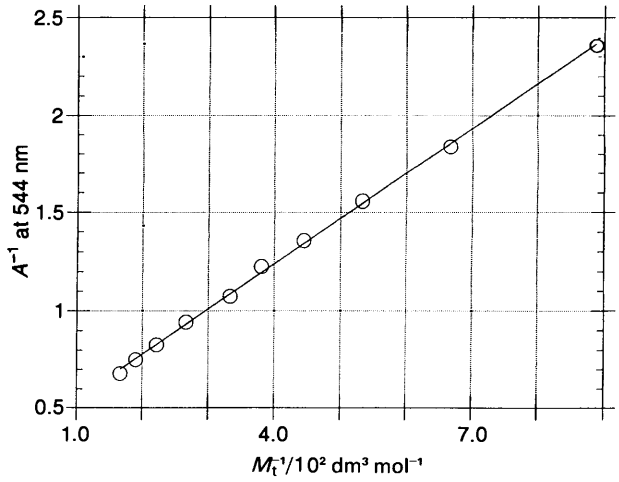


Fig. 3 Absorbance (544 nm) *vs.* Al^{3+} concentration double reciprocal plot for equilibrated solutions of 3′,4′,7-trihydroxy-3-methoxyflavylium chloride in a 0.5 mol dm^{-3} formate buffer at pH 3.75 (T = 25 °C, I = 0.5 mol dm^{-3}). Pigment concentration: 1.5 × 10^{-4} mol dm^{-3}. Al^{3+}:pigment molar ratios: 7.5, 10, 12.5, 15, 17.5, 20, 25, 30, 35, 40. Slope: (23.05 ± 0.25) × 10^{-4}. Intercept: 0.317 ± 0.012. r = 0.9995.

similar absorbance *vs.* pH profiles would be found at 358 and 544 nm since the hemiacetal whose λ_{max} is 276 nm is transparent at both wavelengths (Fig. 2). Therefore, the differences observed bear evidence of (*Z*)-chalcone complexation taking place prior to hemiacetal complexation. A modified eqn. (2) can be used to account for the experimental absorbance *vs.* pH profile recorded at 358 nm. D_1K_{a1} becomes $D_1K_{a1} + D_1'K_iK_h$ to account for free (*Z*)-chalcone formation and a $D_3K_iK_h$-$M_tK_{CM}10^{3pH}$ term is added to the numerator to account for (*Z*)-chalcone–Al^{3+} complexation $\{D_1' = \varepsilon_{CH_2}c$, $D_3 = \varepsilon_{CM}c$ and $K_{CM} = a_H^2[CM]/([CH_2][M])\}$. The curve fitting presented in Fig. 1(*b*) uses the K_{AM} and K_{BM} values determined in the visible range. Because of the impossibility of estimating D_3 in a separate experiment, only the D_3K_{CM} product could be determined. Its value (1.1 × 10^{-6}) is of the order of magnitude of K_{BM} and this once more points to the (*Z*)-chalcone being the major colourless form involved in metal complexation above pH 4.4.

The values for K_h', K_{a1}, K_{AM} and K_{BM} given in Tables 2 and 3 allow one to plot the relative concentrations of coloured and colourless forms as a function of pH in the absence and in the presence of aluminium (Fig. 4). Fig. 1 and Fig. 4 express the influence of the pH on metal–anthocyanin complexation and deserve a few comments. For instance, when the pH of a strongly acidic pigment solution is raised steadily, three distinct pH domains can be distinguished.

From pH 2 *to* pH 3. The first transformation taking place is hydration, whether aluminium is present or not. In both cases, large amounts of colourless forms (mainly, the hemiacetal) are produced and most of the colour is lost at pH 3. In the metal containing solution, competition between protons and metal ions for the complexing sites of the pigment are in favour of the former and no significant complexation occurs.

