× 25 mL). The organic layers were washed with H_2O (20 mL), and the aqueous phase was extracted with AcOEt. The combined organic extracts were dried ($MgSO_4$), filtered, and concentrated in vacuo. Purification by flash chromatography (AcOEt) afforded the desired compound (4.4 mg, 83%): mp 129-131 °C (hexane-AcOEt); ^1H NMR ($CDCl_3$, 490 MHz) δ 5.97 (d, J = 9.7 Hz, 1 H, 4-H), 5.75 (dd, J = 9.7, 5.9 Hz, 1 H, 3-H), 5.57 (br s, 1 H, 5-H), 4.70-4.76 (m, 1 H, 6'-H), 4.39 (dt, J = 8.6, 3.7 Hz, 1 H, 4'-H), 4.24 (br s, 1 H, 8-H), 2.75 (dd, J = 17.6, 5.1 Hz, 1 H, 3'-H), 2.63 (ddd, J = 17.6, 3.7, 1.7 Hz, 1 H, 3'-H), 2.29-2.40 (m, 2 H), 2.14-2.24 (m, 1 H), 1.46-2.07 (complex, 12 H), 0.92 (d, J = 7.1 Hz, 3 H, 2-CH_3); IR ($CHCl_3$) 3580, 3400 (br), 3010, 2950, 2920, 1720, 1390, 1370, 1250, 1075, 1045 cm^{-1}; HRMS (EI) calcd for $C_{18}H_{26}O_4$ 306.1832, found 306.1839. The synthetic compound was indistinguishable (NMR, IR, TLC) from the natural material [obtained from (+)-compactin[28]].

Methyl (1*SR*,2*SR*,4a*RS*,6*SR*,8*SR*,8a*SR*)-8-(*tert*-Butyldimethylsiloxy)-1,2,4a,5,6,7,8,8a-octahydro-6-hydroxy-2-methyl-5-oxo-1-naphthalenecarboxylate (30). To a solution of mCPBA (306.7 mg, 1.42 mmol) in hexanes (20.3 mL), cooled to -20 °C, was added (2 min) a solution of silyl enol ether **7** [prepared from lactone **5** (321 mg, 0.95 mmol), as mentioned above (see preparation of **8**) [LDA, -78 °C, toluene 105 °C (2.5 h), filtration of salt from hexanes solution]] in hexanes (3.5 mL).[29] The reaction mixture was stirred at -20 °C for 5 min and allowed to warm to room temperature. After 2 h, a second portion of mCPBA (100 mg, 0.46 mmol) was added. After 3 h at room temperature, dimethyl sulfide (1.5 mL) was added and the mixture was stirred for 10 min. Most of the acid was removed by filtration and the solvent evaporated. The residue was taken up in THF (35 mL) and treated with 1 N HCl (3.1 mL). After 20 min, the reaction mixture was poured in H_2O (250 mL) and extracted with CH_2Cl_2 (3 × 75 mL). The organic phases were washed with brine (50 mL + 3 mL of 10% $Na_2S_2O_3$). The aqueous layer was extracted with CH_2Cl_2 (50 mL), and the combined organic phases were dried ($MgSO_4$), filtered, and concentrated in vacuo. The crude acid in dry ether (30 mL) was esterified with an excess of a ethereal CH_2N_2 solution (0 °C, 45 min). After concentration, the residue was purified by flash chromatography (hexane-AcOEt, 6:1) to give **30** (263.2 mg, 75%): mp 53.0-54.5 °C (hexane); ^1H NMR ($CDCl_3$, 250 MHz) δ 6.00 (dt, J = 10.1, 1.6 Hz, 1 H, 4-H), 5.77 (ddd, J = 10.1, 4.5, 2.7 Hz, 1 H, 3-H), 4.55 (br s, 1 H, 8-H), 4.52 (ddd, J = 11.9, 7.2, 3.7 Hz, 1 H, 6-H), 3.70 (s, 3 H, 1-CO_2CH_3), 3.57 (br d, J = 11.8 Hz, 1 H, 4a-H), 3.44 (d, J = 3.7 Hz, 1 H, 6-OH), 2.95 (11.5, 6.1 Hz, 1 H, 1-H), 2.62-2.71 (m, 1 H, 2-H), 2.58 (ddd, J = 13.6, 7.2, 3.4 Hz, 1 H, 7-H), 2.02 (~ddd, J = 11.8, 11.5, 1.6 Hz, 1 H, 8a-H), 1.70 (ddd, J = 13.6, 11.9, 2.0 Hz, 1 H, 7-H), 0.91 (s, 9 H, 8-OSi(t-C_4H_9)), 0.85 (d, J = 7.1 Hz, 3 H, 2-CH_3), 0.14, 0.01 (2 s, 2 × 3 H, 8-$OSi(CH_3)_2$); IR ($CHCl_3$) 3480 (br), 3010, 2950, 2930, 2880, 2850, 1730, 1715 (sh), 1470, 1460, 1435, 1375, 1290, 1260, 1245, 1205, 1175, 1145, 1095, 1065, 1010, 970, 960, 930, 865, 840, 815 cm^{-1}; MS m/e 311 $(M - 57)^+$.

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Supplementary Material Available: Experimental conditions for the preparation of racemic compounds **9-17** and for the concluding steps from (+)-**14** to (+)-compactin, spectral data for compounds **9-17**, spectral and rotation data for (+)-**14** to (+)-compactin, and rotation and melting point data for (+)-**3**-(+)-**14** (12 pages). Ordering information is given on any current masthead page.

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The Copigmentation Reaction of Anthocyanins: A Microprobe for the Structural Study of Aqueous Solutions

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Abstract: By means of visible absorption spectrometry, we have demonstrated that, in acidic aqueous solutions, chlorogenic acid (5-*O*-caffeoylquinic acid) gives a loose 1:1 complex with the flavylium cation of malvin (malvidin 3,5-diglucoside) chloride. The molecular interaction taking place between these two chemical species is characteristic of the copigmentation reaction of anthocyanins. For the first time the mechanism associated with this reaction is established. The equation describing the copigment effect is also given. The copigmentation reaction is a very fast process that is extremely influenced by temperature. Increasing the temperature or adding methanol, formamide, or sodium chloride always reduces the copigment effect. In fact, we demonstrate that the extent of copigmentation is strictly under the control of the unique molecular structure of liquid water. Finally, the copigmentation phenomenon, which is widespread in higher plants, constitutes a simple, inexpensive, and very sensitive microprobe for the structural studies of aqueous solutions.

