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ORIGIN OF THE EXCEPTIONAL COLOUR STABILITY OF THE *ZEBRINA* ANTHOCYANIN*

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Key Word Index—*Zebrina pendula*; Commelinaceae; colour stability; acidity constant; visible spectra; pH measurements; acylated anthocyanin; caffeoylferuloyl cyanidin 3,7,3′-triglucoside.

Abstract—The exceptional colour stability near neutrality of the *Zebrina* anthocyanin is related both to a high value of the acidity constant and to the total absence of formation of the colourless pseudobase and chalcone. The aromatic residues of the acylated groups (caffeic and ferulic) probably interact with the positively charged pyrylium nucleus. This intramolecular effect prevents further addition of nucleophiles to the pyrylium ring; in particular, addition of water is largely disfavoured compared to ordinary anthocyanins.

INTRODUCTION

Anthocyanins are the main pigments of flowers and fruits [1]. However, under conditions as similar as possible to natural conditions, i.e. room temperature and slightly acidic aqueous solutions, most anthocyanins are colourless [2]. Therefore, in order to explain plant pigmentation due to anthocyanins, several stabilizing factors of the anthocyanin chromophores have been proposed [2]. Among these, the most important are copigmentation and metal chelation [3]. However, though many compounds have been found to act as copigments, our knowledge of the complexes formed is limited. As for metallic complexes, they can only be formed in anthocyanins with two adjacent hydroxyl groups in the B-ring. Most interesting is the recent discovery of acylated anthocyanins which retain a stable colour in slightly acidic or neutral solutions [4].

The purpose of this paper is to demonstrate both theoretically and experimentally that a stable colour can be obtained near neutrality without the presence of copigments or metal ions. The pigment studied is cyanidin 3,7,3′-triglucoside acylated with caffeic and ferulic acids, recently isolated from the flowers of *Zebrina pendula* Schnizl. [5]. This pigment has the same general structure as that isolated from *Tradescantia reflexa* [4] in that there appear to be three acyl groups present attached through the glucose residues.

RESULTS

Until now, all the anthocyanin pigments we have investigated exist in aqueous acidic solution as a mixture of four species in equilibrium according to the following scheme [6]:

$$AH^+ \rightleftarrows A + H^+ \quad K_a' = ([A]/[AH^+])a_{H^+} \qquad (1)$$

$$AH^+ + H_2O \rightleftarrows B + H^+ \quad K_h' = ([B]/[AH^+])a_{H^+} \qquad (2)$$

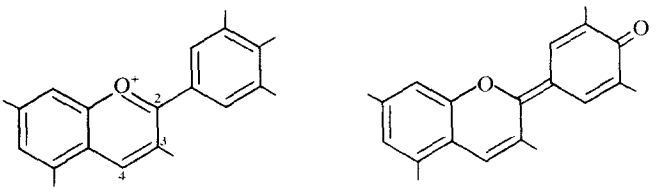
$$B \rightleftarrows C \qquad K_T = [C]/[B]. \qquad (3)$$

a_{H^+} is the activity of the hydronium ion ($pH = -\log a_{H^+}$). AH^+, A, B and C are the flavylium cation, the quinonoidal base, the pseudobase or carbinol and the chalcone, respectively. Equilibrium (1) is a fast diffusion-controlled acid–base reaction. Equilibrium (2) is much slower (half-life of a few minutes) and represents the nucleophilic attack of water on the pyrylium ring. Equilibrium (3) is a slow prototropic change giving rise to the chalcone. When a solution containing these four structures is sufficiently acidified, A, B and C revert to AH^+ according to three kinetically distinct steps.

This is the first time we have encountered an anthocyanin that does not form any pseudobase and/or chalcone. Evidence for the complete absence of these two neutral and colourless species is given in Fig. 1. To an equilibrated solution at 25° and pH 5.5, we rapidly added a few μl of a concentrated hydrochloric acid solution. The new pH value was 1.5. The pH-induced absorbance change was recorded at 540 nm (a wavelength close to the maximum of absorption of the cation). Thus a fast decrease in absorbance related to the transformation of A to AH^+ is observed. After this fast change the absorbance remained at a steady level indicating that in the non-acidified solution (pH 5.5), there are no detectable amounts of B and/or C.

