(23) D. G. Lister and J. K/ Tyler, *Chem. Commun.*, 152 (1966).
(24) E. Fischer, *J. Chem. Phys.*, **19**, 395 (1951); E. D. Bergmann, E. Fischer, E. Zimkin, and S. Pinehaus, *Recl. Trav. Chim. Pays-Bas*, **71**, 213 (1952).
(25) P. M. Kuznesof and D. F. Shriver, *J. Am. Chem. Soc.*, **90**, 1683 (1968); D. R. Armstrong and D. T. Clark, *Chem. Commun.*, 99 (1970); *J. Chem. Soc. A*, 99 (1970); *Theor. Chim. Acta*, **24**, 307 (1971); M. Graffeuil and J.-F. Labarre, *J. Chim. Phys. Phys.-Chim. Biol.*, **68**, 1379 (1971); N. C. Baird and R. K. Dalton, *Inorg. Chem.*, **11**, 17 (1972); J. Gayoso, B. Maouche, and A. Boucekkine, *C. P. Hebd. Seances Acad. Sci., Ser. C*, **276**, 257 (1973); V. M. Scherr and D. T. Haworth, *J. Inorg. Nucl. Chem.*, **35**, 660 (1973).
(26) O. T. Beachley, Jr., *J. Am. Chem. Soc.*, **92**, 5372 (1970).
(27) A similar idea was introduced to rationalize the bonding situation in the silaphenanthrenes [J. M. Gaidis and R. West, *J. Am. Chem. Soc.*, **86**, 5699 (1964)].
(28) A. Yokozeki and K. Kuchitsu, *Bull. Chem. Soc. Jpn.*, **44**, 2926 (1971).
(29) R. Hoffmann, A. Imamura, and W. J. Hehre, *J. Am. Chem. Soc.*, **90**, 1499 (1968).
(30) P. Bischof, J. A. Hashmall, R. Heilbronner, and V. Hornung, *Tetrahedron Lett.*, 4025 (1969).
(31) E. Heilbronner and K. A. Muszkat, *J. Am. Chem. Soc.*, **92**, 3818 (1970).
(32) R. Gleiter, *Angew. Chem., Int. Ed. Engl.*, **13**, 696 (1974).

Mechanism of the Structural Transformations of Anthocyanins in Acidic Media

Raymond Brouillard and Jacques-Emile Dubois*

Contribution from the Laboratoire de Chimie Organique Physique, associé au C.N.R.S., Université Paris VII, 75005 Paris, France.

Received June 16, 1976

Abstract: In acidic aqueous media (pH 1–6), there are three forms of malvin: the flavylium cation AH^+, the carbinol B, and the quinonoidal base A. Equilibrium between the two neutral forms occurs exclusively by way of the flavylium cation with a constant $K_{23} = [B]/[A] = 1.6\ (\pm\ 0.5) \times 10^2$ at 4 °C. Contrary to all previous reports the quinonoidal base does not hydrate. The neutralization of this base by the hydronium ion is endothermic; the associated rate constant k_{21} is $1.4\ (\pm\ 0.1) \times 10^8\ M^{-1}\ s^{-1}$ at 6.5 °C. The rate constant for deprotonation of the cation by the solvent k_{12} is $1.8\ (\pm\ 0.1) \times 10^4\ s^{-1}$ at the same temperature. Hydration of the flavylium cation involves the formation of a C–O bond and a proton transfer. The rate constant k_{13} for nucleophilic addition of water to the flavylium cation is $4.7\ (\pm\ 0.2) \times 10^{-2}\ s^{-1}$ at 4 °C; for the reverse reaction the rate constant k_{31} is $2.6\ (\pm\ 0.1)\ M^{-1}\ s^{-1}$ at the same temperature. The existence of very small amounts of a third neutral form, the chalcone C, a prototropic tautomer of the carbinol B, is considered.