As part of the general effort to improve our knowledge of the phenomena involved in plant pigmentation, we now report results on the copigmentation reaction of anthocyanins. Many factors are known to influence the color of anthocyanins.[1] Among these factors, copigmentation is one of the most important and perhaps the least understood. Copigments have a strong stabilizing effect on the color of anthocyanins. In their absence and, under the physico-chemical conditions prevailing in the natural media in which anthocyanins occur, the common anthocyanins exist es-

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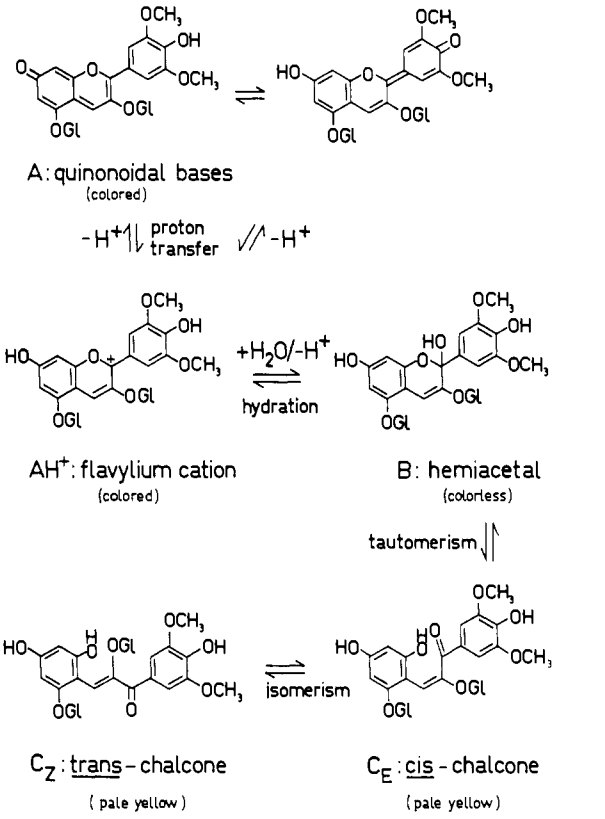
sentially in the colorless forms.[2]

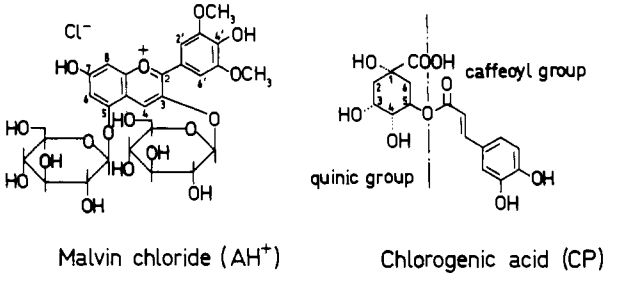
The copigmentation reaction of anthocyanins was first observed by the Robinsons.[3] It occurs when a copigment is added to a sufficiently concentrated slightly acidic aqueous solution of anthocyanin and produces an increase in color intensity and a change in the color which is designated as the "bluing" effect.[4] With the advent of UV–vis absorption spectrophotometry, it was further observed that a copigment almost always produces an increase in absorbance in the visible range (hyperchromic effect) and that the wavelength of maximum absorption shifts toward higher wavelengths (bathochromic effect).[5] Molecules acting as copigments include a large variety of structurally unrelated compounds, such as flavonoids, polyphenols, alkaloids, amino acids, organic acids..., and the anthocyanins themselves. Of these substances, however, only a few flavonoids have been investigated in some detail.[6] Colorless flavonoids and polyphenols are frequently found in association with anthocyanins in the vacuoles of the colored cells of higher plant organs.[7] Therefore, the copigmentation phenomenon is widespread in nature. It also occurs in fruits and vegetable products such as juices and wines.

The intensity of the copigmentation effect has been shown to be dependent upon several factors including the concentrations of anthocyanin and copigment, their chemical structures, the pH of the medium, the solvent, and the temperature.[4,6] The magnitude of the copigment effect, at a given ratio of the concentration of copigment to the concentration of anthocyanin, in buffered water at ambient temperature, is usually estimated by the increase in absorbance, at the visible wavelength of maximum absorption, and by the shift of this maximum wavelength toward higher values.[5,6,8] To date, however, these changes in spectral features of anthocyanin–copigment solutions have been given only empirical treatments without theoretical background.

In this article, a new approach to the study of the anthocyanin–copigment molecular interaction is described and applied to the couple malvin chloride–chlorogenic acid. Malvin chloride (malvidin 3,5-diglucoside) is a common anthocyanin, and its color is largely influenced by the presence of copigments. Chlorogenic acid (5-*O*-caffeoylquinic acid) is a widespread, natural, colorless phenolic molecule, sufficiently soluble in water, and known to be a good copigment. Our method permits to interpret quantitatively the factors involved in the stability of the malvin–chlorogenic acid complex and to give, for the first time, the mechanism associated with the natural copigmentation process. The following factors were investigated and elucidated: (a) the nature of the malvin structure directly involved in copigmentation; (b) the number of chlorogenic acid molecules complexed with the malvin structure; (c) the values of the equilibrium constant characteristic of the stability of the complex in the temperature range of liquid water;[9] (d) the apparent enthalpy and entropy of the copigmentation reaction; (e) the fundamental role played by the very structure of liquid water. Finally, our approach can be used to quantitatively interpret published UV–vis absorption measurements which were not fully explained because of the lack of a theoretical basis. One very recent example is treated in the discussion.

Scheme I





Experimental Section

Commercial malvin and cyanin (malvidin 3,5-diglucoside and cyanidin 3,5-diglucoside) and chlorogenic acid (Roth) purified by polyvinyl polypyrrolidone (Sigma) column chromatography were used. The purity of the chromatographed samples was checked both by UV–vis and ^{1}H NMR spectroscopies according to published procedures.[10]

Malvin or cyanin chloride was dissolved in 0.06 M aqueous phosphoric acid (Prolabo). Immediately after preparation, each solution was thoroughly mixed in the dark at 20 °C for 15–30 min and diluted to the original concentration by addition of 0.20 M aqueous sodium acetate (Prolabo). Phosphoric acid and sodium acetate were found convenient for maintaining constant pH values over the whole acidic pH range. The ionic strength was adjusted to 0.20 M by the addition of sodium chloride (Merck suprapur). Methanol (Merck) and formamide (Fluka) were used without further purification.