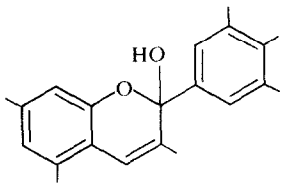
Fig. 2 shows the changes in the visible spectrum of the *Zebrina* anthocyanin for different acidities of an aqueous solution at 25°. At pH 1.37 the pigment exists only as the flavylium cation AH^+ (λ_{max} 537 nm). When the acidity is lowered the cation is instantaneously converted to the quinonoidal base A. Near pH 5.5 transformation to A is

* Part 7 in the series 'The Chemistry of Anthocyanin Pigments'. For Part 6 see Brouillard, R. and El Hage Chahine, J. M. (1980) *J. Am. Chem. Soc.* **102**, 5375.

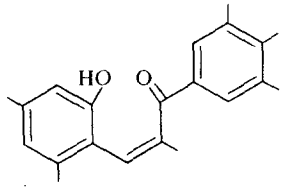


AH^+

A



B



C

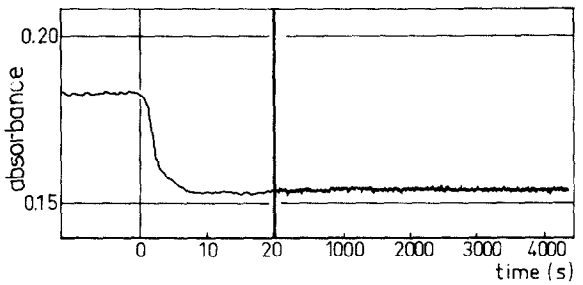


Fig. 1. Absorbance change (540 nm) of a pigment solution initially equilibrated at 25° and pH 5.5 when rapidly acidified (final pH 1.5).

complete (λ_{max} 508, 545 and 585 nm). The acidity constant K'_a has been measured by plotting log $(D_{AH^+} - D/D - D_A)$ against pH. The pH value for a zero value of log $(D_{AH^+} - D/D - D_A)$ corresponds to the pK'_a value. D_{AH^+}, D_A and D are the absorbances of three different solutions: the first containing only the cation, the second only the quinonoidal base and the third containing both these species at the same time, respectively. Thus, $K'_a = 3.1 \times 10^{-4}$ mol/dm^3 at 25° (pK'_a = 3.50).

DISCUSSION

The acidity constant value is unusually high. For malvidin 3-glucoside and malvidin 3,5-diglucoside K'_a is 5.7×10^{-5} mol/dm^3 at 25° and 1.0×10^{-4} mol/dm^3 at

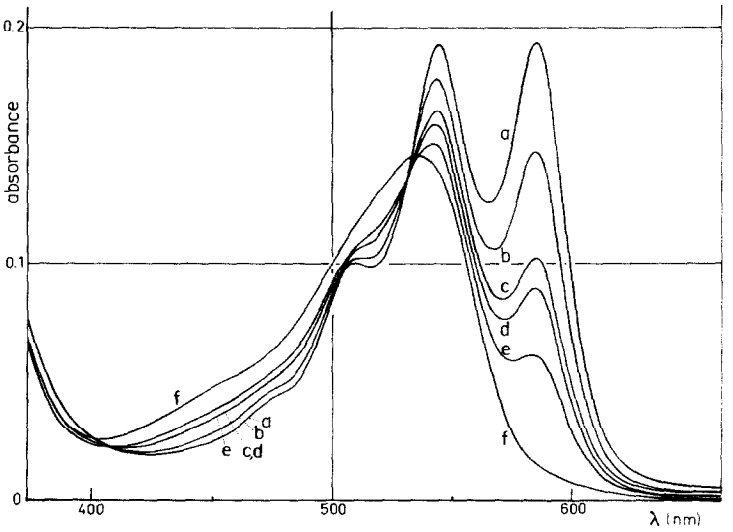


Fig. 2. Absorption spectra in the visible range of a pigment solution at 25° and at various acidities: pH = 5.52 (a); 4.02 (b); 3.48 (c); 3.37 (d); 3.07 (e) and 1.37 (f).

6.5°, respectively [7]. For flavylium cations without a substituent at position 3, values lower than 10^{-4} mol/dm^3 have generally been found [8]. The higher the K'_a value, the greater is the stability of the chromophore A. However, as seen below, other factors contribute to its stability.

The relative amounts of the neutral species A, B and C are given by equations (4) and (5), whatever the pH.

$$[A] = (K'_a/K'_h)\,[B] \quad (4)$$

$$[A] = (K'_a/K'_h K_T)\,[C]. \quad (5)$$

Near neutrality the flavylium cation is generally no longer stable and the overall concentration of the pigment is [A] + [B] + [C]. Therefore any factor preventing the formation of B and C will give rise to coloured solutions and vice-versa as in the following extreme cases.

(a) $K'_h \gg K'_a$, $K'_h K_T$; the only stable species is the pseudobase as is the case for most anthocyanins [7].

