Chemistry of Anthocyanin Pigments. 2.[1] Kinetic and Thermodynamic Study of Proton Transfer, Hydration, and Tautomeric Reactions of Malvidin 3-Glucoside

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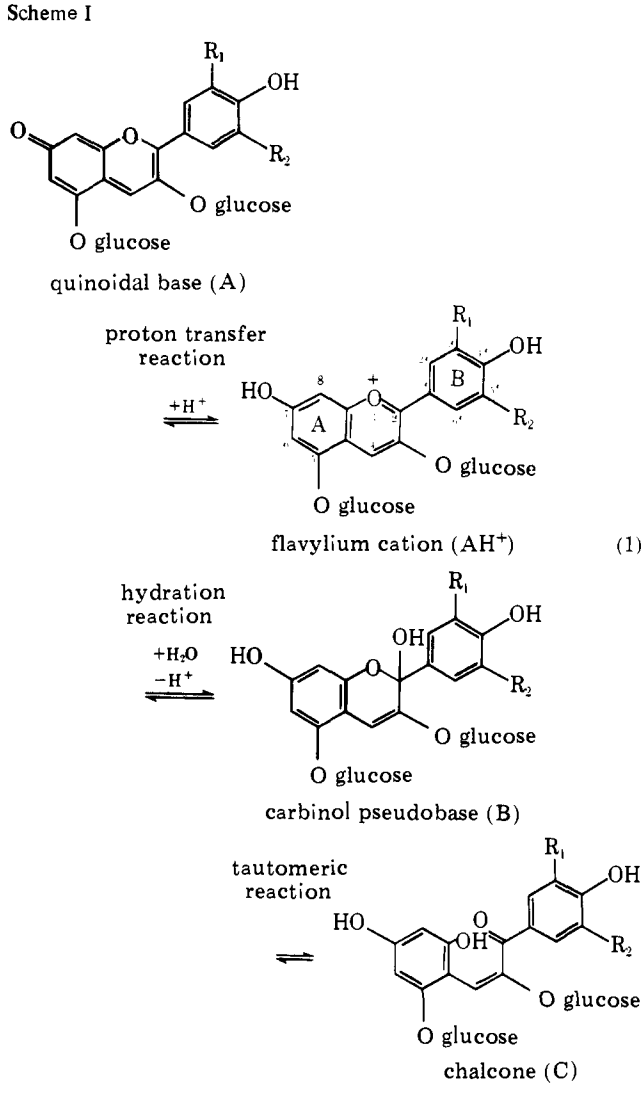
Abstract: At 25 °C in acidic aqueous media, malvidin 3-glucoside exists in four forms in equilibrium: the quinoidal base A, the flavylium cation AH^+, the carbinol pseudobase B, and the chalcone C. Through the loss of a proton, the cation AH^+ yields the base A. The nucleophilic addition of water to this cation yields the pseudobase B. A ring-chain water-catalyzed tautomeric equilibrium occurs between B and the chalcone C. At 25 °C the equilibrium constants for the acid-base, hydration, and tautomeric equilibria are respectively $K_a = K'_{12} = 5.7\ (\pm 1) \times 10^{-5}$ M ($pK'_{12} = 4.25$); $K_h = K'_{13} = 2.5\ (\pm 0.1) \times 10^{-3}$ M ($pK'_{13} = 2.60$), and $K_T = K_{34} = [C]/[B] = 0.12\ (\pm 0.01)$. Cation deprotonation by the solvent is exothermic [$\Delta H^\circ_{12} = -3\ (\pm 2)$ kcal mol^{-1}] whereas cation hydration and pyrylium ring opening are both clearly endothermic [$\Delta H^\circ_{13} = 4.7\ (\pm 0.2)$ kcal mol^{-1} and $\Delta H^\circ_{34} = 3.7\ (\pm 0.2)$ kcal mol^{-1}] and are associated with positive entropy changes [$\Delta S^\circ_{13} = 4\ (\pm 0.7)$ cal deg^{-1} mol^{-1} and $\Delta S^\circ_{34} = 8.5\ (\pm 1)$ cal deg^{-1} mol^{-1}]. The relaxation spectrum comprises three relaxation modes with three very different time constants ($\tau_3 \gg \tau_2 \gg \tau_1$). τ_1 characterizes the proton transfer equilibrium, τ_2 the hydration equilibrium, and τ_3 the tautomeric equilibrium. The rate constants are respectively $k_{12} = 4.7\ (\pm 0.4) \times 10^4$ s^{-1} (deprotonation of the cation by the solvent), $k_{21} = 6.7\ (\pm 0.5) \times 10^8$ M^{-1} s^{-1} (neutralization of the quinoidal base by the hydronium ion), $k_{13} = 8.5\ (\pm 1) \times 10^{-2}$ s^{-1} (hydration of the cation), $k_{31} = 34\ (\pm 3)$ M^{-1} s^{-1} (dehydration of the carbinol pseudobase), $k'_{34} = 4.5\ (\pm 0.3) \times 10^{-5}$ s^{-1} (opening of the pyrylium ring, H_2O catalyst), and $k'_{43} = 3.8\ (\pm 0.2) \times 10^{-4}$ s^{-1} (closure of the pyrylium ring, H_2O catalyst). The finding that a chalcone form exists in tautomeric equilibrium with the carbinol pseudobase leads us to reconsider the thermal degradation of anthocyanin pigments.

We have recently shown[1] that Scheme I (eq 1) expresses the general mechanism involved in the structural transformations of anthocyanins occurring in acidic aqueous media (pH 1-6) at 4 °C, i.e., under conditions similar to those present in natural media. Thus we have clearly established that there is an extremely fast proton transfer between the quinoidal base A and the flavylium cation AH^+ (relaxation time is of the order of 10 μs) which quickly hydrates, thereby yielding the carbinol pseudobase B (relaxation time is about 1 s). We have also suggested the existence of the carbinol-chalcone tautomeric equilibrium, but we were unable to identify the chalcone under our experimental conditions. Indeed, since it was thought that anthocyanins were thermally degraded[2] (starting at room temperature), we carried out our experiments at the lowest possible temperature. As shown in the present article, this is an extremely unfavorable position from which to observe the open tautomeric form.[3] To our knowledge this structure had never before been shown to exist for anthocyanins.[4] In the previous work,[1] we chose a dimonoside (malvidin 3,5-diglucoside, commonly called malvin; $R_1 = R_2$ = methoxy) belonging to one of the two large families of anthocyanins. The second family (which according to Harborne[5] is the one most frequently encountered) is that of the monosides (OH at position 5). Anthocyanin monosides and dimonosides are distinguishable insofar as the relative proportions of the different structures found in Scheme I (eq 1) differ greatly at the same pH. In particular, it is well known that the flavylium cation of monosides is stable at pH's where that of dimonosides is already totally hydrated.[6]

In this work we present kinetic and thermodynamic results for the set of reactions appearing in Scheme I. These results have been obtained for an anthocyanin belonging to the monoside family: malvidin 3-glucoside ($R_1 = R_2$ = methoxy; OH at position 5). By carrying out our measurements at different temperatures, we demonstrate the existence of the open tautomeric form for anthocyanins. This in turn has led us to reconsider the thermal degradation of anthocyanins.[7] Application of the chemical relaxation method[8] has made our findings possible.

Scheme I



Experimental Section

Malvidin 3-glucoside chloride was extracted from a grape belonging to the *Vitis vinifera* genus (Pinot Noir, Alsace). Its separation from the other grape components and its purification were carried out by liquid column chromatography using water-insoluble polyvinyl polypyrrolidone (Sigma) following Hrazdina's procedure.[9] The purity of the isolated sample was checked by cellulose (Merck) and polyamide (Merck) plate chromatography in butanol/acetic acid/water mixtures and no impurity whatsoever was thereby detected. The pigment[10] was identified from its spectral characteristics in methanolic 0.01% HCl,[11] and also by the total lack of reaction between a pigment solution and aluminum chloride.[12]

Kinetic and thermodynamic runs were performed in aqueous solutions with an ionic strength adjusted to 0.2 M by the addition of KNO_3 (Merck suprapur). In order to avoid possible photochemical effects of daylight, the pigment solutions were prepared and stored in inactinic graduated flasks (the walls of the flasks are completely opaque to UV and transmit less than 1% from the UV up to 550 nm) and measurements were carried out in the dark.[13]

Kinetic Measurements. The fastest relaxation phenomenon was recorded by means of a temperature-jump apparatus (Messanlagen Studiengesellschaft). The thermostated solution was permanently circulated between the temperature-jump cell and the pH and temperature measurement cell, so that the pH and temperature could be checked continuously.[14]

The second fastest relaxation was induced by a quick pH jump and measured on a Cary 16 or a Cary 118 spectrophotometer as previously described.[1] This relaxation was recorded at 517.5 nm, which corresponds to the absorption maximum of the flavylium cation.