Visible Absorption Spectra. Visible absorption spectra of buffered pigment solutions, with and without chlorogenic acid, were recorded with a Perkin Elmer Lambda 5 spectrophotometer fitted with a thermostated 1-cm pathlength quartz cuvette with a magnetic stirring device. A 2-mL aliquot malvin or cyanin solution was placed in the thermostated cell, and, after recording of its spectrum, a known weight of solid chlorogenic acid

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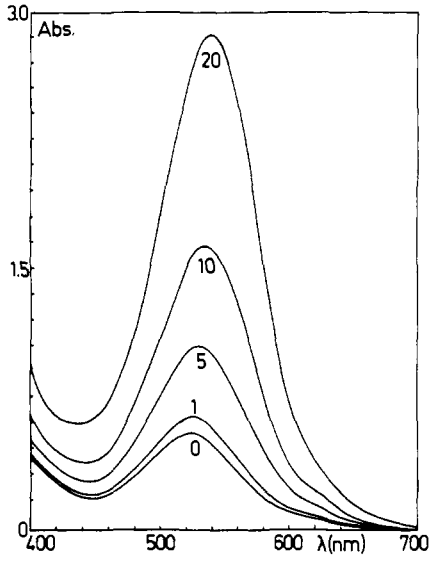

Figure 1. Visible spectra of malvin (7.73×10^{-4} M) and chlorogenic acid at 0, 1, 5, 10 and 20 copigment to pigment molar ratios: pH = 3.65; T = 20 °C; l = 1 cm; solvent, aqueous H_3PO_4–CH_3CO_2Na buffer; ionic strength = 0.20 M.

was added. Whenever necessary the pH of the anthocyanin solution, with and without chlorogenic acid, was adjusted to the desired level by injecting into the sample cell a few microliters of 10 M HCl or 10 M NaOH. The spectrum of the pigment–copigment mixture was recorded after 20–30 min of mixing.

pH and Temperature Measurements. The pH of the solution was measured directly in the thermostated cell with a Metrohm Model 654 pH meter equipped with a small combined glass electrode (EA 125). The buffers used to calibrate the pH meter were pH 7.00 and 4.00 NBS standards (Merck). The temperature of the solution was controlled within 0.2 °C by using a Lauda ultrathermostat water bath.

Results

Effect of pH and Chlorogenic Acid on the Visible Absorption Spectra of Malvin. The structural transformations of malvin alone in aqueous acidic solutions are shown in Scheme I.[1] Malvin occurs as an equilibrium mixture of the colored flavylium cation AH^+, the colored quinonoidal bases A, the colorless hemiacetal B, and the pale yellow chalcones C_E and C_Z. At very low pH, the flavylium structure dominates, whereas, from pH 2 up to neutrality, the other structures are the more abundant. Due to the high value of the equilibrium constant of the hydration reaction, malvin itself cannot confer much color to a solution whose pH ranges from about 3 to neutrality.[11] Indeed, only a few percent of the overall concentration of malvin remains stable in the strongly colored forms in these slightly acidic solutions. Such a result is clearly demonstrated by the weak absorbance of a pH 3.65 solution of malvin as shown by spectrum 0 in Figure 1. This absorption is characteristic of the flavylium cation AH^+. The shoulder around 610 nm indicates that at pH 3.65, a small amount of the quinonoidal bases A is already present. Under such conditions malvin essentially exists in the form of the fast ring–chain tautomeric equilibrium $B \rightleftharpoons C_E$.[12] At 20 °C C_Z is a very minor component.

In this study, we found that addition of chlorogenic acid to solutions of malvin enhances the color at pH values from about 2 to neutrality. The pH value for maximal color enhancement was found to be close to 3.6. The largest increase in absorbance observed, at the wavelength of maximum absorption, is 12.2-fold and this was for a 2.58×10^{-4} M malvin solution at pH 3.60 and 20 °C and at the copigment-to-pigment molar ratio of 150. It will be made clear in the discussion, however, that this is far from being the highest increase obtainable. Figure 1 shows stable typical visible absorption spectra of malvin alone and mixtures of malvin

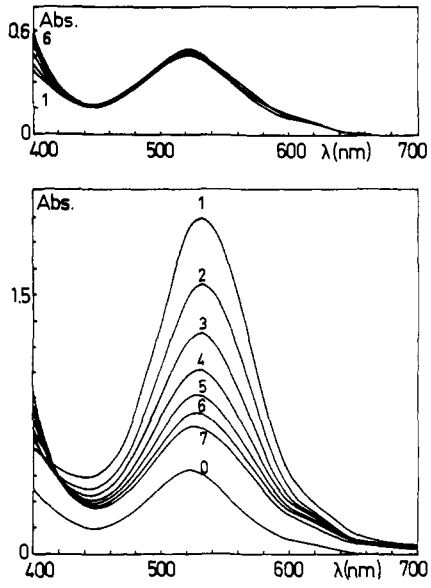

Figure 2. Upper part: visible spectra of malvin (7.73×10^{-4} M) at 10 (1), 20, 30, 40, 50 and 60 °C (6): pH = 3.67; l = 1 cm; solvent, aqueous H_3PO_4–CH_3CO_2Na buffer; I = 0.20 M. Lower part: visible spectra of malvin alone (0), and malvin with chlorogenic acid at 10 (1), 20 (2), 30 (3), 40 (4), 50 (5), 60 (6), and 70 °C (7). Concentration of malvin: 7.73×10^{-4} M; pH = 3.65; l = 1 cm; solvent; aqueous H_3PO_4–CH_3CO_2Na buffer; I = 0.20 M; copigment-to-pigment molar ratio, 10.