Since the work of Willstätter[1] and Robinson,[2] it has been known that anthocyanins are responsible for a variety of beautiful plant colors. Although they are found in every part of a plant, they are most obvious in flowers and fruits.[3] Extending our knowledge of the biological phenomena involved requires a good understanding of the physicochemical properties of these compounds and especially of their reactivity toward chemical agents found in nature.

Depending on the degree of acidity or alkalinity, anthocyanins adopt different chemical structures. Each of these structures presents, in the visible region, a characteristic absorption spectrum. At present, it is generally accepted that there is an equilibrium between the flavylium (2-phenylbenzopyrylium) cation AH^+ and the carbinol B in acidic media (eq 1).

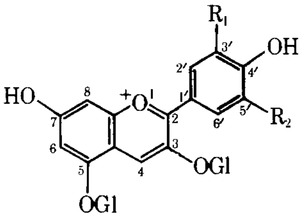
$$AH^+ + H_2O \rightleftarrows B + H^+ \tag{1}$$

Sondheimer[4] was the first to measure the equilibrium constant of this reaction in the case of pelargonidin 3-monoglucoside ($R_1 = R_2 = H$; OH at position 5). The mechanism generally proposed[5] supposes the existence of a transient species, the anhydro base A, obtained by deprotonation of the flavylium cation. This quinonoidal base would be converted into the carbinol B by addition of water (mechanism I, eq 2).

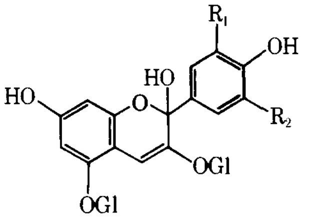
Mechanism I

$$AH^+ \underset{}{\overset{-H^+}{\rightleftarrows}} A \overset{+H_2O}{\rightleftarrows} B \tag{2}$$

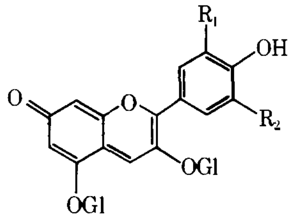
This mechanism has been elaborated on the basis of somewhat superficial observations; i.e., when the acidity of the medium is rapidly and sufficiently diminished, the flavylium cation instantaneously turns into the anhydro base which, *apparently*, slowly hydrates into the carbinol.[6] The following reasons, however, cause us to doubt the validity of this mechanism:



AH^+
flavylium cation



B
carbinol base



A
quinonoidal base or anhydro base

(a) According to mechanism I (eq 2), any shift from left to right is always assumed to make anhydro base show up, no matter what the final medium acidity value (the proton transfer reaction is very fast as compared to the hydration reaction). However, it has been shown[5] that there is an acidity threshold (pH 3–4), below which anhydro base formation is no longer observed and that, despite this fact, the final product is indeed the carbinol.

(b) The flavylium cation–carbinol equilibrium exists even when the cation lacks a hydroxyl group. Some authors[7] consider that in this last case direct reaction of the cation with hydroxyl ion takes place.