The slowest relaxation phenomenon was also induced by a pH jump and recorded by means of a spectrophotometer fitted with a thermostated sample cell, again at 517.5 nm.

Equilibrium Measurements. The equilibrium measurements were carried out at different temperatures using the same spectrophotometers as above.

pH Measurements. In both kinetic and thermodynamic experiments, the pH was measured with a Knick pH meter fitted with a Metrohm combined electrode (EA 125). The buffered solutions used for pH meter standardization were either pH 4.01 and 6.86 NBS standards (Beckman) or 0.1 and 0.01 N hydrochloric acid solutions (Merck titrisol).

Results

A. Kinetic Results. At the most acidic pH's, the pigment only appears stable when in the form of the flavylium cation–carbinol pseudobase equilibrium. When such a solution is subjected to a fast pH jump ($\simeq$ 1 s) to a suitably distant pH (final pH near 5), three kinetically distinct stages are observed for the overall equilibration process of the system. The absorbance at 517.5 nm (absorption maximum of the flavylium cation) undergoes an initial decrease as fast as the pH jump; this is followed by a second decrease which is clearly slower than the pH jump and which can be observed on an ordinary spectrophotometer (Figure 1). The third step, much slower than the two previous ones, can be recorded by changing the wavelength (360 nm) and using a spectrophotometer with a high signal to noise ratio. Under these conditions a slow increase of very low amplitude (a few hundredths of an OD unit) of the absorbance is noted (Figure 2). We ascribe this latter stage to the appearance of the open chalcone form. Further evidence for the existence of this form has been obtained by rapidly dissolving a few micrograms of malvidin 3-glucoside in a pH 4 solution and recording the evolution of the absorption spectrum as a function of time (Figure 3). First of all, within a few minutes, the greater part of the cation is seen to disappear and to give way to the colorless carbinol with an isosbestic point appearing near 360 nm. Further observation shows a slow but regular increase in absorption near 350 nm with the disappearance of the isosbestic point.[15] The absorption increase in this spectral region is characteristic of chalcone formation.[16]

(a) The Fast Relaxation (τ_1). We have previously shown[1] that this very fast relaxation is due to an equilibrium between the flavylium cation (AH^+) and the quinoidal base (A). This is an acid–base type reaction in which the proton transferred from the cation to a water molecule is the hydroxyl proton at position 7. The temperature dependence of τ_1^{-1} is shown in Figure 4; at 25 °C the plot of τ_1^{-1} against hydronium ion and quinoidal base concentrations is linear and can be expressed by

$$\tau_1^{-1}(s^{-1}) = 4.7 \times 10^4 + 6.7 \times 10^8([A] + [H^+]) \quad (2)$$

(b) The Intermediate Relaxation (τ_2). For the most acidic media (pH <3), τ_2^{-1} is a linear function of the hydronium ion concentration (Figure 5) and, at 25 °C, corresponds to

$$\tau_2^{-1}(s^{-1}) = 8.5 \times 10^{-2} + 34[H^+] \quad (3)$$

When the acidity of the medium is lowered (pH >3), the variation of τ_2^{-1} as a function of the hydronium ion concentration ceases to be linear and appears as in Figure 6. In particular, when $[H^+] \rightarrow 0$, $\tau_2^{-1} \rightarrow 0$. This kinetic behavior is identical with that already described in detail for malvidin 3,5-diglucoside.[1]

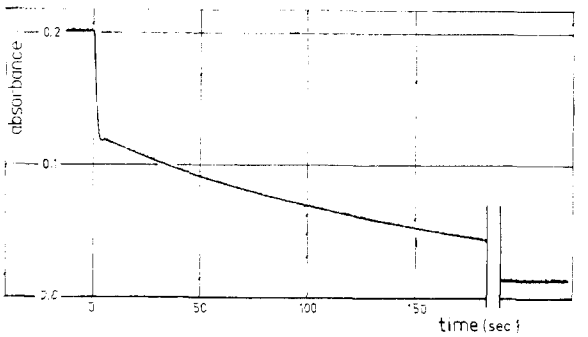


Figure 1. Absorbance variation at 517.5 nm of a pigment solution in its equilibrium state at 25 °C and pH 3.07 when submitted to a rapid pH jump (final pH 5.20). The most rapid phenomenon (τ_1) is related to the flavylium cation-quinoidal base transformation and cannot be measured in this way. Under such conditions its amplitude corresponds approximately to $[AH^+]_0/\Delta\epsilon$. The least rapid phenomenon is related to the reversible cation hydration reaction and follows an exponential decrease whose time constant is τ_2. Its amplitude corresponds approximately to $[AH^+]_0\epsilon_A l$; $[AH^+]_0$ = the equilibrium cation concentration at 25 °C and pH 3.07; $\Delta\epsilon = \epsilon_{AH^+} - \epsilon_A$; ϵ_{AH^+} and ϵ_A are the molecular extinction coefficients of the flavylium cation and the quinoidal base, respectively, at 517.5 nm; l = 1 cm; analytical concentration is 3.3×10^{-5} M. Thus we obtain ϵ_{AH^+} of the order of 27 000 M^{-1} cm^{-1} and ϵ_A of the order of 14 000 M^{-1} cm^{-1}.

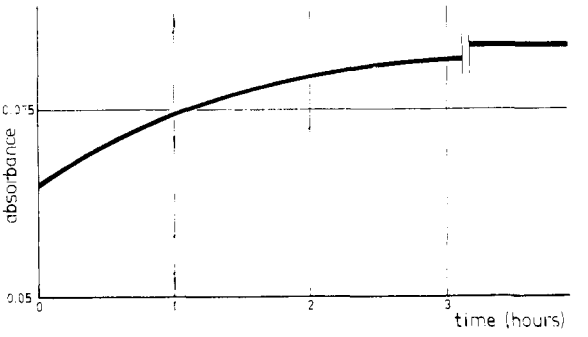


Figure 2. Absorbance variation at 360 nm of a pigment solution in its equilibrium state at 25 °C and pH 2.04 when submitted to a rapid pH jump (final pH 6.2). The increase in absorption is due to the establishment of the tautomeric equilibrium. The total amplitude corresponds, as a good approximation, to $[AH^+]_0(\epsilon_C - \epsilon_B)l\,[K_{34}/(1 + K_{34})]$; K_{34} is the tautomeric equilibrium constant at 25 °C; $[AH^+]_0$ is the equilibrium cation concentration at 25 °C and pH 2.04; ϵ_C and ϵ_B are the molecular extinction coefficients at 360 nm of chalcone and carbinol pseudobase, respectively; l = 1 cm; analytical concentration is 2.6×10^{-5} M. We thus obtain $\epsilon_C - \epsilon_B$ of the order of 9000 M^{-1} cm^{-1}. Zero time corresponds to 4 min after the partial neutralization of the solution.