(b) $K'_h K_T \gg K'_a$, K'_h; the only stable species is the chalcone as is the case for 7,4'-dihydroxyflavylium chloride, recently investigated (Brouillard, R., unpublished results).

(c) $K'_a \gg K'_h$, $K'_h K_T$; the quinonoidal base is perfectly stable.

In the last case, it should be noticed that if K'_h and $K'_h K_T$ are lower than *ca* 1 % of K'_a, the very small amounts of B and C formed when complete equilibrium is reached cannot be detected and K'_h and K_T cannot be measured. The main reason for this is that after a pH jump the quinonoidal base is at the same time the kinetic and thermodynamic product. The reverse is not true; in particular for cases (a) and (b) K'_a can always be measured by performing appropriate pH jumps generating A in large amounts. Case (c) applies to the *Zebrina* anthocyanin. For this type of pigment pK'_h should be greater than 5 and K_T lower than 1. Thus the high value of the acidity constant and lack of formation of the pseudobase and the chalcone are at the origin of the exceptional colour stability of the *Zebrina* anthocyanin and other structurally related pigments [4].

Since the hydration reaction, which is so easy with ordinary anthocyanins, does not occur at all, there must be some unusual structural factor preventing the nucleophilic addition of water to the pyrylium ring. The results here indicate that the aromatic residues of the acylated groups interact with the positively charged pyrylium ring in such a way that the reactivity of the carbon at position 2 with nucleophilic reactants is greatly diminished. This is not surprising, since it has been shown that cinnamic acid derivatives act as copigments with ordinary anthocyanins [9]. The main effect of these copigments is to protect the pyrylium ring against the approach of water molecules. Therefore, the existence of hydrophobic interactions between the pyrylium ring and the aromatic rings of the caffeic and ferulic esters is a satisfactory explanation for the extraordinary colour stability of the *Zebrina* anthocyanin. Such an interaction has been recently postulated in the case of the sky-blue anthocyanin complex from *Commelina* [10]. On the other hand, the hydrophilic parts of the molecule, i.e. the hydroxyl groups of the aglycone and the sugars, remain completely unaffected. All pigments exhibiting such a high colour stability are characterized by the presence of two acyl groups [4]. Probably one group is situated above the pyrylium ring and the other beneath. Molecular models show that the existence of such a conformation is highly plausible.

EXPERIMENTAL

The *Zebrina* anthocyanin was used without further purification. All measurements were done in distilled H_2O suitably acidified (HCl) at 25°. Solns were prepared by directly dissolving the pigment into the spectrophotometer sample cell.

Visible spectra. The visible spectra were recorded using a Cary 118 spectrophotometer fitted with a reference cell and a thermostated sample cell with a magnetic stirring device. The path length of both cells was 1 cm.

pH measurements. The pH was measured directly within the sample cell using a pH meter fitted with a combined glass electrode. The buffered solns used for pH meter standardization were either pH 4.01 and 6.86 N.B.S. standards or 0.01 N HCl solutions. The pH change was achieved by injecting, into the sample cell, a few μl of a conc acidic (HCl) aq. soln. The mixing time was *ca* 2 sec.

Kinetic measurements. For a suitable pH jump, the change in absorbance was recorded at a wavelength close to the λ_{max} of the flavylium cation using the same spectrophotometer as above.

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REFERENCES

1. Harborne, J. B. (1967) *Comparative Biochemistry of the Flavonoids*. Academic Press, London and New York.
2. Timberlake, C. F. and Bridle, P. (1975) in *The Flavonoids* (Harborne, J. B., Mabry, T. J. and Mabry, H., eds.). Chapman & Hall, London.
3. Asen, S. Norris, K. H. and Stewart, R. N. (1969) *Phytochemistry* **8**, 653.
4. Yoshitama, K. (1978) *Bot. Mag. Tokyo* **91**, 207.
5. Stirton, J. Z. and Harborne, J. B. (1980) *Biochem. Syst. Ecol.* **8**, 285.
6. Brouillard, R. and Dubois, J. E. (1977) *J. Am. Chem. Soc.* **99**, 1359.
7. Brouillard, R. and Delaporte, B. (1977) *J. Am. Chem. Soc.* **99**, 8461.
8. Sperling, W., Werner, F. C. and Kuhn, H. (1966) *Ber. Bunseges. Phys. Chem.* **70**, 530.
9. Asen, S., Stewart, R. N. and Norris, K. H. (1972) *Phytochemistry* **11**, 1139.
10. Goto, T., Hoshino, T. and Takase, S. (1979) *Tetrahedron Letters* 2905.