These indications point to the existence of a pathway dif-

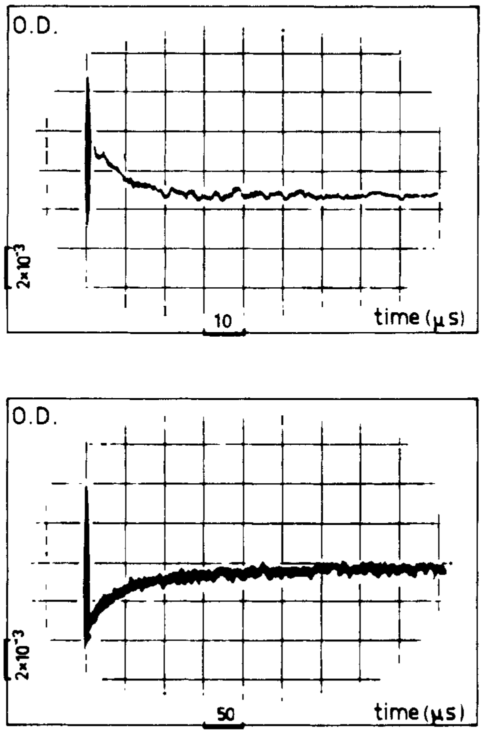


Figure 1. T-Jump relaxation spectra of malvin in aqueous solution: analytical concentration = 1.2 × 10^{-3} M; initial temperature = 3.9 °C; final temperature = 6.4 °C. Upper part: wavelength = 570 nm; pH 3.75; vertical scale 2 × 10^{-3} ODU per division; horizontal scale 10 µs per division. Lower part: wavelength = 525 nm; pH 4.47; vertical scale 2 × 10^{-3} ODU per division; horizontal scale 50 µs per division.

ferent from that predicted by mechanism I for the flavylium cation–carbinol equilibrium. The main objective of the present work was to establish the general mechanism of anthocyanins structural transformations in acidic media. We have used the chemical relaxation method[8] and chosen for study malvin chloride (R_1 = R_2 = methoxy), this being one of the commonest anthocyanins.

Experimental Section

The purity of commercial malvin chloride (Roth) was checked by paper chromatography in butanol/acetic acid/water mixtures by published procedures.[9] Kinetic and thermodynamic runs were performed in aqueous solutions with ionic strength 0.2 M adjusted by the addition of KNO_3 (Merck suprapur).

Kinetic Measurements. The fast relaxation phenomenon was recorded by means of a T-jump apparatus (Messanlagen Studiengesellschaft) as described previously.[10] Since the initial temperature in the T-jump cell was 4 °C and the magnitude of the temperature jump was 2.5 °C, the relaxation constants thus measured are for a temperature of 6.5 (± 0.5) °C.

Slow relaxation measurements were performed on a Cary 16 UV spectrometer fitted with a thermostatted sample cell. The equilibrium was perturbed by a sudden change in the hydronium ion concentration and the absorbance at the cation absorption maximum (520 nm) was recorded as a function of time. This pH-jump was generally performed by injecting a small amount of concentrated HCl or concentrated NaOH into the UV cell containing the malvin solution. Another technique was also used: Into an acidic solution X containing no malvin a small amount of an equilibrated malvin solution of acidity very different from that of solution X is injected via a microsyringe. After each test, the pH was directly measured in the UV sample cell. The relaxation constants thus measured are for 4 (± 0.5) °C.

pH Measurements. In both types of kinetic experiment (T-jump and pH-jump), the pH was measured with a Knick pH meter at 4 (± 0.5) °C. A combined electrode (Metrohm EA 125) was directly immersed in the UV cell. The buffered solutions used for pH-meter standardization were either pH 4.01 and 6.86 N.B.S. standards (Beckman) or 0.1 and 0.01 N hydrochloric acid solutions (Merck titrisol).

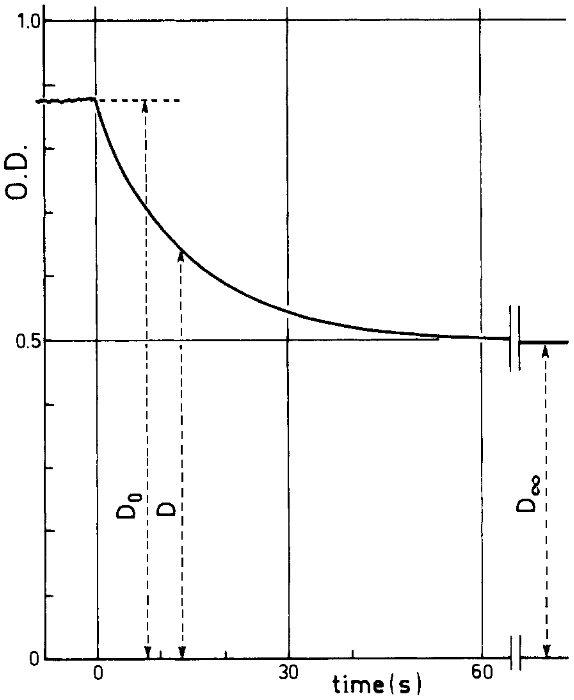


Figure 2. pH-Jump relaxation spectrum of malvin in aqueous solution: analytical concentration = 5.2 × 10^{-5} M; temperature = 4.1 °C; initial pH 1.76; final pH 2.20; wavelength = 520 nm; optical path = 1 cm; D_0, initial optical density; D, optical density at time t; D_∞, optical density at infinity.