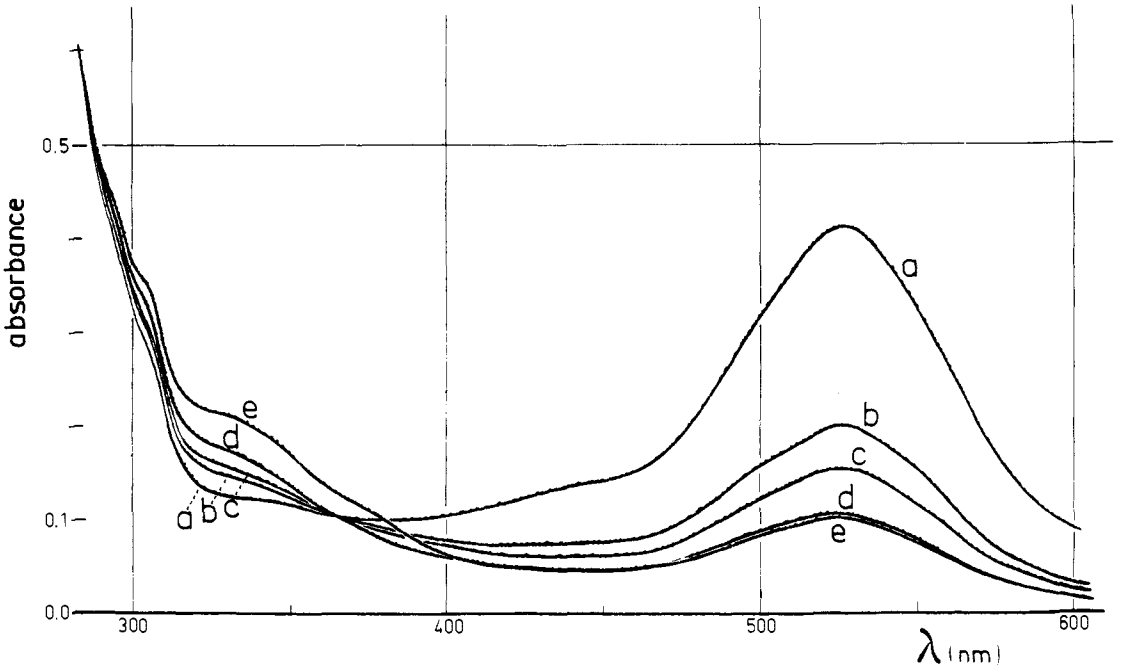


Figure 3. Variation with time of the absorption spectrum of a solution obtained by instantaneous dissolution of pigment in the sample cell at 25 °C and pH 4. The time interval between the beginning of two consecutive spectra for the spectra a to d is about 1 min. Spectrum e was recorded 3 h after spectrum a and shows no further detectable change. The very slight drop in absorption near 525 nm between spectra d and e is worthy of note; this drop corresponds to the effect of the establishment of the tautomeric equilibrium on the flavylium cation and quinoidal base concentrations. Analytical concentration is about 6×10^{-5} M.

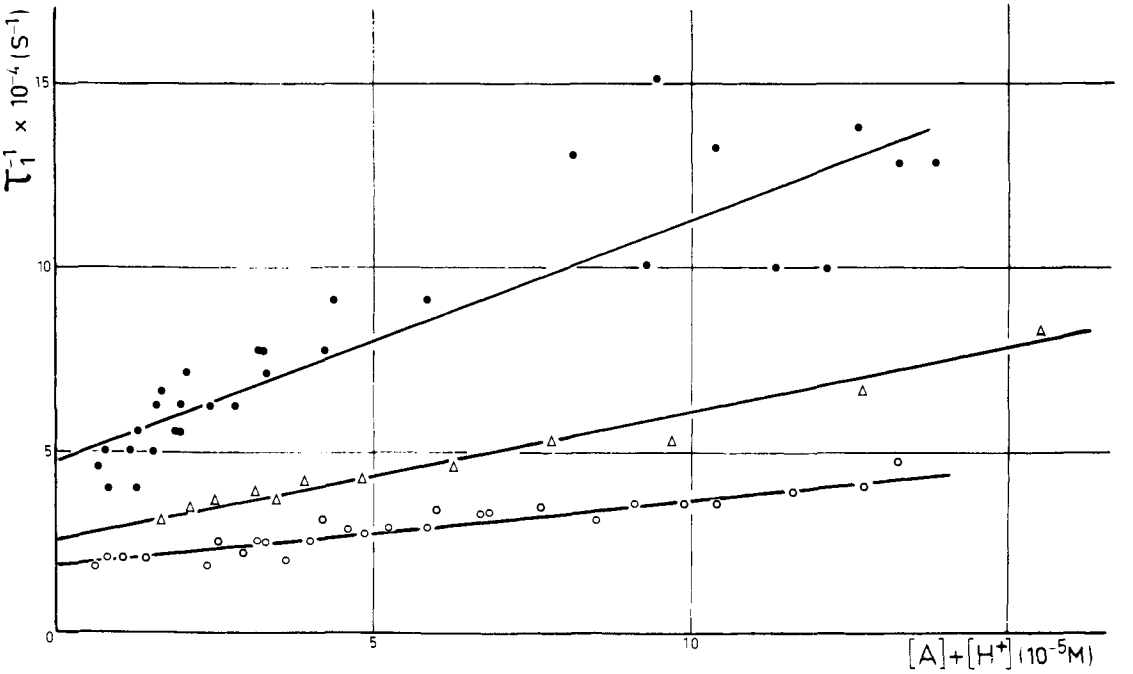


Figure 4. Plot of τ_1^{-1} vs. the sum of the quinoidal base and hydronium ion concentrations at different temperatures. Intercepts: 1.8 (±0.1) × 10^4, 2.5 (±0.2) × 10^4, and 4.7 (±0.4) × 10^4 s^{-1}. Slopes: 1.8 (±0.1) × 10^8, 3.3 (±0.2) × 10^8, and 6.7 (±0.5) × 10^8 M^{-1} s^{-1}. r = 0.95, 0.98, and 0.91 at 6.5, 11.5, and 25 °C, respectively. Analytical concentration = 0.9 × 10^{-3} M.

(c) The Slow Relaxation (τ_3). When, at a given temperature (e.g., 25 °C) and acidity (pH 4–6), a pigment solution having reached the state of chemical equilibrium is rapidly acidified, in addition to the two fast relaxation modes, a third mode of relaxation with time constant τ_3 is observed (Figure 7).[17] Figure 8 shows that τ_3^{-1} does not depend significantly on the hydronium ion concentration and that, at 25 °C, it corresponds to a constant value of 3.8 (±0.2) × 10^{-4} s^{-1}.

B. Thermodynamic Results. (a) Equilibrium Constants. Equilibrium constants K'_{12}, K'_{13}, and K_{34},

$$K'_{12} = \frac{[A]}{[AH^+]} a_{H^+}; \quad K'_{13} = \frac{[B]}{[AH^+]} a_{H^+}; \quad K_{34} = \frac{[C]}{[B]}$$

where a_{H^+} represents the activity of the hydronium ion, were measured as follows.

(1) K'_{12} is given by the ratio of the rate constants for deprotonation of the flavylium cation and protonation of the quinoidal base (Figure 4). Thus at 25 °C, K'_{12} = 5.7 (±1) × 10^{-5} M.

(2) K'_{13} is obtained by using

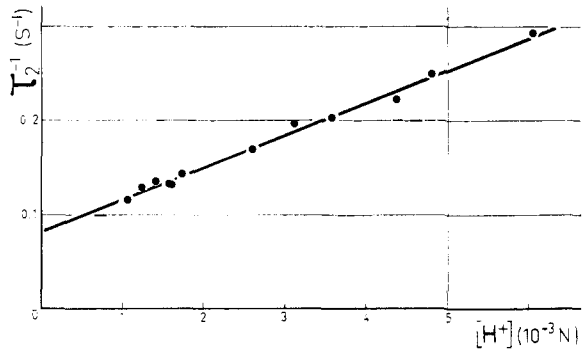


Figure 5. Plot of τ_2^{-1} vs. hydronium ion concentration at 25 °C. Intercept = 8.5 (±1) × 10^{-2} s^{-1}; slope = 34 (±3) M^{-1} s^{-1}; r = 0.996. This case corresponds to [H$^+$] ≫ K_{12}.