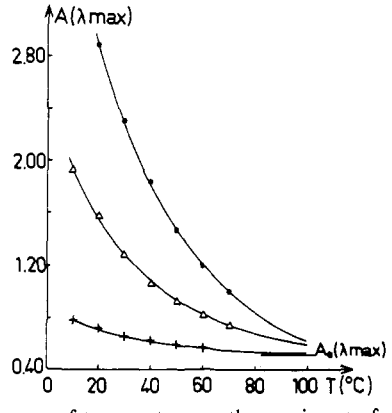

Figure 3. Influence of temperature on the maximum of absorption of malvin (7.73×10^{-4} M) with chlorogenic acid in aqueous H_3PO_4–CH_3CO_2Na buffer at pH 3.65: I = 0.20 M; l = 1 cm; copigment-to-pigment molar ratio; 20 (●), 10 (△) and 2 (+). A_0 is the absorbance, at the maximum of absorption in the visible, in the absence of chlorogenic acid.

and chlorogenic acid. Chlorogenic acid is a colorless compound which does not absorb in the spectral range shown in Figure 1. The magnitudes of both the increase in absorbance and of the bathochromic shift are strongly dependent on the concentration of chlorogenic acid. Moreover, for a given analytical concentration of chlorogenic acid, the increase in absorbance, is also markedly dependent upon the analytical concentration of the pigment. Similar results have been previously reported for other anthocyanins and copigment couples.[5]

Effect of Temperature on the Visible Absorption Spectrum of Malvin with and without Chlorogenic Acid. Figure 2 (lower part) illustrates that raising the temperature strongly reduces the color intensifying effect produced by chlorogenic acid. On the other hand, the thermal effect is larger at low temperatures than at elevated temperatures. In contrast, a malvin solution, at the same pH but without chlorogenic acid, gives visible absorption spectra almost identical, whatever the value of the temperature (Figure 2, upper part). The small changes observed in the latter case, relate only to the displacement of the equilibria shown in Scheme I under the influence of the temperature variations and the very small changes in the pH values of the buffer. A plot of the absorbance, at the wavelength of maximum absorption vs tem-

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(12) Brouillard, R.; Lang, J., in preparation.

Scheme II

$$AH^+ \rightleftharpoons (B+C_E)+H^+ \quad K_h$$
$$AH^+ + nCP \rightleftharpoons AH(CP)_n^+ \quad K$$

perature, of three different malvin–chlorogenic acid solutions is shown in Figure 3. Extrapolation of the curves indicates that, close to 100 °C, the copigment effect should be considerably weaker, whatever the amount of chlorogenic acid. The temperature effect, on the copigmentation of anthocyanins by tannins, was mentioned as early as 1931 by the Robinsons,[3] but this is the first report of its quantitative determination. Moreover, such a large thermal effect has probably never been observed on a UV–vis absorption band. It is also important to note that, when the solution at 70 °C (spectrum 7 of Figure 2) is cooled down to 10 °C, it results in the immediate and quantitative appearance of spectrum 1 of the same figure. One can conclude that no decomposition of malvin occurs during the decrease of the absorbance with the temperature.

Effect of Ionic Strength and Solvent on the Visible Absorption Spectrum of Malvin–Chlorogenic Acid Solutions. Increasing the ionic strength slightly reduces the effect of chlorogenic acid on the intensity of the color of malvin. At constant temperature, pH, and copigment and pigment concentrations, the copigment effect sharply decreases with the content of methanol or ethanol. The same experiment was repeated by adding formamide to the solution, and the drop in the copigment effect was even larger than with methanol or ethanol.

Discussion

Mechanism of the Copigmentation Reaction between Malvin and Chlorogenic Acid. As stated previously, the copigment effect of chlorogenic acid on malvin reaches its highest level at pH values close to 3.6. At this pH, malvin exists essentially in the hemiacetal B and the chalcone C_E structures in fast equilibrium with each other (Scheme I).[12] A small quantity of the flavylium cation AH^+ and very small quantities of the fast equilibrating quinonoidal bases A as well as tiny amounts of the chalcone C_Z are also present.[11] As illustrated in Figure 1, the addition of chlorogenic acid to a solution of malvin at equilibrium produces a large increase in the absorbance of the flavylium form AH^+. Apart from a small bathochromic shift, no other spectral feature appears. This prompted us to postulate the mechanism shown in Scheme II. For the sake of clarity, the very small amounts of A and C_Z, at pH 3.65 and 20 °C, have been omitted from Scheme II. CP represents the free chlorogenic acid molecule. The extent of its ionization into its conjugated base will be discussed later. n is the number of chlorogenic acid molecules linked to the flavylium cation in the complex. When equilibrium is attained, Scheme II is characterized by two equilibrium constants K_h and K. K_h is expressed as $(([B] + [C_E])/[AH^+])a_{H^+}$, where a_{H^+} is the activity of the hydronium ion. Until now the K_h constant has always been written as $([B]/[AH^+])a_{H^+}$, because the existence of the very fast equilibrium between B and C_E was unknown. On the basis of recently reported results on the synthetic 4′-methoxy-3-methylflavylium perchlorate cation,[13] however, we, very recently, demonstrated the existence for malvin of a fast base-catalyzed equilibrium between the hemiacetal B and the *cis*-chalcone C_E.[12] In the pH range investigated for measuring K_h, the latter equilibrium is always much faster than the hydration equilibrium of the flavylium cation AH^+. Moreover, since K_h measurements have only been obtained by monitoring the change with respect to pH of the absorbance in the visible characteristic of the sole flavylium cation, K_h values reported to date always refer to the corrected definition given above. K, the equilibrium constant for the reaction of complexation, is expressed as $[AH(CP)_n^+]/([AH^+][CP]^n)$. It gives the strength of the association between chlorogenic acid and the flavylium form of malvin. Knowledge of the three equilibrium concentrations $[AH(CP)_n^+]$, $[AH^+]$, and [CP] should give the values of n and K. We now demonstrate that such a precise

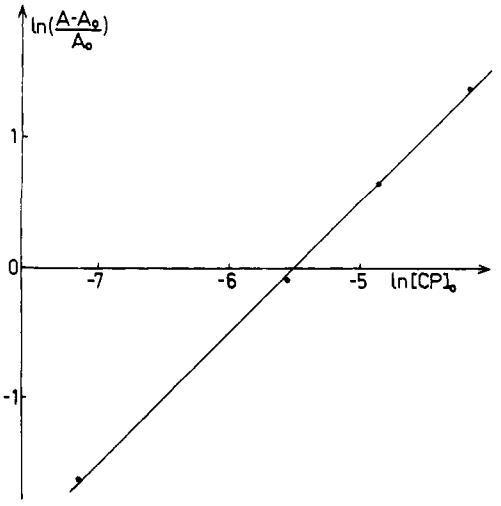

Figure 4. Plot of ln $((A - A_0)/A_0)$ vs ln $[CP]_0$ at 20 °C. Experimental conditions are those of Figure 1: λ = 525 nm; r = 0.9997; slope = 1.00 (±0.02); intercept = 5.53 (±0.09).

determination is not necessary and that both K and n values can be readily obtained from spectra such as those shown in Figure 1, according to the following new procedure.