Equilibrium Measurements. The equilibrium measurements were carried out at 4 (± 0.5) °C by absorption spectroscopy using a Cary 15 spectrometer fitted with a thermostatted sample cell.

Results

Until this study there were no kinetic data for the structural transformations of anthocyanins.[11] However, they have been qualified as "fast", "immediate", and even "instantaneous" reactions.[6b,12]

Our results allowed us to show that, in a pH range 1–6, there is a relaxation spectrum made up of two kinetically distinct processes. When a malvin solution is submitted to a very fast temperature jump ($\simeq 10^{-6}$ s) and the optical density is recorded as a function of time, one obtains a relaxation curve characterizing the fastest process (Figure 1). If this same solution is then submitted to a suitable pH-jump, in addition to the fast process, a second and much slower relaxation process is observed (Figure 2).[13]

The Fast Relaxation. The upper part of Figure 3 gives the difference with respect to the wavelength between the values of the extinction coefficients of the flavylium cation and the anhydro base. The lower part of the figure shows the variation of the amplitude of the fast relaxation with the wavelength. The amplitude of the fast relaxation process is directly related to the difference between the molecular extinction coefficients of the two forms of malvin leading to the fast equilibrium. The similarity of the two differential spectra thus obtained indicates that the fast relaxation process is simply due to the acid–base equilibrium between the flavylium cation and the anhydro base. When the temperature is increased, this equilibrium shifts from the base toward the acidic cation, indicating that the neutralization of the anhydro base by the hydronium ion is endothermic. The rate of this shift is sensitive to the acidity and increases as the acidity increases. The plot of τ_I^{-1} against hydronium ion concentration is linear (Figure 4) and can be expressed by eq 3.

$$\tau_I^{-1}(s^{-1}) = 1.8 \times 10^4 + 1.4 \times 10^8[H^+] \qquad (3)$$

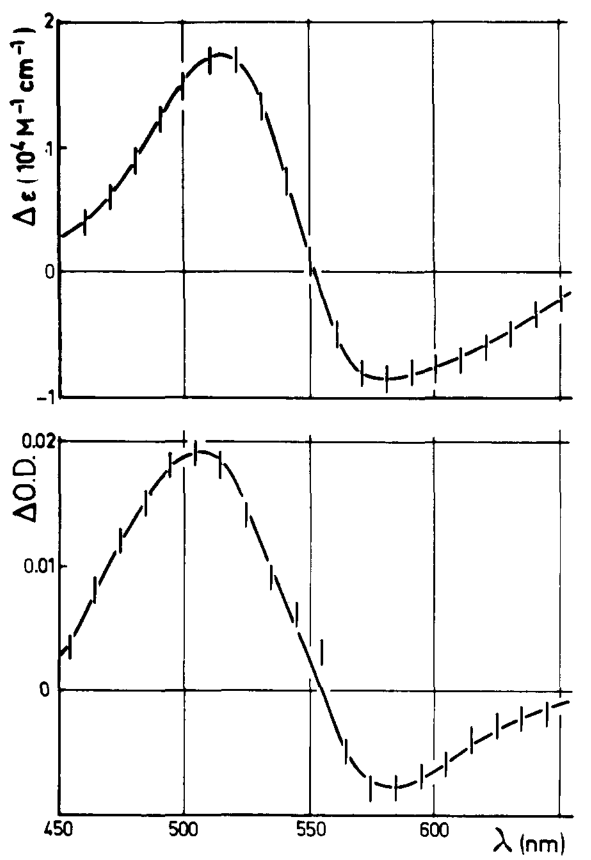


Figure 3. Upper part: $\Delta\epsilon = (\epsilon_{AH^+} - \epsilon_A)$ vs. wavelength; $T = 4\ (\pm\ 0.5)$ °C. Lower part: T-Jump induced variations in optical density of a malvin solution vs. the wavelength; initial temperature = 4 °C; final temperature = 8 °C; pH 3.46.