From pH 3 *to* pH 4.4. In the absence of aluminium, the fading continues resulting in an almost colourless solution. Above pH 4, the remaining pale-red colour begins to turn into pale-purple because of the partial conversion of the flavylium ion into the quinonoidal bases. By contrast, large colour changes occur in the metal containing solution. Above pH 3, Al^{3+} becomes able to remove the phenolic protons of the flavylium B ring and increasing amounts of **AM** chelate appear as the pH is raised. This complexation process is in competition with hydration and complex formation actually leads to conversion of free colourless forms into coloured forms (dehydration). The colour

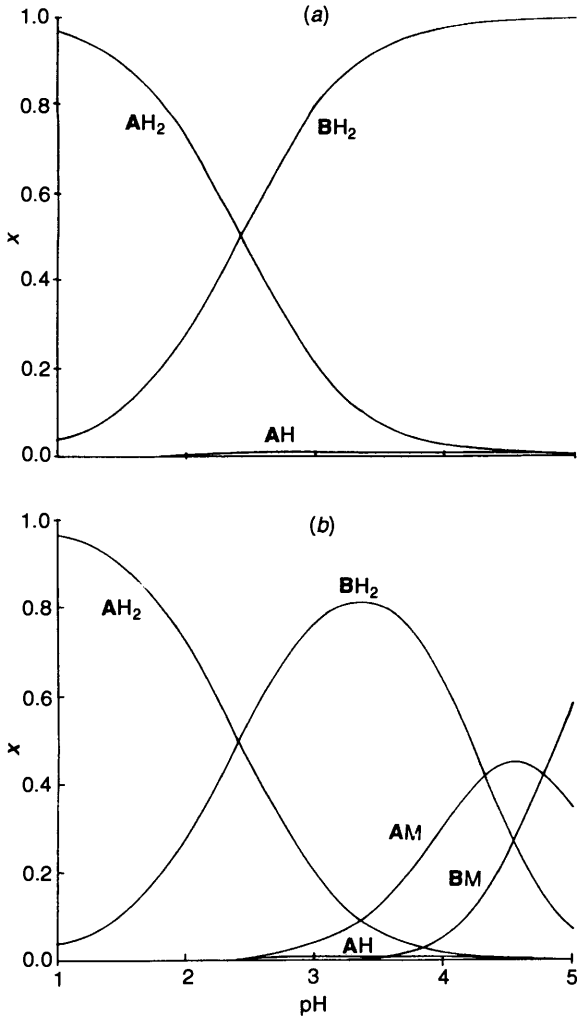
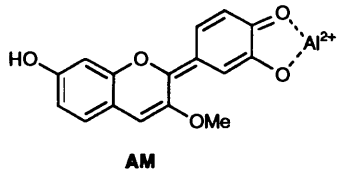


Fig. 4 Distribution diagrams for equilibrated solutions of 3′,4′,7-trihydroxy-3-methoxyflavylium chloride (T = 25 °C, I = 0.5 mol dm^{-3}); (*a*) in the absence of aluminium. (*b*) in the presence of aluminium. Al^{3+} concentration: 2 × 10^{-3} mol dm^{-3}. The diagrams are independent of the pigment concentration as long as Al^{3+} is in large excess with respect to the pigment.

is not only strongly intensified but also deeply changed. Indeed, in the pH domain where the flavylium ion is the dominant coloured form, complexation is accompanied by the loss of two phenolic protons so that the chromophore adopts a quinonoidal structure responsible for the deep-purple colour.

Above pH 4.4. In the absence of aluminium, the quinonoidal bases replace the flavylium ion but the solution remains poorly coloured. In the presence of aluminium, the proton activity becomes weak enough for the phenolic protons of the still abundant hemiacetal and chalcones to be replaced by aluminium and strong catechol–aluminium complexation begins to operate. As a consequence, the overall hydration equilibrium is shifted towards hemiacetal and chalcones and the colour begins to decay.