At pH 3.65, the equilibrium concentration of the flavylium cation $[AH^+]_0$, in the absence of chlorogenic acid and neglecting the very small amounts of A and C_Z, is given by eq 1, where C_0

$$[AH^+]_0 = C_0 \times 10^{-pH}/(K_h + 10^{-pH}) \quad (1)$$

represents the overall concentration of malvin. Taking $K_h = 8 \times 10^{-3}$ M at 20 °C and a 0.2 M ionic strength,[14] one obtains $[AH^+]_0 = 0.027C_0$. With a copigment-to-pigment molar ratio of 20 (Figure 1), there is an approximate 5-fold increase in the absorbance at 530–540 nm. At these wavelengths, the molar absorption coefficient of the flavylium ion, in its free state, is almost identical with its molar absorption coefficient when attached to chlorogenic acid. Therefore, the concentration of the remaining free malvin forms is $C_0 - 5(0.027 \times C_0) = 0.87C_0$. One can now deduce the equilibrium concentration of the free flavylium form, in the case of the copigment-to-pigment molar ratio of 20. It is roughly equal to $0.027 \times 0.87C_0 = 0.024C_0$, a value not very different from the initial $0.027C_0$ value. The reason for this almost constant value of the equilibrium concentration of AH^+ is that the B and C_E species represent a large reservoir for the flavylium species whose concentration is actually buffered. In the K expression, $[AH^+]$ can be replaced, with a good approximation, by $[AH^+]_0$. The equilibrium concentration of chlorogenic acid can be expressed by eq 2, where $[CP]_0$ is its analytical concentration.

$$[CP] = [CP]_0 - n[AH(CP)_n^+] \quad (2)$$

Inspection of Figure 1 demonstrates that if the K value is not too large and n a small integer, [CP] differs from $[CP]_0$ by less than 1%. Therefore, to a reasonably good approximation, the K expression becomes $K = [AH(CP)_n^+]/([AH^+]_0[CP]_0^n)$. In the absence of chlorogenic acid, and in a region where the quinonoidal bases do not contribute too much to the absorbance, that is for wavelengths close to or lower than the maximum of absorption of the free AH^+ cation, the absorbance is given by eq 3, where

$$A_0 = \epsilon_{AH^+}[AH^+]_0 l \quad (3)$$

ϵ_{AH^+} is the molar absorption coefficient of AH^+ and l the optical pathlength. In the presence of chlorogenic acid, eq 3 becomes eq 4, where $\epsilon_{AH(CP)_n^+}$ is the molar absorption coefficient of AH^+

$$A = \epsilon_{AH^+}[AH^+]l + \epsilon_{AH(CP)_n^+}[AH(CP)_n^+]l \quad (4)$$

in the complex. Replacing $[AH^+]$ by $[AH^+]_0$, the K expression

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can be easily transformed into eq 5, where r_1 is $\epsilon_{AH(CP)_n^+}/\epsilon_{AH^+}$ and

$$(A - A_0)/A_0 = Kr_1[CP]_0^n \quad (5)$$

is therefore depending upon the wavelength of observation. *$(A - A_0)/A_0$ is the key experimental parameter measuring the magnitude of the copigment effect under given conditions.* Eq 5 is easier to apply when in the form of eq 6. A plot of ln $((A - A_0)/A_0)$ as a function of ln $[CP]_0$, at a suitable wavelength, should be linear with a slope identical to n. The result, which

$$\ln ((A - A_0)/A_0) = \ln (Kr_1) + n \ln [CP]_0 \quad (6)$$

is surprisingly good, is shown in Figure 4. The relationship is linear and the slope is 1.00 (±0.02). One can conclude that one molecule of chlorogenic acid associates with one molecule of malvin in the flavylium form. It was previously observed that molecular complexes, in a 1:1 molar ratio, could be precipitated from aqueous methanolic acidic solutions when apigeninidin and pentamethylcyanidin chlorides were copigmented by quercetin-5′-sulfonic acid.[5c] If K is to be determined, r_1 must first be determined. We did this by measuring A_0 and A for a 0.2 M aqueous HCl solution containing no chlorogenic acid and an excessive amount of chlorogenic acid, respectively. In this experiment A corresponds to the absorbance of an entirely complexed flavylium cation. With eq 3 and 4 one calculates $r_1 = A/A_0 = 0.8$ at 525 nm. From the intercept of the linear relationship and the value of r_1 at 525 nm, K is estimated to be 390 (±50) M^{-1} at 20 °C and a 0.2 M ionic strength. The large uncertainty in the value of K probably originates from the difficulty in obtaining reliable measurements for r_1 due to some diffusion of light for the more concentrated chlorogenic acid solutions. Chlorogenic acid bears a carboxylic group whose pK_a has been measured by us to be 3.45(±0.05) at 20 °C. Thus K at pH 3.65 (Figure 4) relates to a mixture of the neutral chlorogenic acid and the negatively charged chlorogenic base in an approximately 4:6 molar ratio. It is, therefore, an apparent complexation constant. The influence of the acidity of the medium on the K constant is currently under investigation. Similar experiments at different concentrations of malvin and with molar ratios of copigment to pigment up to 150 yielded also good linear relationships with slopes close to unity.

An interesting conclusion, which can be drawn from this work, is that the effect of the copigment is to suppress the hydration reaction, when the flavylium cation AH^+ is in the complex. It corresponds to a real protection of the flavylium nucleus against nucleophilic attack of water at C-2. Such an idea was first put forward by one of us six years ago, but at that time experimental evidence was lacking.[9]

This is the first time that a mechanism, shown in Scheme II, is proposed and established for the copigmentation reaction of anthocyanins. In previous works, only suggestions were made on the nature of the driving force bringing the pigment and the copigment molecules together. Some authors proposed that hydrogen bonding promotes the association,[4,5a,b] while others were in favor of hydrophobic interactions.[1,8] It will be seen later in the discussion that the main driving force is the hydrophobic interaction, which does not preclude hydrogen bonding to occur when it is possible.