The Slow Relaxation. After a pH-jump, the slow equilibration is a pseudo-first-order process. Under these conditions, since a large perturbation can be applied to the system, an excellent signal-to-noise ratio is obtained (Figure 2). For each kinetic run τ_2^{-1} was obtained by plotting log $(D_0 - D_\infty)/(D - D_\infty)$ vs. time. Figure 5 shows the variation of τ_2^{-1} with hydronium ion concentration. It is clear that the slow equilibration rate depends strongly on the acidity; the greater the acidity, the greater the rate.

We are now able to quantitatively explain observations reported in previous studies. For malvin it is known that the flavylium cation is red, that the anhydro base is blue, and that the carbinol is colorless. If the acidity of a colorless solution previously equilibrated at pH 5-6 is modified so that the new acidity value is pH 1-2, the red color of the flavylium cation appears without delay. This agrees well with our measurement of τ_2: if $[H^+] = 0.1$ N, then $\tau_2 = 3.3$ s at 4 °C and 0.6 s at 20 °C. Thus for temperatures close to room temperature, the time required for the appearance of the red color is about the same as that for mixing if the mixing is not performed in the quickest possible way. What happens if the opposite operation is attempted, i.e., going from a highly acidic red medium to a moderately acidic and colorless one? For pH values between 3 and 4, the rate of equilibration, although slower, is still high since τ_2 varies between 30 and 70 s from pH 3 to 4. If the acidity is further decreased (pH 5-6), the τ_2 values become very large ($\simeq 10^3$ s) and there is time enough to record the whole anhydro-base absorption spectrum by conventional means.

The variation of τ_2^{-1} with the acidity does not obey any simple relationship; depending on the pH, three distinct cases can be observed. For the most acidic media (Figure 5a) and the least acidic media (Figure 5c), the acidity dependence is linear as expressed by eq 4 and 5, respectively.

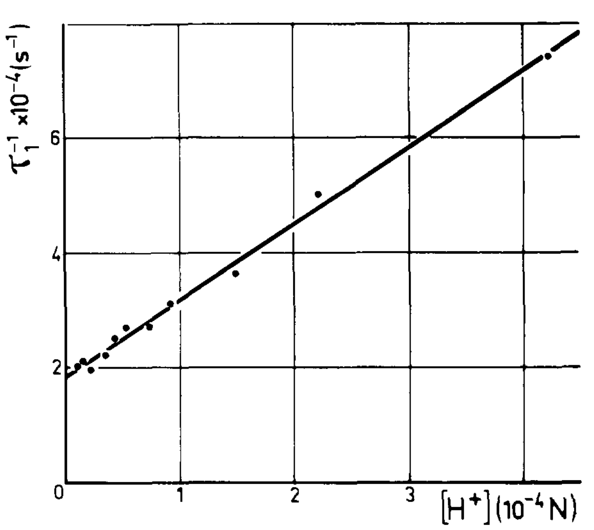


Figure 4. Plot of τ_1^{-1} vs. hydronium ion concentration. Analytical concentration = 1.2×10^{-3} M; initial temperature = 4 °C; final temperature = 6.5 °C; wavelength = 485, 495, 505, and 525 nm; intercept = 1.8 ($\pm$ 0.1) $\times\ 10^4$ s^{-1}; slope = 1.4 ($\pm$ 0.1) $\times\ 10^8$ M^{-1} s^{-1}; $r = 0.996$.