Upon deprotonation of the flavylium ion, two tautomeric quinonoidal forms can be produced (Scheme 2). In the case of the 3′,4′,7-trihydroxy-3-methoxyflavylium ion, AH(4′) is expected to prevail because of hydrogen bonding between the C-4′–O carbonyl group and the O–H group at C-3′. Semi-empirical quantum mechanics calculations lead to a revision of that view. Indeed, in the AH(4′) most stable conformation, the B-ring and the benzopyrylium nucleus are coplanar and unfavourable steric repulsions occur between the methoxy group in the 3-position and the B-ring. By contrast, the B-ring in AH(7) can adopt a more favourable position by shifting out of the benzopyrylium plane with a corresponding C-2′–C-1′–C-2–O-1 torsion angle of about 20°. As a consequence, the AH(7) most stable conformation *in vacuo* appears to be about 1.4 kcal mol^{-1} more stable than the corresponding AH(4′) conformation. These results are in close agreement with recent theoretical work on the relative stabilities of anthocyanidin quinonoidal forms.[26] Concerning the deprotonation of the AH(7) form of 3′,4′,7-trihydroxy-3-methoxyflavylium chloride, it is much more likely to occur at the OH group at C-4′ than at the OH group at C-3′ because the phenolate formed upon deprotonation of the OH group at C-7 [A(7,4′) in Scheme 2] exists under another mesomeric form [A(4′,7) in Scheme 2] which is stabilized by hydrogen bonding. By contrast, the phenolates A(7,3′) and A(4′,3′) formed upon deprotonation of the OH group at C-3′ in AH(7) and AH(4′) respectively, bear negative charges that are essentially localized. The quantum chemical calculations confirm that view and give energy values for A(7,3′) and A(4′,3′) that are respectively 12.7 and 19.1 kcal mol^{-1} higher than the common value for the A(7,4′) and A(4′,7) mesomeric forms. As a consequence, the A(7,4′)–A(4′,7) form is the only anionic quinonoidal form to be taken into consideration. Finally, formation of a dianionic species upon deprotonation of the OH group at C-3′ of the A form would certainly require fairly alkaline conditions and has not been investigated. The visible absorbance *vs.* pH plot for metal-containing solutions of pigment is best reproduced within the hypothesis of two protons being lost in the flavylium structure upon complexation (Fig. 1). Therefore, the only reasonable structure for complex **AM** is that given in Scheme 5.



AM

Scheme 5 The aluminium complex of 3′,4′,7-trihydroxy-3-methoxyflavylium chloride

When the pH is raised, the replacement by Al^{3+} of the two phenolic protons of the anthocyanin B-ring becomes easier for both the flavylium ion and the colourless forms. However, the phenolic proton at C-7 in the flavylium ion is exceptionally acidic because the corresponding conjugated base is not a simple phenolate but the quinonoidal compound AH(7) strongly stabilized by the large delocalization of its π-electrons. AH(7) is in fast equilibrium with AH(4′) which, although probably in a minority, is able to chelate Al^{3+} upon loss of only one proton. For comparison, the first pK_a value of 3′,4′,7-trihydroxy-3-methoxyflavylium chloride is 4.40 whereas it amounts to 9.20 for catechol[27] (1,2-dihydroxybenzene). In fact, the proton affinity of simple catecholate ligands such as the hemiacetal and chalcones is certainly too strong for complexation to occur significantly below pH 4. On the contrary, the first phenolic proton being easily removed, complexation of the flavylium ion requires the deplacement by Al^{3+} of only one weakly acidic proton, the phenolic proton at C-3′. Therefore, around pH 4, Al^{3+} binds selectively to the 3′,4′,7-trihydroxy-3-methoxyflavylium ion and colour stabilization ensues. The selectivity can be quantified by the K_{AM}:K_{BM} ratio whose value is about 220 and essentially reflects much easier deprotonations in the flavylium structure than in the catechol group of the colourless forms. The log K'_{AM} value of 0.36 ± 0.12 found here for the replacement by Al^{3+} of the phenolic proton at C-3′ in AH is of the order of that measured for maltol[28] (−0.13 at 25 °C and 0.6 mol dm^{-3} ionic strength) which bears a similar complexing site. Moreover, the log K_{BM} value of −6.39 ± 0.05 is in very good