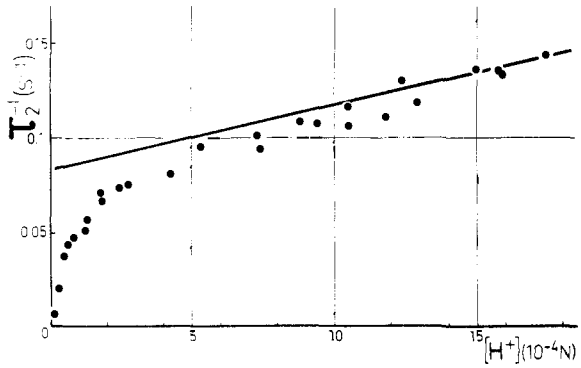
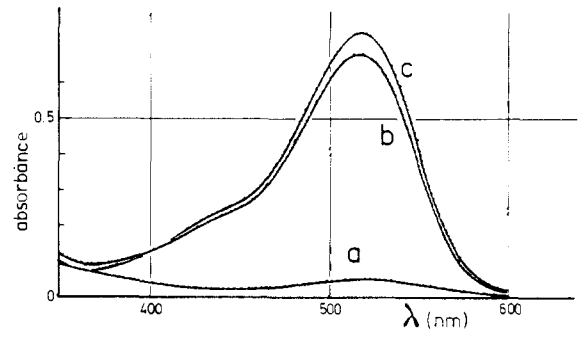


Figure 6. Plot of τ_2^{-1} vs. hydronium ion concentration at 25 °C for the case [H$^+$] ≃ K_{12}. The line is that of Figure 5.

$$\log \frac{D_{AH^+} - D}{D - D_B} + pK'_{13} = pH$$

D_{AH^+} and D_B, respectively, represent the absorbance, at a given wavelength, of a solution containing only the cation or the carbinol pseudobase at the same analytical concentration. D is thus the absorbance of a solution whose acidity is such that it contains both the cationic and the carbinol forms in equilibrium, excluding the presence of any appreciable amounts of the quinoidal and chalcone forms.[18] This is made possible by first preparing a solution that is sufficiently acid so as to contain practically only the flavylium cation. A few microliters of a 2 N sodium hydroxide solution are then injected so that the new pH value is about 2–3. Under these conditions, formation of the quinoidal base is quite negligible ($K'_{12} \ll K'_{13}$). Moreover, the spectrum at equilibrium is recorded as soon as the kinetic relaxation described by τ_2 has ended, and the pH is again modified if necessary. The whole experiment is carried out rapidly so that the formation of chalcone is as insignificant as possible (Figure 9).[19] In this way we measured at 25 °C that K'_{13} = 2.5 (±0.1) × 10^{-3} M. This finding is in good agreement with that obtained by calculating K'_{13} from the ratio of the rate constants for cation hydration and carbinol dehydration. However, this latter method leads to a greater uncertainty in the determination of K'_{13}:K'_{13} = 2 (±0.5) × 10^{-3} M at 25 °C.

(3) K_{34} is determined in the following manner: into a solution of pigment at pH about 4, left for 5 h in a thermostated bath, and whose spectrum is completely stable, a few microliters of an approximately 10 N hydrochloric acid solution are injected so that the new pH is of the order of 1 (Figure 10).[20] K_{34} then corresponds to the ratio between the amplitude of the slow equilibration process and the amplitude of the intermediate equilibration process. Indeed, as soon as the pH jump has occurred, the fraction of pigment in the carbinol form is rapidly transformed into the cation (τ_2 = 0.3 s at 25 °C when [H$^+$] = 0.1 N); secondly, pigment initially in the chalcone form slowly evolves toward this same cation in a process whose slow step is the chalcone–carbinol reaction and whose relaxation time is τ_3. The amplitudes measured correspond to differences in absorbance at 517.5 nm. Thus, at 25 °C, K_{34} = 0.12 (±0.01). This result indicates that at 25 °C and for media which are not too acidic, the malvidin 3-glucoside occurs to a significant extent in the chalcone structure.



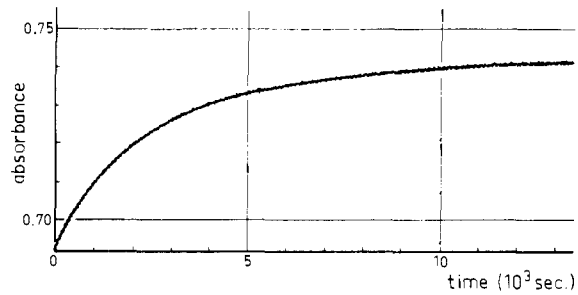


Figure 7. Upper part: variation of the absorption spectrum in the visible range of a pigment solution initially at equilibrium at pH 3.90 (a), and afterwards acidified to pH 1.07, the spectrum being recorded immediately after acidification (b). The spectrum (c) was recorded 5 h after spectrum (b). Analytical concentration = 3 × 10^{-5} M. T = 25 °C. Lower part: slow increase in absorption at 517.5 nm of a solution initially at equilibrium at pH 3.90 and 25 °C and acidified subsequently to pH 1.07. The exponential curve corresponds to the relaxation rate τ_3 for the passage from spectrum (b) to spectrum (c). The recording begins approximately 12 min after acidification.

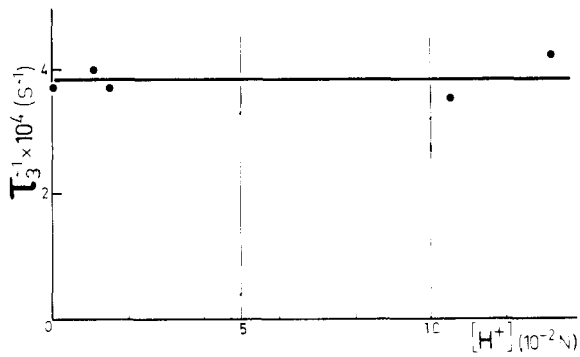


Figure 8. Plot of τ_3^{-1} vs. the hydronium ion concentration at 25 °C.

(b) Variation of the Equilibrium Constants with Temperature. The values of the equilibrium constants at 25 °C and the enthalpy and eventually entropy variations characterizing the three reactions in Scheme I (eq 1) appear in Table I. Both ln K'_{13} and ln K_{34} are linear functions of $1/T$ in the temperature ranges investigated (3.5–25 °C for K'_{13}; 15–63 °C for K_{34}). It should be noted that the study of the $1/T$ dependence of K'_{12}

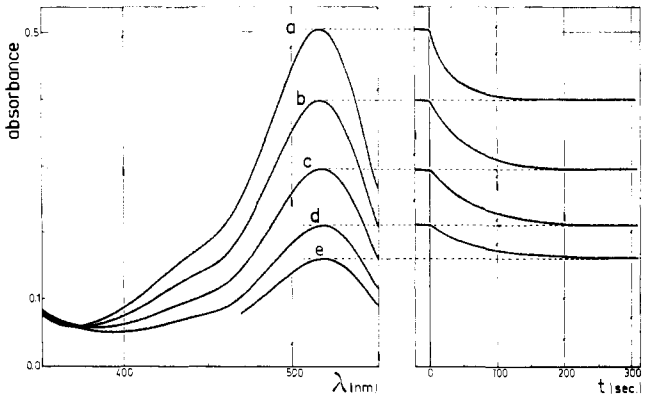


Figure 9. Left: absorption spectra in the visible region of a pigment solution at 4 °C and at different pH's: 2.15 (a), 2.62 (b), 2.91 (c), 3.09 (d), and 3.29 (e). Analytical concentration 3.3×10^{-5} M; optical path length = 1 cm. Right: kinetic behavior of rapid absorption changes associated with acidity changes ($T = 4$ °C). The curves obtained are exponential with time constant τ_2. The reduction in the rate of equilibration of the hydration equilibrium with lowering of acidity is quite evident.