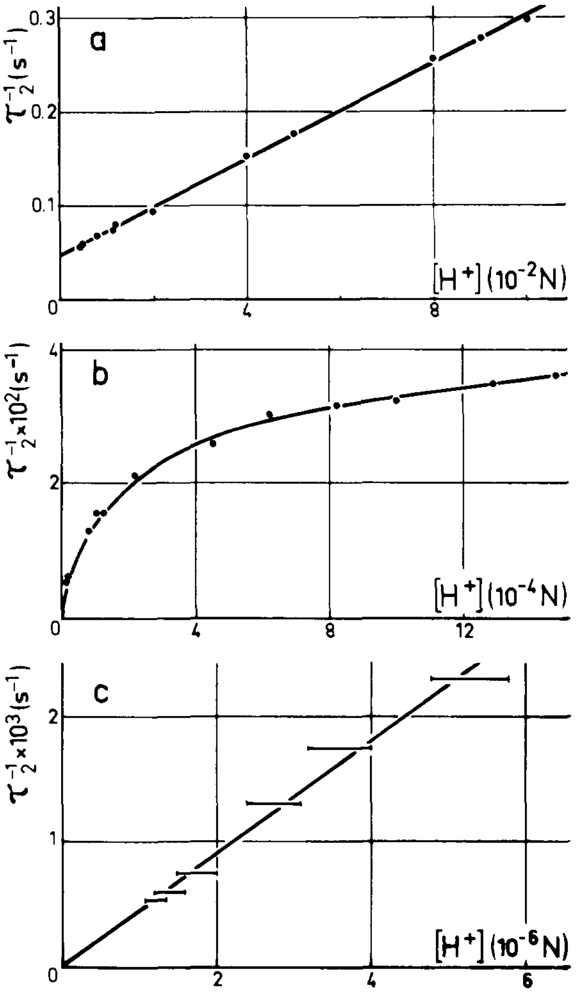


Figure 5. Plot of τ_2^{-1} versus hydronium ion concentration. $T = 4\ (\pm\ 0.5)$ °C. (a) $[H^+] \gg K_{12}$: intercept = 4.7 ($\pm$ 0.2) $\times\ 10^{-2}$ s^{-1}; slope = 2.6 ($\pm$ 0.1) M^{-1} s^{-1}; $r = 0.999$. (b) $[H^+] \simeq K_{12}$. (c) $[H^+] \ll K_{12}$: intercept = 0; slope = 4.5 ($\pm$ 0.5) $\times\ 10^2$ M^{-1} s^{-1}. $K_{12} = 1.1 \times 10^{-4}$ M at 4 °C. In case (c) the acidity is measured immediately before and after each kinetic run.

$$\tau_2^{-1}(\mathrm{s}^{-1}) = 4.7 \times 10^{-2} + 2.6[H^+] \qquad (4)$$

$$\tau_2^{-1}(\mathrm{s}^{-1}) = 4.5 \times 10^2[H^+] \qquad (5)$$

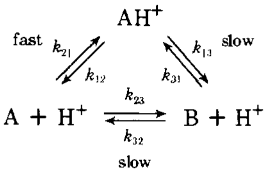
For moderately acidic media (Figure 5b) the dependence is apparently complicated and shows that there is a transition zone between the two linear relationships. It thus well explains the large change in slope (from 2.6 to 4.5 × 10^2 M^{-1} s^{-1}) observed between eq 4 and 5.

Discussion

(A) Mechanistic Considerations. The least complicated complete mechanism able to account for the flavylium cation–carbinol equilibrium will be a triangular one (mechanism II, eq 6) characterized by the existence of two parallel pathways: one which we call direct and one involving the anhydro base as an intermediate. Other mechanisms (I, eq 2; and III, eq 21) will appear as special cases of the triangular one.