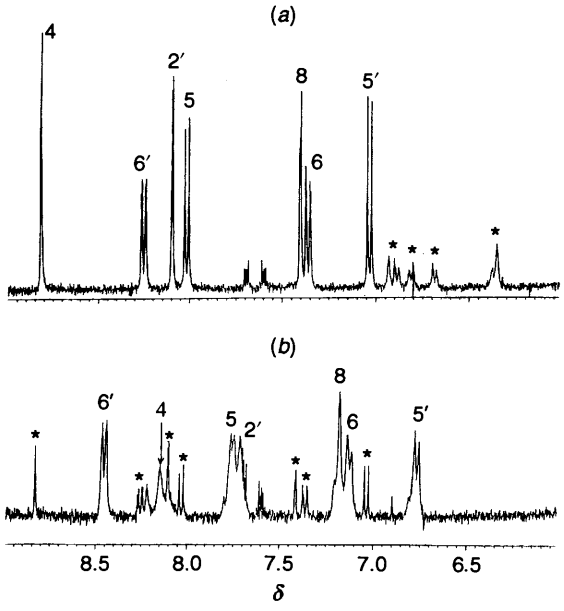
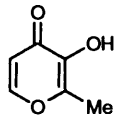


Fig. 5 ^{1}H NMR spectra (400 MHz, 27 °C, Me_4Si as external reference) of equilibrated solutions of 3',4',7-trihydroxy-3-methoxyflavylium chloride in methanol. Pigment concentration: 4×10^{-3} mol dm^{-3}. (*a*) Free flavylium (signals labelled * are attributed to a colourless methanol adduct, probably the methylacetal). (*b*) Aluminium complex. Al^{3+} concentration: 2×10^{-2} mol dm^{-3} (signals labelled * are due to minor amounts of free flavylium).



Maltol

agreement with that found for catechol[27] (−6.34 at 25 °C and 0.6 mol dm^{-3} ionic strength). The binding selectivity in favour of the coloured forms does not hold above pH 4.4 and competitive complexation of the colourless forms occurs. This is due to Al^{3+} having an intrinsically higher affinity for catechol-type ligands than for α-hydroxyketo ligands such as maltol and anthocyanin quinonoidal forms. For instance, the replacement by Al^{3+} of the remaining phenolic proton in the monocatecholate ion has a thermodynamic constant whose log value is $-6.34 + 9.20 = 2.86$ at 25 °C and 0.6 mol dm^{-3} ionic strength, *i.e.* about 2.5 log units higher than our log K'_{AM} value. This opposite selectivity reflects stronger aluminium–ligand interactions in the colourless forms which can be detected in a pH range where competition between Al^{3+} and protons no longer plays a dominant role. The (*Z*)-chalcone is probably more easily deprotonated at the 4'-position (4-position in the usual chalcone site numbering) than the other colourless forms because of a larger electronic delocalization occurring in the corresponding, almost planar phenolate. This could explain why the (*Z*)-chalcone seems to exhibit a higher affinity for Al^{3+} than do the two other colourless forms.