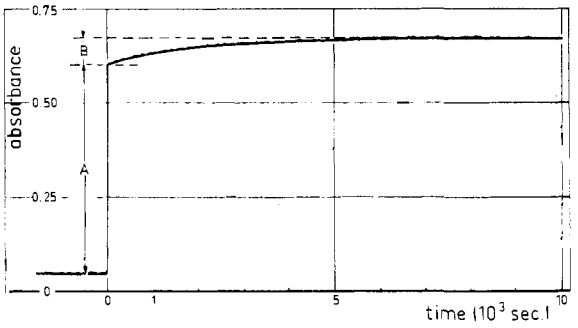


Figure 10. Absorption variation (517.5 nm) of a pigment solution initially equilibrated at 25 °C and pH 4.02 when rapidly acidified (final pH 1.13). A and B represent the total amplitudes of rapid (τ_2) and slow (τ_3) relaxations and are directly proportional to the quantities of carbinol pseudobase and chalcone present in solution at pH 4.02 at the time of acidification. Analytical concentration 2.6×10^{-5} M; $l = 1$ cm; $T = 25$ °C.

is highly delicate; the value assigned to ΔH°_{12} in Table I, though only approximate, nevertheless clearly indicates that the neutralization of the quinoidal base is endothermic.[21]

Table I. Thermodynamic Constants Associated with the Structural Transformations of Malvidin 3-Glucoside in a 0.2 M Ionic Strength Aqueous Medium

$AH^+ \rightleftharpoons A + H^+$	$AH^+ \rightleftharpoons B + H^+$	$B \rightleftharpoons C$
$K'_{12} = 5.7\ (\pm 1) \times 10^{-5}$ M[a]	$K'_{13} = 2.5\ (\pm 0.1) \times 10^{-3}$ M[a]	$K_{34} = 0.12\ (\pm 0.01)$[a]
$[pK'_{12} = 4.25\ (\pm 0.1)]$	$[pK'_{13} = 2.60\ (\pm 0.02)]$	
$\Delta H^\circ_{12} = -3\ (\pm 2)$[b]	$\Delta H^\circ_{13} = 4.7\ (\pm 0.2)$[b]	$\Delta H^\circ_{34} = 3.7\ (\pm 0.2)$[b]
ΔS°_{12}[c]	$\Delta S^\circ_{13} = 4\ (\pm 0.7)$[d]	$\Delta S^\circ_{34} = 8.5\ (\pm 1)$[d]

[a] At 25 °C, cf. text for definitions. [b] kcal mol^{-1}. [c] No value is given because the experimental uncertainty is too great. [d] cal deg^{-1} mol^{-1}.

Discussion

A. Kinetic Study. (a) Relaxation Spectrum. For the sake of clarity the three reactions of Scheme I are represented symbolically by

$$AH^+ \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} A + H^+ \quad (4)$$

$$AH^+ \underset{k_{31}}{\overset{k_{13}}{\rightleftharpoons}} B + H^+ \quad (5)$$

$$B + \text{catalyst} \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} C + \text{catalyst} \quad (6)$$

This set of reactions will be characterized by a relaxation spectrum consisting of three relaxation times:[8]

$$\tau_1^{-1} = k_{12} + k_{21}([A] + [H^+]) \quad (7)$$

$$\tau_2^{-1} = k_{43}(1 + K_{34})[\text{Cat}] + k_{13}\frac{[H^+]}{K_{12} + [A] + [H^+]} + k_{31}[H^+]\left(1 + \frac{[B]}{K_{12} + [A] + [H^+]}\right) \quad (8)$$

$$\tau_3^{-1} = k_{43}\left(1 + \frac{K_{34}(K_{13} + [B])}{K_{12} + K_{13} + [A] + [B] + [H^+]}\right)[\text{Cat}] \quad (9)$$

$$(K_{12} = k_{12}/k_{21};\ K_{13} = k_{13}/k_{31} \text{ and } K_{34} = k_{34}/k_{43})$$

τ_1 represents the relaxation time of the fastest process, τ_3 that of the slowest one, and τ_2 that of the intermediate one and we observe experimentally that $\tau_1^{-1} \gg \tau_2^{-1} \gg \tau_3^{-1}$. The acid–base equilibrium is significantly faster than hydration which in turn is significantly faster than the tautomeric equilibrium.[22]

(b) Rate Constants for the Proton Transfer, Hydration, and Tautomeric Equilibria of Malvidin 3-Glucoside. After studying the theoretical kinetic equations (eq 7–9) and the experimental ones (Figures 4, 5, 6, and 8), it is possible to deduce the rate constants of the three reactions shown in Scheme I. However, to obtain both the rate constants of the tautomeric equilibrium a further equilibrium measurement is required.

According to eq 2 and 7, the rate constants of the proton-transfer equilibrium are at 25 °C: $k_{12} = 4.7\ (\pm 0.4) \times 10^4$ s^{-1} and $k_{21} = 6.7\ (\pm 0.5) \times 10^8$ M^{-1} s^{-1}. At 6.5 °C, these values become $k_{12} = 1.8\ (\pm 0.1) \times 10^4$ s^{-1} and $k_{21} = 1.8\ (\pm 0.1) \times 10^8$ M^{-1} s^{-1}. These latter two values are very close to those observed at 6.5 °C for malvidin 3,5-diglucoside[1] where $k_{12} = 1.8 \times 10^4$ s^{-1} and $k_{21} = 1.4 \times 10^8$ M^{-1} s^{-1}. The presence of an *O*-glucose group at position 5 in the place of an OH group has only a minimal influence both on the position [$pK'_{12} = 4.0\ (\pm 0.1)$ for malvidin 3,5-diglucoside[1] and 4.10 (± 0.05) for malvidin 3-glucoside at 6.5 °C] and on the kinetics of the proton transfer equilibrium.

The relaxation time τ_3 of the tautomeric equilibrium is independent of the pH (the most acidic pH's); this is shown in Figure 8. When $[H^+] > 10^{-3}$ N, eq 9 reduces to a quasi-constant term which is independent of both the acidity of the solution and the pigment concentration. Indeed, as $K_{13} \gg K_{12}$, [A], eq 9 reduces to

$$\tau_3^{-1} = k_{43}(1 + \alpha)[\text{Cat}] \quad (10)$$

where

$$\alpha = K_{34}\frac{K_{13} + [B]}{K_{13} + [B] + [H^+]} \quad (11)$$

For $[H^+] = 10^{-4}$, 10^{-3}, and 10^{-2} N, the values of α at 25 °C are respectively 0.12 (K_{34}), 0.09 and 0.03 (for [B] values of the order of 10^{-5} M). Within the limits of experimental error, τ_3^{-1} can be considered to correspond to k_{43}[Cat] for the most acidic media.[23] Under such conditions the catalyst of the tautomeric equilibrium is therefore the solvent itself and the reaction is base catalyzed.[24] Thus the rate constant of the cyclization reaction $k'_{43} = k_{43}$ [H_2O], obtained at 25 °C is equal to 3.8 (± 0.2) $\times 10^{-4}$ s^{-1}. At the same temperature, the rate constant

for the pyrylium ring-opening reaction will be $k'_{34} = K_{34}k'_{43} = 4.5\ (\pm 0.3) \times 10^{-5}\ s^{-1}$.

The relaxation characterizing the establishment of the hydration equilibrium is, in a sufficiently acidic medium, a linear function of the hydronium ion concentration (Figure 5, eq 3). Since $[H^+] \gg K_{12}$, [A], [B] and since the term $k_{43}(1 + K_{34})[Cat] = 4.2 \times 10^{-4}\ s^{-1}$ at 25 °C is completely negligible with respect to the other two terms, then eq 8 reduces to

$$\tau_2^{-1} = k_{13} + k_{31}[H^+] \qquad (12)$$

By identification, k_{13}, the rate constant for nucleophilic addition of water to the flavylium cation, is $8.5\ (\pm 1) \times 10^{-2}\ s^{-1}$ and k_{31}, the dehydration rate constant of the carbinol, is 34 $(\pm 3)\ M^{-1}\ s^{-1}$ at 25 °C. For the least acidic media (pH >3), the relaxation of the hydration equilibrium is correctly described by

$$\tau_2^{-1} = k_{13}\frac{[H^+]}{K_{12} + [A] + [H^+]} + k_{31}[H^+]\left(1 + \frac{[B]}{K_{12} + [A] + 1h^+]}\right) \qquad (13)$$

This equation has already been established for malvidin 3,5-diglucoside and enabled us to demonstrate that the hydration of anthocyanin pigments takes place on the flavylium cation and not, as had been thought till then, on the quinoidal base.[1] We also measured k_{13} and k_{31} at 4 °C and found them to be $0.95\ (\pm 0.1) \times 10^{-2}\ s^{-1}$ and $6.2\ (\pm 0.3)\ M^{-1}\ s^{-1}$, respectively. For malvidin 3,5-diglucoside[1] at the same temperature, $k_{13} = 4.7\ (\pm 0.2) \times 10^{-2}\ s^{-1}$ and $k_{31} = 2.6\ (\pm 0.1)\ M^{-1}\ s^{-1}$. The presence of an *O*-glucose group instead of a hydroxyl group at position 5 considerably increases the hydration rate and significantly slows down the reverse reaction. This is expressed by a large shift of the hydration equilibrium (for the diglucoside[1] and for the monoglucoside at 4 °C, pK'_{13} is respectively 1.86 and 2.86).[25] This latter result has been interpreted to mean that it is impossible for anthocyanin dimonosides to contribute appreciably to the plant pigmentation since the pH of these natural media is around 3-6.[26] The set of rate constants for the reactions in Scheme I appears in Table II.