(a) Formation of B by Two Competitive Pathways.[14]

Mechanism II



(6)

Insofar as the three species AH^+, A, and B are present in detectable concentrations, mechanism II (eq 6) will be characterized by two relaxation times.[15] Linearized rate equations are given by eq 7:

$$-dx_1/dt = a_{11}x_1 + a_{12}x_2$$

$$-dx_2/dt = a_{21}x_1 + a_{22}x_2 \qquad (7)$$

where:

$$x_1 = \Delta[AH^+] \qquad (8)$$

$$x_2 = \Delta[B] \qquad (9)$$

$$a_{11} = k_{12} + k_{21}([A] + [H^+]) + k_{13} + k_{31}[B] \qquad (10)$$

$$a_{12} = (k_{21} - k_{31})[H^+] \qquad (11)$$

$$a_{21} = k_{23} - k_{13} - k_{31}[B] \qquad (12)$$

$$a_{22} = k_{23} + k_{32} + k_{31}[H^+] \qquad (13)$$

Eq 14 is the characteristic equation:

$$\begin{vmatrix} a_{11} - \tau^{-1} & a_{12} \\ a_{21} & a_{22} - \tau^{-1} \end{vmatrix} = 0 \qquad (14)$$

The reciprocals of the relaxation times obey eq 15 and 16, where τ_1 represents the relaxation constant of the faster process and τ_2 that of the slower one.

$$\tau_1^{-1} + \tau_2^{-1} = a_{11} + a_{22} \qquad (15)$$

$$\tau_1^{-1} \times \tau_2^{-1} = a_{11}a_{22} - a_{12}a_{21} \qquad (16)$$

A simplification is possible here because the acid-base equilibrium between the flavylium cation and the anhydro base is very fast compared to the two hydrolysis equilibria. This leads to $a_{11} \gg a_{22}$. Moreover, it is easy to show that: the last two terms in eq 10 are negligible relative to the first two terms ($k_{12}, k_{21} \gg k_{13}, k_{31}$); a_{12} reduces to $k_{21}[H^+]$; $[H^+] \gg [A]$, at most, [A] represents only a very small fraction of the analytical concentration.

Consequently, if the triangular mechanism is the correct one, the measured relaxation times should obey eq 17 and 18:

$$\tau_1^{-1} = k_{12} + k_{21}[H^+] \qquad (17)$$

$$\tau_2^{-1} = k_{32} + k_{23}\left(\frac{K_{12}}{K_{12} + [H^+]}\right) + k_{13}\left(\frac{[H^+]}{K_{12} + [H^+]}\right) + k_{31}[H^+]\left(1 + \frac{[B]}{K_{12} + [H^+]}\right) \qquad (18)$$

where:

$$K_{12} = k_{12}/k_{21} \qquad (19)$$

Equation 18 is the sum of four terms. Each term corresponds to the particular contribution of each of the slow reactions to the overall slow equilibration process.

(b) B is Formed Through A only. As already pointed out, mechanism I (eq 2) is the generally accepted one. The kinetic expression for the faster process is identical with that of eq 17. The reciprocal slow relaxation time for this mechanism is given by eq 20, obtained from eq 18 by setting $k_{13} = k_{31} = 0$.

$$\tau_2^{-1} = k_{32} + k_{23}\left(\frac{K_{12}}{K_{12} + [H^+]}\right) \qquad (20)$$

(c) B is Formed by the Direct Reaction only.

Mechanism III

$$A + H^+ \underset{\underset{\text{fast}}{k_{12}}}{\overset{k_{21}}{\rightleftarrows}} AH^+ \underset{\underset{\text{slow}}{k_{31}}}{\overset{k_{13}}{\rightleftarrows}} B + H^+ \qquad (21)$$

The kinetic originality of mechanism III (eq 21) lies in the fact that the slow hydrolysis equilibrium of the flavylium cation is coupled with the fast proton transfer equilibrium between the same cation and the anhydro base. The reciprocal fast relaxation time for this mechanism is given by eq 17. Equation 22 which expresses τ_2^{-1} as a function of the hydronium ion and the carbinol concentrations is obtained from eq 18 by setting $k_{23} = k_{32} = 0$.

$$\tau_2^{-1} = k_{13}\left(\frac{[H^+]}{K_{12} + [H^+]}\right) + k_{31}[H^+]\left(1 + \frac{[B]}{K_{12} + [H^+]}\right) \qquad (22)$$

When no anhydro base is present, eq 22 reduces to eq 23. This means either that $K_{12} = 0$ for compounds not forming an anhydro base or that $[H^+] \gg K_{12}$.