Investigation in Methanol.—^{1}H NMR analysis of the aluminium complex in CD_3OD (Fig. 5) provides additional evidence for the structure given in Scheme 5. For instance, the complex gives signals which are strongly diamagnetically shifted in comparison to the corresponding signals of the free flavylium ion, the exception being the 6'-H signal which upon complexation moves to a higher δ value. These results are in agreement with a π-electron rich quinonoidal structure for the pigment in the complex. The singular behaviour of 6'-H is due probably to its being located on the electron-deficient β-

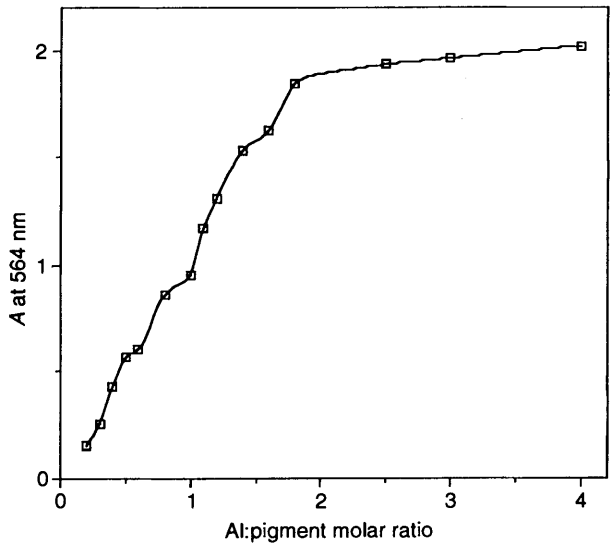


Fig. 6 Plot of the visible absorbance (564 nm) of equilibrated solutions of 3',4',7-trihydroxy-3-methoxyflavylium chloride in methanol *vs.* the Al^{3+}:pigment molar ratio (T = 25 °C). Pigment concentration: 10^{-4} mol dm^{-3}.

position of an α,β unsaturated carbonyl moiety in the complex. The spectroscopic study of complexation as a function of the metal concentration points to a metal–pigment interaction much stronger in methanol than in water and, for instance, the complex is formed almost quantitatively when two molar equivalents of aluminium are added to a 5×10^{-5} mol dm^{-3} solution of pigment in methanol (Fig. 6). Attempts to fit the experimental absorbance *vs.* metal concentration curve with a simple theoretical curve assuming 1:1 complexation were unsuccessful, probably because 1:2 and 1:3 metal–pigment complexes take part at low metal:pigment molar ratios. The relative permittivity (dielectric constant) of methanol being much lower than that of water, electrostatic attraction between entities bearing opposite charges is much stronger in methanol than in water.[29] Therefore, strong metal–pigment complexation in methanol points to a complexation driving force dominated by strong electrostatic interactions between a highly positively charged aluminium ion and the electron-rich coloured forms of the pigment.

Kinetic Investigation.—When a pH-jump (quick addition of concentrated NaOH with negligible dilution) is applied to an equilibrated pigment solution at pH 2.5 with consecutive recording of the visible absorbance at a fixed wavelength, very contrasting behaviour is observed depending on whether the pigment solution contains aluminium or not. In the absence of aluminium, a decrease in visible absorbance occurs according to apparently first-order kinetics due to the hydration process and acid catalysis is exhibited [Fig. 7(*a*)]. The corresponding apparent rate constant can be expressed as eqn. (4) (see ref. 12).

$$k = \frac{k_1 + k_2K_{a1} + k_2 10^{-pH}}{1 + K_{a1}10^{pH}} \quad (4)$$

In the presence of aluminium, both hydration and complexation have to be taken into account in the theoretical treatment of the kinetic data. At pH lower than 3.8, two distinct kinetic steps are observed in the relaxation process after a pH-jump (when monitoring at a wavelength intermediate between the wavelengths of maximum absorption for the free flavylium and the complex, *e.g.* 520 nm): a fast decrease in the visible absorbance during the first 5 or 10 s, which is due to hydration, followed by a slow increase in the visible absorbance featuring complexation. Both phenomena can be considered to be