There is still some controversy whether a copigment stabilizes the flavylium cation or the quinonoidal bases.[14] Visible absorption spectra recorded at different pH values show that at low pH, the flavylium cation seems to be stabilized, whereas near neutrality it appears to be the quinonoidal bases. Our results clearly indicate that both forms are stabilized but not in the same way. Chlorogenic acid directly interacts with the flavylium cation, and, consequently, more flavylium ions are present at a given pH value. In turn, the amount of quinonoidal bases increases due to the fact that the acidities of the hydroxyl groups at C-7 and C-4′ of the flavylium form are certainly little affected by the complexation process. Indeed, these hydroxyl groups are hydrogen bonded to the water molecules of the first solvation shell, and the proton-transfer reactions from these groups to those of water molecules can hardly be influenced by the presence of a copigment. It has also been demonstrated that quinonoidal bases do not hydrate.[11]

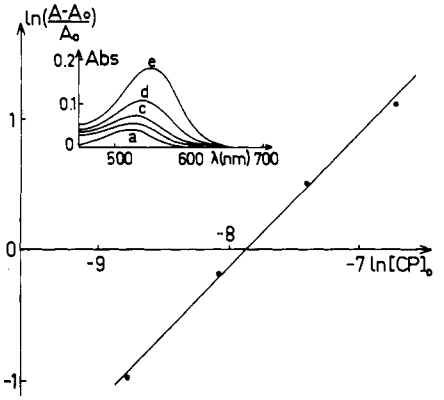

Figure 5. Upper part: visible spectra of malvin (3.00×10^{-5} M) and rutin at 0 (a), 5.1 (b), 10.2 (c), 20 (d) and 40 (e) copigment to pigment molar ratios: pH = 3.10; T = 25 °C. Lower part: plot of ln $((A - A_0)/A_0)$ vs ln $[CP]_0$: λ = 530 nm; r = 0.998; slope = 1.01 (±0.04); intercept = 7.98 (±0.34).

The situation is completely different for the flavylium cation, which, unprotected, strongly hydrates to the mixture of the hemiacetal and the chalcones.

How long does it take for the system described in Scheme II to reach equilibrium? Our results show that the copigmentation reaction itself is a very fast process, whose kinetics is certainly out of reach of the Joule-heating T-jump technique. For instance, to a pH 3.42 malvin solution with a large excess of chlorogenic acid, kept at 16.5 °C and an ionic strength of 0.2 M, we applied a fast transient temperature jump of 3.5 °C. At 500 and 530 nm we observed a very fast increase in the absorbance, whereas at 555 and 590 nm a very fast decrease was observed. With the solution without chlorogenic acid, such signals were not seen. At 500 and 530 nm ϵ_{AH^+} is larger than ϵ_{AHCP^+} and the opposite is observed at 555 and 590 nm. One can deduce that the dissociation of the complex is endothermic. The rate-limiting step of Scheme II is the hydration equilibrium whose kinetics is strongly influenced by the presence of the complexation equilibrium. If the flavylium cation of malvin and chlorogenic acid are mixed at room temperature, complete equilibrium is reached within seconds, in the more acidic solutions, whereas several hours are required for solutions close to neutrality. At 20 °C and pH 3.65, a typical experiment shows that, after addition of chlorogenic acid to a previously equilibrated solution of malvin, it takes about 1 min for color to change from its initial pale hue to its much more colored final aspect. Such a delay represents only the time needed for the $B + C_E$ mixture to reform the AH^+ consumed by the copigment, and it has nothing to do with the kinetics of the complexation reaction!

We also applied our method to results reported in the literature. Unfortunately, we were able to locate only one study in which the temperature had been carefully measured. That study deals with complexation of malvin with rutin (quercetin 3-rutinoside), a common colorless flavonoid known to be a good copigment. Figure 5 shows both the spectra recorded by Sadlowski[14] and the associated plot of ln $((A - A_0)/A_0)$ vs ln $[CP]_0$. Agreement with eq 6 is excellent. The relationship is linear with a slope of 1.01 (±0.04). From the same study the r_1 value was also obtained and this allowed us to calculate the K value for the malvin–rutin complex, which at 25 °C and a 0.2 M ionic strength is approximately 4000 M^{-1}. The malvin–rutin complex is also a one-to-one complex, but the tendancy of rutin to associate with malvin is much greater than that of chlorogenic acid. Sadlowski using a different method for the determination of n also reported a value close to 1 (1.33). His method is different from ours in that it is based on spectra recorded in 0.49 M HCl where the pigment is completely in the flavylium form. Sondheimer and his associates[9] found a 1:1 ratio in the case of the pelargonidin–caffeine complex. Their measurements have also been done at a low pH value (pH = 2.05) without taking into consideration the existence of both the hydration and tautomeric equilibria of the pelargonidin an-

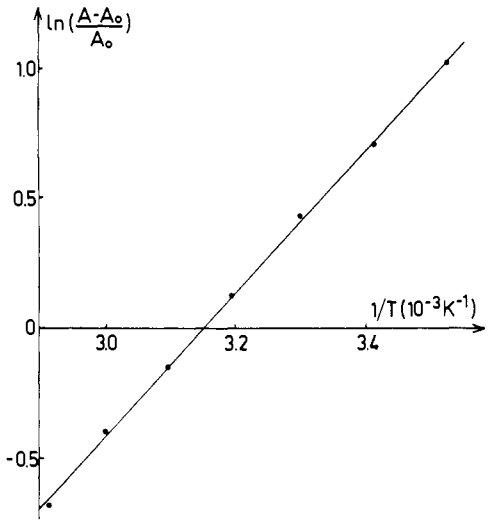

Figure 6. Plot of ln $((A - A_0)/A_0)$ as a function of the reciprocal of the temperature. Experimental conditions are those of Figure 2: λ = 525 nm; r = 0.9995; slope = 2,772 (±38); intercept = −8.73 (±0.12).