B. The Carbinol–Chalcone Tautomeric Equilibrium.[27] For flavylium cations lacking a substituent at position 3, the hydration reaction has generally been observed to lead to the tautomeric open form.[16] An *O*-glucose group at position 3[28] shifts the tautomeric equilibrium toward the cyclic tautomer.[29] This structural effect is identical with that observed in the general case of ring-chain tautomerism: the presence of a substituent on the chain always displaces the equilibrium toward the cyclic form.[30] This large shift in the tautomeric equilibrium makes it very difficult, because of the presence of other structures (A, AH^+), to observe the rarest form. Whether or not it is possible to demonstrate its presence depends, of course, on the value of the equilibrium constant and on the techniques used.[31]

The thermodynamic measurements on this equilibrium show that the ring-opening reaction is endothermic ($\Delta H^\circ_{34} = 3.7$ kcal mol^{-1}) and that this reaction is accompanied by a large and positive entropy change ($\Delta S^\circ_{34} = 8.5$ cal $deg^{-1}\ mol^{-1}$). Thus our results are in excellent agreement with those from studies of the temperature dependence of the equilibrium constant for ring-chain tautomerism: the ring-opening reaction is always clearly endothermic and the entropy increase is always large (ca. 8-10 cal $deg^{-1}\ mol^{-1}$).[32] Any decrease in temperature will thus lead to a large decrease in the amount of chalcone present at equilibrium. This thermal effect is represented in Figure 11. With this knowledge, it is easy to understand why low temperatures are highly unsuitable both thermodynamically and kinetically for observing the chalcone tautomer. Since the thermodynamic conditions determine the amplitude of a relaxation process, the slow relaxation amplitude will be diminished at low temperatures and might even be of the same order of magnitude as the spectrophotometer noise, thereby escaping observation. As for the third relaxation rate (τ_3), this will be slowed down so much that it would take more than 10 h to observe, thereby necessitating very high spectrophotometer stability.[33] It is therefore easier and wiser to study the reactivity of anthocyanins at 25 °C than at temperatures near 0 °C. However, it must still be shown that there is really no thermal degradation of anthocyanins at room temperatures.

Table II. Rate Constants[a] of the Structural Transformations of Malvidin 3-Glucoside in a 0.2 M Ionic Strength Aqueous Acidic Medium at 25 °C

$k_{12} = 4.7\ (\pm 0.4) \times 10^4\ s^{-1}$	$k'_{34}{}^b = 4.5\ (\pm 0.3) \times 10^{-5}\ s^{-1}$
$k_{21} = 6.7\ (\pm 0.5) \times 10^8\ M^{-1}\ s^{-1}$	$k'_{43}{}^b = 3.8\ (\pm 0.2) \times 10^{-4}\ s^{-1}$
$k_{13} = 8.5\ (\pm 1) \times 10^{-2}\ s^{-1}$	
$k_{31} = 34\ (\pm 3)\ M^{-1}\ s^{-1}$	

[a] Cf. text for definitions. [b] $k'_{34} = k_{34}[H_2O]$; $k'_{43} = k_{43}[H_2O]$.

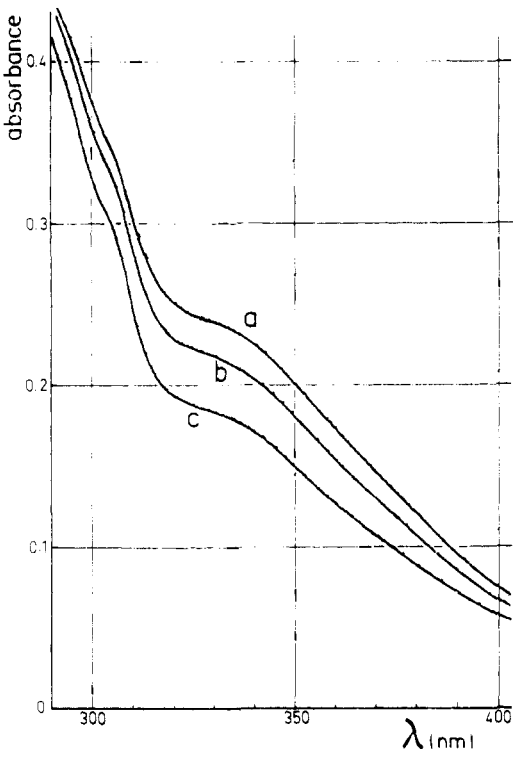


Figure 11. Absorption variation with temperature of a pigment solution at pH 4 in the neighborhood of the absorption maximum of the chalcone form. T = 54 (a), 40.5 (b), and 25 °C (c). Analytical concentration = 4.5×10^{-5} M.

C. Concerning the Thermal Degradation of Anthocyanins. On the basis of experiments whose principle is outlined below, some authors[2] have concluded that there is a thermal degradation of anthocyanins. Pigment solutions are prepared at various acidities (pH 1-7) and immediately plunged into thermostated baths (generally between 20 and 90 °C). For each pH and for each temperature a sample is taken immediately from the resulting solution, cooled, and acidified (pH <1). The absorbance of this sample at the absorption maximum of the flavylium cation, the only stable form under these conditions, is then measured. This initial absorbance measurement, corresponding to the initial amount of pigment in the solution, serves as a reference. Other samples are then taken at different intervals of time and subjected to the same treatment as the reference sample (cooling, acidification, and measurement of the absorbance). The drop in absorbance for a given time interval (generally several hours) increases as the

temperature is raised, whatever the pH. This is how Hrazdina[2] measured differences in absorbance of about 0.02 for temperatures near 20 °C, and of about 0.3–0.4 for temperatures near 100 °C for malvidin 3,5-diglucoside (observation time was 7 h; pH 1.5–6 and analytical concentration $\simeq 3 \times 10^{-5}$ M). The difference in absorbance is thus interpreted as proof of the instability of anthocyanins when subjected to heat. In light of our findings, we wish to point out the following:

(1) Since the three reactions represented by Scheme I are all endothermic (Table I), any rise in temperature will highly favor the chalcone form over the three other forms, particularly over the carbinol pseudobase.

(2) Whatever the temperature or the pH, all the solutions are left long enough for the set of reactions in Scheme I to reach equilibrium.

(3) When a given sample is being treated (cooling and acidification), it is evident that the quinoidal base and the carbinol pseudobase immediately transform into the flavylium structure (cf. τ_1, τ_2, K'_{12}, and K'_{13}). What happens to the chalcone is totally different: this form is converted only slowly to carbinol at room temperature and therefore its reaction is quenched by the cooling procedure. The absorbance measured then does not take into account the amount of pigment (which grows as the temperature increases) present in this form at the time of cooling and acidification. As for the reference sample taken as soon as solution is achieved, the amount of chalcone formed can only be minimal, since the tautomeric equilibrium has not had enough time to occur.[34]

We therefore feel that the differences in absorbance thus measured are a consequence of both the strong endothermicity of the pyrylium ring-opening reaction and the relaxation kinetics involved in establishing the slow tautomeric equilibrium at room temperature.[35]

Conclusion

At room temperature in moderately acidic media (pH 4–6), anthocyanins exist mainly in the form of a ring-chain tautomeric equilibrium between the carbinol pseudobase and the chalcone. The position of this equilibrium under these conditions always favors the carbinol pseudobase. At 25 °C the amount of chalcone is about two times greater for malvidin 3-glucoside than for malvidin 3,5-diglucoside. In more acidic media (pH <4), owing to the existence of the hydration equilibrium, the flavylium cation and the carbinol pseudobase predominate. The hydration reaction of the cation is clearly faster for the diglucoside than for the monoglucoside. At equilibrium and at any pH whatsoever, the quinoidal base is only present to a very small extent. The kinetic and thermodynamic characteristics of the proton transfer equilibrium are almost identical for both pigments.