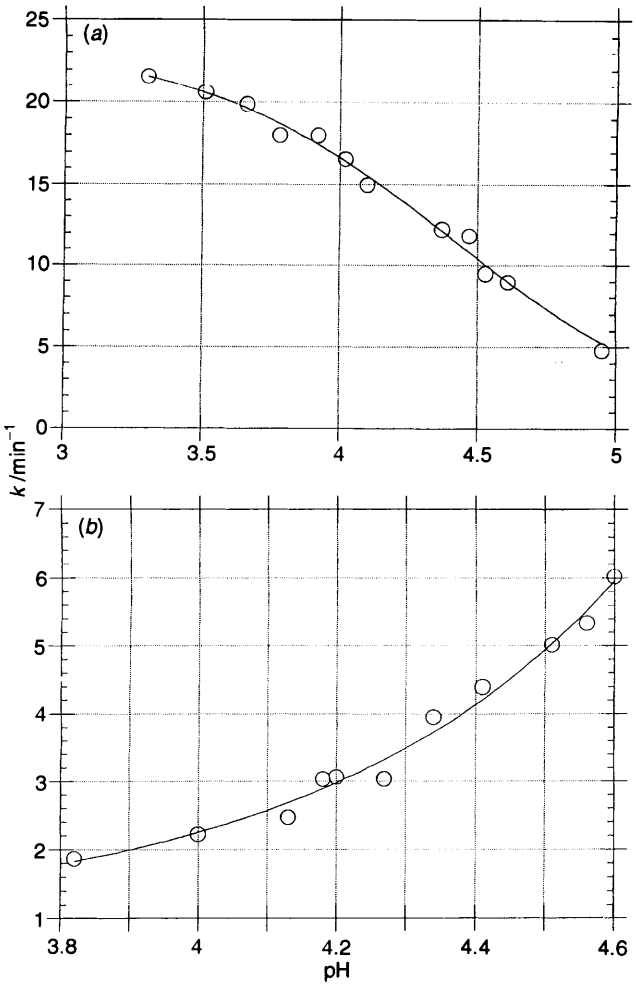


Fig. 7 First-order apparent rate constant *vs.* pH plot for the relaxation process initiated by pH-jump on an equilibrated solution of 3′,4′,7-trihydroxy-3-methoxyflavylium chloride in a 0.05 mol dm^{-3} formate buffer at pH 2.50 (T = 25 °C, I = 0.5 mol dm^{-3}); (*a*) In the absence of aluminium. Pigment concentration: 7.5 × 10^{-5} mol dm^{-3}. (*b*) In the presence of aluminium. Pigment concentration: 10^{-4} mol dm^{-3}. Al^{3+}:pigment molar ratio: 50. The solid lines are the results of the curve fittings according to eqn. (3) [(*a*), r = 0.996] and eqn. (4) [(*b*), r = 0.992].

kinetically independent. At pH higher than 3.8, hydration becomes slower whereas complexation becomes faster and both phenomena are now coupled. A single kinetic step is observed, *i.e.*, a first-order monotonous increase in the visible absorbance which becomes faster when the pH is raised [Fig. 7(*b*)]. Complexation thus appears as the rate-determining step and hydration as a fast equilibrium. Aluminium–anthocyanin complexation can take place according to three different mechanisms. (*i*) Al^{3+} binds to the flavylium ion with consecutive fast removal of the two phenolic protons at C-3′ and C-4′. (*ii*) Al^{3+} binds to the neutral quinonoidal form **AH**(4′) with consecutive fast removal of the phenolic proton at C-3′ and/or to **AH**(7) with, in addition, fast proton transfer from C-4′ to C-7. (*iii*) Al^{3+} binds to the very minor anionic quinonoidal form **A**(7,4′)–**A**(4′,7) with consecutive fast proton transfer from C-3′ to C-7. Any mechanism involving the anionic quinonoidal forms **A**(7,3′) and **A**(4′,3′), which are much higher in energy than **A**(7,4′)–**A**(4′,7), can be safely discarded.