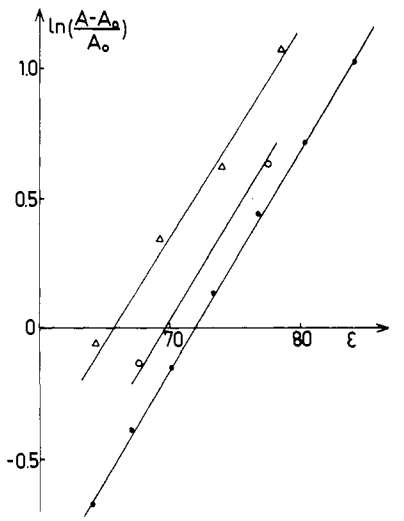

Figure 7. Plot of ln $((A - A_0)/A_0)$ vs static dielectric permittivity: pH = 3.65; λ = 525 nm. (●) in buffered H_2O at different temperatures and constant ionic strength. Experimental conditions are those of Figure 2: r = 0.9996; slope = 8.42 (±0.10) × 10^{-2}; intercept = −6.05 (±0.07). (△) in buffered MeOH:H_2O mixtures at 25 °C and constant ionic strength. (○) in buffered NaCl:H_2O mixtures at 25 °C. Values of the static dielectric permittivity have been taken from ref 18.

thocyanidin. In order to get the n value, our method does not require to measure separately the AH^+ and $AHCP^+$ spectra. It is only in the case when one wants to obtain K that the values of these spectra are required.

It is unfortunate that most of the experimental values reported in the literature on the copigment effect are never the $(A - A_0)/A_0$ values, at a given constant wavelength, but the $\{A(\lambda_{max}) - A_0(\lambda_{max})\}/A_0(\lambda_{max})$. This obvious experimental quantity is of no use in the determination of K and n. Nonetheless, attempts to correlate the quantity $A(\lambda_{max}) - A_0(\lambda_{max})$ with the magnitude of the bathochromic shift ($\Delta\lambda_{max}$) measured at the wavelength of maximum absorption have been made.[8]

It is readily seen from eq 5 that K and $[CP]_0$ quantitatively act in the same manner. For instance, the effect produced by an increase in K can also be obtained by an appropriate increase in the concentration of the copigment. If both factors are maximized, extremely large values for the ratio $(A - A_0)/A_0$ should be obtained. Without doubt, *K is the key theoretical parameter in the copigmentation phenomenon.* Once a given pigment to copigment couple has been chosen, how does K depend on the medium in which the reaction takes place? We now try to give the answer to this important question.

Influence of the Medium: The Copigment Effect is a Microprobe for the Structural Study of Liquid Water. As previously mentioned, the Robinsons[3] discovered that both temperature and composition of the aqueous solvent influence the extent of the copigmentation process. Surprisingly, since that time, no quantitative studies on these much important factors have been undertaken. Our results, quantitatively demonstrate that increasing the temperature leads to a drastic weakening of the association of the flavylium cation with chlorogenic acid (Figures 2 and 3). Such an extraordinary thermal effect is found only in the existence of the complexation reaction since spectra of solutions of malvin, without chlorogenic acid, remain almost unchanged over the temperature range 10–60 °C (Figure 2—upper part). If the molar enthalpy change, ΔH, of the copigmentation process is independent of temperature, eq 6 predicts that a plot of ln $((A - A_0)/A_0)$ as a function of the reciprocal of the temperature should be linear with a slope equal to $-\Delta H/R$. Indeed, such a linear relationship exists (Figure 6), and ΔH is −23.0 (±0.4) kJ·mol^{-1}. One can note that measuring this apparent enthalpy change does not necessitate knowledge of the molecular absorption coefficients of any of the species present in the system. The experimental set is simple and straightforward. From eq 6 and from the value of the intercept of the linear relationship in Figure 6, we estimate the entropy decrease associated with the complexation reaction to be −28.5 (±1) J mol^{-1} K^{-1}.

How can we take into account such a large thermal effect? What is the meaning of the value of ΔH and of the value of ΔS? Water is a unique solvent whose properties greatly differ from the properties of other solvents even when they are polar solvents.[15] Particularly its structure is thought to be a three-dimensional network of hydrogen-bonded H_2O molecules associated according to a quasi-tetrahedral geometry.[16] This type of structure is closer to the structure of ice I than to the structure of water in the vapor phase. Copigmentation is a natural process occurring in vivo in the vacuoles of the colored cells of higher plants.[17] Vacuoles are essentially aqueous solutions. One of the most important physico-chemical properties of liquid water is its static dielectric permittivity ϵ, whose unusual high value is due to the peculiar structure of water. The dielectric permittivity is known to vary linearly with the reciprocal of the temperature. It is therefore not surprising that a plot of ln $((A - A_0)/A_0)$ versus ϵ is linear (Figure 7). Values taken for the dielectric permittivity, at different temperatures, are those of pure water.[18] They differ, by a constant factor, from the true values of our solution. Knowledge of this factor is not necessary to our reasoning. From the linear plot of the logarithm of the copigment effect as a function of dielectric permittivity and using eq 6, one obtains eq 7. Whether

$$d(\ln K)/d\epsilon = 0.084 \qquad (7)$$

the van't Hoff relationship or eq 7 governs the copigment effect in water can be decided by performing experiments either at T constant and ϵ variable or at ϵ constant and T variable. Figure 7 shows the effect of varying ϵ and keeping temperature constant. In one case methanol was added to water, and in the other case sodium chloride was used.[18] Straight lines, parallel to the one obtained by changing temperature, were observed. Furthermore, experiments conducted at a constant ϵ value and different temperatures yielded identical copigment effects. One can conclude that the effect produced by a change in the temperature is only indirect. The degree of association between malvin and chlorogenic acid does not depend directly on the temperature but on the structure of liquid water. The fast absorbance change observed in the T-jump experiment is in fact produced by a dielectric permittivity jump. Strong support to our views is given by two facts. (a) The value of the molar enthalpy change for dissociation of the complex [23.0 (±0.4) kJ·mol^{-1}] corresponds exactly to the