At this point of our knowledge about the reactivity of anthocyanins, there are two fundamental reasons which dictate a major revision of the findings and conclusions reported in the literature: (a) Up till now all authors, on the sole basis of appearances, have accepted that the interconversion of AH^+, A, and B follows the mechanism $AH^+ \rightleftharpoons A \rightleftharpoons B$; whereas we have actually shown[1] and confirmed in the present work that this mechanism does not correspond to what really occurs. The mechanism which we propose ($A \rightleftharpoons AH^+ \rightleftharpoons B$) accounts fully for our observations as well as for those reported in the literature. (b) The existence of the chalcone form and thus of the tautomeric equilibrium has been completely ignored.

Above all, the properties of anthocyanins solutions can be understood only when one knows that placing the cation in the most acidic media (pH <3) rapidly sets up the flavylium cation–carbinol equilibrium with the formation of barely detectable amounts of quinoidal base and chalcone and that placing this same cation in slightly acidic or basic media (pH >3) immediately yields the quinoidal base or its ionized form A^-. Effectively kinetic competition between the deprotonation and hydration reactions always greatly favors proton loss.[36] In other words, the totality of the cation initially present is deprotonated before even a minute fraction of it is hydrated.[37] However, since the hydration equilibrium constant is much greater than the constant for the proton-transfer equilibrium, A and/or A^- are unstable and, depending on the pH of the medium, rearrange into B and C or into ionized forms of B and C.

Acknowledgments. We wish to express our gratitude to Professor J. E. Dubois for his helpful advice and for the many fruitful discussions. We also acknowledge with thanks the help of Drs. J. S. Lomas and M. Dreyfus for their constructive comments and are grateful to Mr. Blanck for having supplied us with the sample of Pinot Noir.

Appendix. Conditions Required for Linearization of the Rate Equations

It can be shown that, whatever the amplitude of the perturbation, eq I, II, and III correspond to the rate equations of the proton transfer, hydration, and tautomeric equilibria, respectively.

$$-\frac{d\Delta[AH^+]}{dt} = \tau_1^{-1}\Delta[AH^+](1 + r_1) \qquad (I)$$

$$-\frac{d\Delta[B]}{dt} = \tau_2^{-1}\Delta[B](1 + r_2) \qquad (II)$$

$$-\frac{d\Delta[C]}{dt} = \tau_3^{-1}\Delta[C] \qquad (III)$$

with

$$r_1 = \frac{-\Delta[AH^+]}{K_{12} + [A] + [H^+]} \qquad (IV)$$

$$r_2 = \frac{\tau_2 k_{31}[H^+]}{K_{12} + [A] + [H^+]}\Delta[B] \qquad (V)$$

For temperature jumps of a few degrees, the term r_1 is perfectly negligible.[8b] Moreover, the rate equation for the tautomeric equilibrium (eq III) is strictly first order regardless of the size of the perturbation. Taking into consideration eq 13, it is possible to express eq V in a simple and useful form:

$$\Delta[B] = r_2(K_{12} + K_{13} + [A] + [B] + [H^+]) \qquad (VI)$$

The linearization condition will be completely satisfied for eq II, *whatever the degree of advancement of the relaxation process*, if $|r_2^\circ| \leq 10^{-2}$ (r_2° is defined as the value of r_2 at time t = 0).[38] Since $K_{13} \gg K_{12}$, [A], [B], this leads to the inequality[39]

$$|\Delta[B]_0| \leq 10^{-2}(K_{13} + [H^+]) \qquad (VII)$$

It will be noticed that the larger K_{13} or $[H^+]$, the greater $|\Delta[B]_0|$ can be. For example, in the case of malvidin 3-glucoside, the total variation in the concentration of B thus permitted during a kinetic run goes from 10^{-3} M ($[H^+] = 10^{-1}$ N) to 3.1×10^{-5} M ($[H^+] = 10^{-5}$ N or less). The first of these extreme values is equal to about 30 times the analytical concentration normally used in this work. A pH jump of any size could thus be applied to our system.

References and Notes

(1) Part 1: R. Brouillard and J. E. Dubois, *J. Am. Chem. Soc.*, **99**, 1359 (1977).
(2) G. Hrazdina, A. J. Borzell and W. B. Robinson, *Am. J. Enol. Vitic.*, **21**, 201 (1970); N. Ioncheva and S. Tanchev, *Z. Lebensm.-Unters.-Forsch.*, **155**, 257 (1974), and references cited therein.
(3) A systematic study of the factors involved in the carbinol–chalcone ring-chain tautomerism is totally lacking. However it has been shown that, for some compounds not substituted at position 3, the ring-closure reaction is photochemically catalyzed [W. Sperling, F. C. Werner, and H. Kuhn, *Ber. Bunsenges. Phys. Chem.*, **70**, 530 (1966); L. Jurd, *Tetrahedron*, **25**, 2367

(1969)]
(4) P. Ribéreau-Gayon [*Rev. Gen. Bot.*, **70**, 531 (1959)] postulates the existence of a chalcone form in order to account for the discoloration of aqueous anthocyanin solutions by bisulfite. L. Génevois' work [*Bull. Soc. Chim. Biol.*, **38**, 7 (1956)] is probably behind this idea; indeed Génevois has shown that some of the reactions characterizing the ketone function occur with anthocyanins and their aglycones. In answer to Ribéreau-Gayon, L. Jurd [*J. Food Sci.*, **29**, 16 (1964)] convincingly shows that, for compounds not substituted at position 3, the bisulfite action occurs on the flavylium cation. Furthermore, Jurd claims to have demonstrated the absence of any chalcone form whatsoever for the anthocyanins.
(5) J. B. Harborne, "Comparative Biochemistry of the Flavonoids", Academic Press, New York, N.Y., 1967, p 22.
(6) C. F. Timberlake and P. Bridle, *J. Sci. Food Agric.*, **18**, 473 (1967).
(7) Even today industry seems to be unaware that anthocyanins are a very abundant raw material. Among the many things they could be used for the most urgent seems to be their substitution for synthetic food colorings. The great advantage of natural pigments is that they are totally innocuous, man having consumed them from the beginning of time without apparent ill effects. Evidently such a test with synthetic colorings is out of the question.
(8) (a) M. Eigen and L. De Maeyer, "Technique of Organic Chemistry", Vol. 8, A. Weissberger, Ed., Interscience, New York, N.Y., 1963; (b) C. F. Bernasconi, "Relaxation Kinetics", Academic Press, New York, N.Y., 1976.
(9) G. Hrazdina, *J. Agr. Food Chem.*, **18**, 243 (1970).
(10) Malvidin 3-glucoside is the most abundant pigment in the Pinot Noir grape. Moreover, it should be noted that *Vitis vinifera* only possesses monoside anthocyanin pigments [R. A. Fong, R. E. Kepner, and A. D. Webb, *Am. J. Enol. Vitic.*, **22**, 150 (1971); J. Ribéreau-Gayon, E. Peynaud, P. Sudraud, and P. Ribéreau-Gayon, "Sciences et Techniques du Vin", Vol. 1, Ed. Dunod, Paris, 1972, p 477].
(11) J. B. Harborne, *Biochem. J.*, **70**, 22 (1958).
(12) T. A. Geissman and L. Jurd, *Arch. Biochem. Biophys.*, **56**, 259 (1955).
(13) During the temperature-jump measurement of the fastest relaxation process, the solution alternately passes from darkness (temperature-jump cell) to ambient light (pH and temperature measurement glass cell). This does not in any way affect the kinetics of this relaxation which is only related to the quinoidal base–flavylium cation proton transfer reaction upon which the ambient light has no observable effect.
(14) M. Dreyfus, G. Dodin, O. Bensaude, and J. E. Dubois, *J. Am. Chem. Soc.*, **97**, 2369 (1975).
(15) Given the number and nature of the different equilibria in Scheme I it should be pointed out that the data appearing in the literature and establishing the presence or absence of isosbestic points for the anthocyanins and for the flavylium salts are valid only when all of the experimental conditions (concentration, temperature, pH, light exposure, compounds present in the solution,etc.) are included.
(16) L. Jurd, *J. Org. Chem.*, **28**, 987 (1963).
(17) This experiment is different from that by which the existence of the open chalcone tautomeric form was demonstrated (Figure 2). Here the observation is carried out at the absorption maximum of the flavylium cation (517.5 nm). At this wavelength, there is no absorption of the chalcone form and the risk of catalysis by the analysis light is thereby removed.
(18) Since the carbinol form is colorless, the choice of a wavelength close to the absorption maximum of the flavylium cation leads to $D_B = 0$.
(19) Furthermore, we show that under these conditions the equilibrated formation of chalcone only very slightly affects the measurement of K_{13}. Indeed, the relative decrease in concentration of the cation ($\delta\,[AH^+]/C_0$) during the period of tautomeric equilibration is expressed by