The only mechanism giving an apparent rate constant increasing with the pH assumes complexation occurring through direct coordination of the anionic quinonoidal base to Al^{3+} (Scheme 6). In Scheme 6, the hydration step is the

$$AH_2^+ + H_2O \rightleftharpoons BH_2 + H^+ \qquad K_h$$

$$AH_2^+ \rightleftharpoons AH + H^+ \qquad K_{a1}$$

$$AH \rightleftharpoons A^- + H^+ \qquad K_{a2}$$

$$A^- + M^{3+} \underset{k_{2M}}{\overset{k_{1M}}{\rightleftharpoons}} AM^{2+} \qquad RDS^*$$

Scheme 6

conversion of the flavylium ion into the quickly equilibrating hemiacetal and (*E*)-chalcone (gathered into the BH_2 notation). In the pH range investigated (pH 3.8 to 4.6 after pH-jump), an approximation is made to neglect aluminium–BH_2 complexation. The general rate equation for the relaxation process following a first order perturbation[30] of the complexation equilibrium can be written as eqn. (5). For the fast proton

$$d\Delta c_{AM}/dt = k_{1M}(c_M^*\Delta c_A + c_A^*\Delta c_M) - k_{2M}\Delta c_{AM} = -k\Delta c_{AM} \quad (5)$$

transfer and hydration equilibria, the mass law leads to the expressions (6)–(8). For each species i (**M**, AH_2, **AH**, **A**, **AM**,

$$c_{AH}^*\Delta c_H + c_H^*\Delta c_{AH} = K_{a1}\Delta c_{AH_2} \quad (6)$$

$$c_A^*\Delta c_H + c_H^*\Delta c_A = K_{a2}\Delta c_{AH} \quad (7)$$

$$c_{BH_2}^*\Delta c_H + c_H^*\Delta c_{BH_2} = K_h\Delta c_{AH_2} \quad (8)$$

BH_2), Δc_i is the time-dependent deviation from c_i^*, the concentration of i in the final equilibrium state (reached after perturbation). k, k_{1M} and k_{2M} are the first-order apparent rate constant, the second-order rate constant of complex formation and the first-order rate constant of complex dissociation, respectively. In the formic acid–formate ion buffer, the final pH value is fixed through the instantaneous formic acid–formate ion equilibrium *i.e.* just after mixing the pigment and NaOH solutions. Thus, during the relatively slow relaxation process, the pH can be considered constant, *i.e.* $\Delta c_H = 0$. Finally, mass conservation applied to aluminium ion and pigment gives eqns. (9) and (10). A combination of these equations first gives

$$\Delta c_{AH_2} + \Delta c_{AH} + \Delta c_A + \Delta c_{BH_2} + \Delta c_{AM} = 0 \quad (9)$$

$$\Delta c_M + \Delta c_{AM} = 0 \quad (10)$$

eqn. (11). Considering the ratio of the second term to the first

$$k = k_{2M} + k_{1M}c_A^* + k_{1M}c_M^*K_{a1}K_{a2}/(K_hc_H^*) \quad (11)$$

one, we may write: $k_{1M}c_A^*/k_{2M} = K''_{AM}c_A^* = c_{AM}^*/c_M^* < c/c_M^*$ (c is the overall pigment concentration). Since aluminium chloride is in large excess with respect to the pigment, the concentration of free metal ion at equilibrium c_M^* can be taken as equal to the overall metal concentration M_t which is much larger than c. As a consequence, the second term in the last equation can be neglected with respect to the first one, thus giving expression (12) for the apparent first-order rate constant

$$k = k_{2M} + k_{1M}(K_{a1}K_{a2}/K_h)M_t10^{pH} \quad (12)$$

(c_H^* is approximated to 10^{-pH}). Finally, using $K''_{AM} = k_{1M}/k_{2M} = K_{AM}/(K_{a1}K_{a2})$, we get eqn. (13). If the

* Rate determining step.