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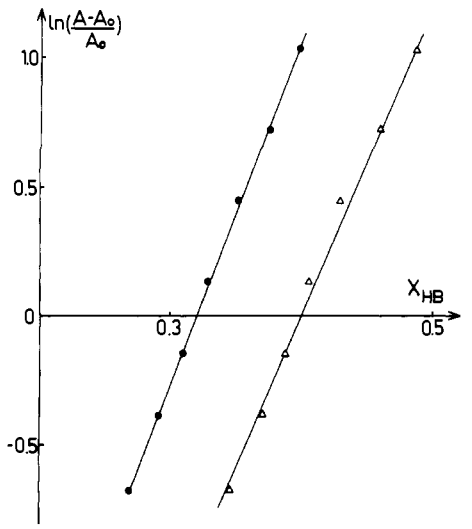

Figure 8. Plot of the logarithm of the copigment effect vs the fraction X_{HB} of intact hydrogen bonds in water: λ = 525 nm. Experimental conditions for measuring the copigment effect are those of Figure 2. (●) r = 0.9994. X_{HB} values are taken from ref 22. (△) r = 0.994. X_{HB} values are taken from ref 23.

average hydrogen bond energy of ordinary ice.[16,19] In the case of liquid water, a widely quoted value is 21 kJ·mol^{-1} (~5 kcal·mol^{-1}) estimated by Pauling.[20] One can then state that the largest contribution to the enthalpy change observed for the copigmentation reaction is to be found in the structure-breaking effect produced by the temperature on the tetrahedral network of the associated water molecules. In this way, ΔH should only slightly depend on the chemical nature of the pigment and the copigment. Effectively, for cyanin chloride, another very common anthocyanin, when associated to chlorogenic acid, we found $-\Delta H$ to be 22.2 (±1.1) kJ·mol^{-1}. Moreover, it has been demonstrated that the entropy increase associated with the dissociation of the malvin–chlorogenic acid complex is 28.5 (±1) J mol^{-1} K^{-1} (6.8 cal mol^{-1} K^{-1}). This value is close to those determined by Stevenson[21] for the equilibrium between the hydrogen-bonded water molecules and the free water molecules in the liquid state. According to two different approaches, he reported ΔS values to be 8 (±1) and 6.2 cal mol^{-1} K^{-1}. (b) A plot of the logarithm of the copigment effect, measured at different temperatures, versus the fraction X_{HB} of intact hydrogen bonds in water, measured at the same temperatures, is linear (Figure 8). Although there are differences in the X_{HB} values, according to different theoretical approaches,[22,23] results in Figure 8 unambiguously demonstrate that the extent of the malvin–chlorogenic acid association is under the control of the very peculiar, hydrogen-bonded molecular structure of liquid water.

What is the nature of the intermolecular force which brings together malvin and chlorogenic acid? The decrease of the copigment effect with the decrease of the dielectric permittivity suggests that it is a solvent effect related to the polarity of the medium. In liquid water, the polar H_2O molecules are engaged within a tridimensional approximately tetrahedral network and are not free to orientate in a purely random fashion.[19] Does the copigment effect depend more on the polarity of a water molecule or more on their tetrahedral network? Methanol as well as sodium chloride reduces polarity and creates disorder. We also added formamide to an aqueous copigmented solution, and we observed a strong decrease of the copigment effect as compared to the solution without formamide. Formamide increases polarity and also perturbs the tetrahedral network of water. We now come to the following conclusion: it is not so much the polarity of a free water molecule which is important for copigmentation to take place but rather the unique feature of the association of water molecules. Any factor breaking the latter structure (increase of the temperature, presence of a cosolvent, ionic salt...) at the same time weakens the copigment effect. However, as long as the very peculiar network of water molecules constitutes a dominant structural parameter, the pigment and the copigment are forced into close contact, according to a mechanism which can be designated as a hydrophobic interaction. In this respect, copigmentation appears to be a solvent–solute interaction characteristic of aqueous solutions only. Unexpectedly, the copigmentation reaction constitutes an easy to handle novel microprobe for the structural studies of water, the unique and most important solvent of the chemistry of life.

Conclusion

Prior to this work, the copigment effect has always been estimated from the increase in the absorbance, at the visible wavelength of maximum absorption and from the bathochromic shift of this maximum absorbance. These two empirical parameters bear no direct quantitative relationship to the copigmentation reaction itself. It is, therefore, not surprising that no mechanism accounting for the formation of the anthocyanin–copigment complex has been given. Our method permits to measure quantitatively the copigment effect and gives the molecularity of the complexation reaction. This method is simple and does not necessitate the use of expensive equipment or sophisticated mathematical treatments. We have also demonstrated that the large thermal effect is only apparent and that it is the very structure of water, in the liquid state, which governs the molecular association between the flavylium cation of the anthocyanin and the copigment. In the absence of water, the copigment effect probably does not exist. Another interesting point is that the copigmentation reaction possesses characteristics frequently found among biological reactions:[15] it occurs in water, and it is sensitive to pH, temperature, and composition of the aqueous medium.

Copigments known today are limited to substances thought to be found within the cell vacuoles containing the anthocyanins. In our opinion, many more biochemical and chemical substances, natural and synthetic, capable of acting as copigments will be discovered. To date, the copigmentation phenomenon has only been reported in anthocyanins. It may be worth asking, however, if it could also occur in other classes of flavonoids. Experimental procedures for studying the copigment effect in colorless flavonoids are far from obvious. Indeed, among the flavonoids, only the anthocyanins strongly absorb light in the visible region. This unique spectroscopic feature is of immense value, since it constitutes a microprobe in studies on anthocyanins. If other flavonoids have to be tested, one should first turn to those aurones and chalcones which possess an absorption band extending in the visible region.

We also believe that our findings are valuable to the food and beverage industry which requires natural red colors to replace banned or toxicologically questionable azo dyes to color many food products.[24] Common anthocyanins, which are unprotected against hydration, cannot be used as food colors. Copigmentation can provide such a protection of the color, and, furthermore, it is a unique model which may aid in the synthesis of new anthocyanins with protecting groups covalently bound to the anthocyanin itself. Another important application of this work is in the aging process of red wines.[25] Tannins and anthocyanins are both involved in this economically important process. Tannins are good copigments,[3] and since copigmentation takes place in water with 10% ethanol, the copigmentation reaction is believed to be the most important step in the development and stability of wines containing both anthocyanins and tannins, a fact which has not been recognized until now.

Registry No. CP, 327-97-9; AH$^+$, 16727-30-3; H_2O, 7732-18-5.

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