$$a_{H^+}\left(\frac{K'_{13}K_{34}}{(K_{13}+a_{H^+})[K_{13}(1+K_{34})+a_{H^+}]}\right)$$

whose value at 25 °C is about 10^{-2} for pH near 2–3 (cf. Figure 3).
(20) At around pH 4 there are significant amounts of quinoidal and flavylium forms at equilibrium. This does not appreciably affect our determination of K_{34}.
(21) To our knowledge, there are two other methods for determining K'_{12}: the first one is based on the measurement of the instantaneous absorbance of the quinoidal form generated by appropriate pH jumps. The quinoidal base is thus the product immediately formed, to the exclusion of the carbinol and chalcone forms. For each pH it is therefore indispensable that the absorbance measurements for zero time (pH jump) be extrapolated. The second method is based on the knowledge of the relaxation kinetics governing the establishment of the flavylium cation–carbinol equilibrium for the least acidic media,[1] the relaxation time being expressed by

$$\tau_2^{-1} = \left(\frac{k_{13}}{K_{12}} + \beta k_{31}\right)[H^+]$$

where $\beta \simeq 1$. These two methods, though lengthier, are not more precise. It should also be noted that it is possible, in principle, to estimate ΔH°_{12} from the amplitude of the fast relaxation.
(22) [A], [B], and [H$^+$] refer to the equilibrium state. In the Appendix, it is shown that linearization conditions are completely satisfied for temperature jumps of a few degrees (proton transfer equilibrium) and for pH jumps of any size (hydration and tautomeric equilibria).
(23) The hydronium ion and the water molecule are likely to be the effective catalysts of the tautomeric equilibrium.
(24) For this prototropic tautomeric equilibrium, we had previously suggested the possibility of acid catalysis bringing the protonated forms of both tautomers into play.[1] Our present results show that in fact this transformation occurs by an ionization mechanism wherein the solvent acts as the base. There is a remarkable analogy here with the prototropic tautomeric equilibrium of β-dicarbonyl compounds whose acidity is such that the reaction in an aqueous medium is only base catalyzed [R. Brouillard and J. E. Dubois, *J. Org. Chem.*, **39**, 1137 (1974); R. Brouillard, Thèse de Doctorat ès Sciences, Université Paris VII, 1974].
(25) This difference between anthocyanin dimonosides and monosides seems to be systematic.[6]
(26) S. Asen, R. N. Stewart, and K. H. Norris, *Phytochemistry*, **11**, 1139 (1972).
(27) To date, there is no information regarding the configuration of the chalcone tautomer. The representation of this tautomer in Scheme I is purely schematic. For compounds not substituted at position 3, and diversely hydroxylated and/or methoxylated on rings A and B, Jurd[16] has observed that the trans structure is the more stable one.
(28) Although anthocyanins are never substituted at position 4, it seems reasonable to think that the introduction of a substituent at this position would have major consequences on the hydration and tautomeric equilibria.
(29) Our measurements show that the open form is more abundant for malvidin 3-glucoside than for malvidin 3,5-diglucoside (K_{34} at 25 °C is respectively 0.12 and 0.06); this indicates that the *O*-glucose group at position 5 has a significant effect on the tautomeric equilibrium.
(30) G. S. Hammond, "Steric Effects in Organic Chemistry", M. Newman, Ed., Wiley, New York, N.Y., 1956, p 463; P. Jones, *Chem. Rev.*, **63**, 461 (1963); J. Castells and A. Colombo, *Chem. Commun.*, 1062 (1969); R. Chiron and Y. Graff, *Bull. Soc. Chim. Fr.*, 2145 (1971); R. Escale and J. Verducci, *ibid.*, 1203 (1974).
(31) In the case of the 8,4′-dimethoxyflavylium cation, Jurd[16] has observed during a pH jump from 1 to 5.4, the kinetic formation of the cyclic tautomer (rapid discoloration of the solution) and its slow transformation into a thermodynamically more stable open form. It should be noted that, since formation of a quinoidal base is impossible owing to the absence of a hydroxyl group, the kinetic product resulting from such a pH jump is the carbinol pseudobase. Since, for this type of compound, the position of the tautomeric equilibrium is very favorable to the chalcone and since $\tau_3 \gg \tau_2$, the cyclic tautomer evolves slowly toward the open form which is the thermodynamic product. It is an entirely different matter for those compounds likely to form a quinoidal base: indeed, during the pH jump, the kinetic product is no longer the carbinol pseudobase, but rather the quinoidal base and, under these conditions, the solution could not become immediately colorless since the quinoidal base (as well as the very small fraction of cation that is still present) is evolving less rapidly (see τ_2^{-1}, eq 13 and Figure 6) toward the only really stable state at this pH for this type of compound, i.e., the chalcone form. This latter behavior is observed[16] for the following cations: 3′-methoxy-4′,7-dihydroxy- and 4′-methoxy-7-hydroxyflavylium.
(32) L. Dorman, *J. Org. Chem.*, **32**, 255 (1967); J. Whiting and J. Edward, *Can. J. Chem.*, **49**, 3799 (1971).
(33) We have seen that it is possible to observe the slow relaxation process by two slightly different methods: one of the methods is based on a pH jump toward less acidic media and generates the two tautomeric forms starting from a pigment solution only in the flavylium form; the second method is based on a pH jump toward more acidic media and generates the flavylium cation starting from a pigment solution essentially in the tautomeric equilibrium. In the first case, the recording is carried out at a wavelength of 360 nm, which is near the absorption maximum of the chalcone form; in the latter case it is carried out at 517.5 nm, which is the absorption maximum of the flavylium structure. Moreover, all things being equal, since

$$\epsilon_{cation}^{517.5\ nm} > (\epsilon_{chalcone}^{360\ nm} - \epsilon_{carbinol}^{360\ nm})$$

the amplitude of this relaxation mode is greater for the pH jumps generating the flavylium form.
(34) The anthocyanins are always isolated in the form of flavylium salts which subsequently are dissolved in suitable media.
(35) It is certain that for the highest temperatures and for sufficiently long periods of observation (over 5 h), other unequilibrated but as yet not well identified reactions also contribute significantly to the decrease in absorbance [L. Jurd, "The Chemistry of Plant Pigments", C. O. Chichester, Ed., Academic Press, New York, N.Y., 1972, p 123; J. B. Adams, *J. Sci. Food Agric.*, **24**, 747 (1973)].
(36) There are perhaps flavylium cations for which $K'_{12} \simeq K'_{13}$ or even $K'_{12} > K'_{13}$. The quinoidal base would then be as stable as or more stable than the carbinol pseudobase and would constitute an important fraction of the equilibrium mixture. A cation of this type would give rise to solutions which would be colored regardless of the pH.
(37) In a basic medium, the hydroxyl ion instantly reacts with the cation, thereby yielding first A, then A^-. We can reasonably assume that the very slow overall equilibration of the pigment solution involves at some point a nucleophilic addition of the hydroxyl ion to the flavylium cation.
(38) Since $r_2 = r_2^\circ e^{-t/\tau_2}$ for $|r_2^\circ| \ll 1$, $|r_2^\circ|$ is the largest value of $|r_2|$ for a given experiment.
(39) In the case of the proton-transfer equilibrium, one obtains for the linearization condition $|\Delta[AH^+]_0| \leq 10^{-2}(K_{12} + [A] + [H^+])$. Thus, calculation of the largest permitted jump could be readily achieved by mere examination of the acidity constant and the analytical concentration.