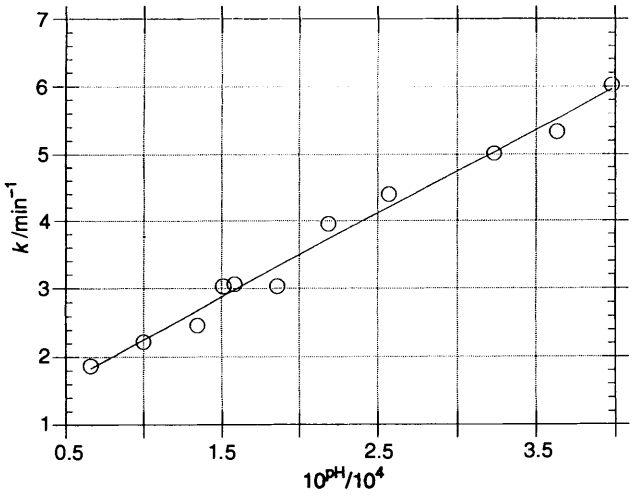


Fig. 8 First-order apparent rate constant *vs.* 10^{pH} plot for the relaxation process initiated by pH-jump on an equilibrated solution of 3′,4′-7-trihydroxy-3-methoxyflavylium chloride and Al^{3+} in a 0.05 mol dm^{-3} formate buffer at pH 2.50 (T = 25 °C, I = 0.5 mol dm^{-3}). Pigment concentration: 10^{-4} mol dm^{-3}. Al^{3+}:pigment molar ratio: 50. Slope: (1.25 ± 0.05) × 10^{-4}. Intercept: 1.00 ± 0.12. r = 0.992.

$$k = k_{2M} + k_{2M}(K_{AM}/K_h)M_t10^{pH} \quad (13)$$

mechanism proposed in Scheme 6 is correct, a k *vs.* 10^{pH} plot must give a straight line. This is well verified as can be seen on Fig. 8. In addition, the slope:intercept ratio $(K_{AM}/K_h)M_t$ provides an independent way to determine K_{AM}. A log value of −4.12 ± 0.09 has thus been estimated which is in good agreement with that determined from thermodynamic measurements (−4.04 ± 0.06). The intercept of the k *vs.* 10^{pH} plot reported in Fig. 8 gives: k_{2M} = 1.00 ± 0.12 min^{-1}. From the $\log K''_{AM}$ value given in Table 3, a rough estimate for the k_{1M} value can be drawn: k_{1M} = (1.0 ± 0.6) × 10^8 dm^3 mol^{-1} min^{-1}. The values of the apparent first order rate constant of the relaxation process initiated by pH-jump range from approximately 2 to 6 min^{-1}, depending on the final pH [Fig. 7(*b*)]. These values are at least one order of magnitude lower than that for water exchange on the aluminium ion [31] and seem to rule out any dissociative mechanism in which the complexation kinetics would be controlled by Al^{3+} desolvation. It is more likely that complexation occurs through direct Al^{3+}–anthocyanin quinonoidal base interaction with consecutive or concerted water removal from the Al^{3+} coordination sphere.

Conclusions

This work is the first quantitative investigation of metal–anthocyanin complexation in aqueous solution. 3′,4′,7-Trihydroxy-3-methoxyflavylium chloride, a valuable synthetic model of natural anthocyanins, has been found to bind aluminium selectively through its coloured forms for pH values ranging from pH 3.0 to pH 4.4, *i.e.*, in acidity conditions which are frequently encountered in the natural medium of anthocyanins. Complexation is strong enough to induce impressive colour changes from pale-red to deep-purple which are interpreted by a large conversion of colourless forms into a coloured complex in which the pigment adopts a quinonoidal structure. In addition to previous works on copigmentation, this work highlights the versatility of flavylium ions in colour stabilizing complexation processes and it is hoped, provides the basis for investigations dealing with complexation of anthocyanins with other highly charged metal ions such as the Mg^{2+} and Ca^{2+} which are very abundant in the plant vacuoles[4] and the Fe^{3+} ion. In this latter case, anthocyanin complexations could be followed by oxidative processes which are of major significance in polyphenol biochemistry.

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