$$\tau_2^{-1} = k_{13} + k_{31}([B] + [H^+]) \qquad (23)$$

(B) The Mechanism of Structural Transformations of Anthocyanins in Acidic Media. (a) The Fast Relaxation. τ_1 is related to the setting up of the equilibrium between the flavylium cation and the anhydro base.[16] According to eq 3, the rate constant for deprotonation of the cation by the solvent k_{12} is 1.8 (± 0.1) × 10^4 s^{-1}; the rate constant k_{21} of the neutralization of the anhydro base by the proton is 1.4 (± 0.1) × 10^8 M^{-1} s^{-1}. This latter value is much lower than that expected for a reaction whose rate is diffusion controlled ($k_{21} \simeq 10^{10}$ M^{-1} s^{-1}). This result may be related to the major structural reorganization which occurs when a quinonoidal structure changes to a flavylium one.[17] The equilibrium constant K_{12} is 1.3 (± 0.2) × 10^{-4} M at 6.5 (± 0.5) °C and is typical of the first pK of the flavylium cation (pK_{12} = 3.9). This value is in good agreement with those measured for some flavylium cations hydroxylated at position 7 or 4′ or at both positions at the same time.[7] We shall show that it is absolutely necessary to know K_{12} if one is to understand the slow relaxation process.

Showing that there is a fast acid-base equilibrium between AH^+ and A does not allow us to distinguish among the postulated mechanisms I, II, and III.

(b) The Slow Relaxation. If we examine the slow equilibration (Figure 5), we can see that the theoretical expression found in eq 20 cannot in any way account for the experimental data. *Thus mechanism I (eq 2) is not the mechanism behind the structural transformations of anthocyanins in acidic media.* It is clear that only eq 18 and 22 are likely to be in agreement with the experimental facts. We shall consider two extreme cases depending on the acidity: $[H^+] \gg K_{12}$ and $[H^+] \ll K_{12}$.

For $[H^+] \gg K_{12}$ (Figure 5a), eq 18 becomes eq 24 and eq 22 becomes eq 25, assuming that $[B] \ll [H^+]$.

$$\tau_2^{-1} = k_{32} + \frac{k_{23}K_{12}}{[H^+]} + k_{13} + k_{31}[H^+] \tag{24}$$

$$\tau_2^{-1} = k_{13} + k_{31}[H^+] \tag{25}$$

If this theoretical result is compared to eq 4, one can see that mechanism III (eq 21) correctly describes the structural transformations of malvin for the most acidic media. The triangular mechanism (eq 6) is also compatible insofar as k_{32} and $k_{23}K_{12}/[H^+]$ are negligible relative to k_{13} and $k_{31}[H^+]$. By identification $k_{13} = 4.7\ (\pm\ 0.2) \times 10^{-2}\ s^{-1}$ and $k_{31} = 2.6\ (\pm\ 0.1)\ M^{-1}\ s^{-1}$ at 4 (± 0.5) °C. Thus the value of the constant for the flavylium cation-carbinol equilibrium is $K'_{13} = (k_{13}/k_{31}) \times \gamma_{H^+} = 1.4\ (\pm\ 0.3) \times 10^{-2}$ M, where γ_{H^+} is the activity coefficient of the hydronium ion. Expressed as a logarithm this becomes: $pK'_{13} = 1.85$ at 4 (± 0.5) °C. This value is in excellent agreement with that determined by spectroscopic equilibrium measurements at the same temperature: $pK'_{13} = 1.86\ (\pm\ 0.02)$. Moreover, Timberlake and Bridle[6c] reported a value of 1.91 (± 0.07) for the same equilibrium at 20 °C and 0.1 M ionic strength ($NaClO_4$). The following partial conclusion can be drawn; for $[H^+] \simeq K'_{13}$, the interconversion of the flavylium cation and the carbinol takes place by *the direct reaction only*.

Now that the existence of the direct pathway is firmly established, it remains to be seen if it coexists with the hydration of the anhydro base or if this hydration reaction does not exist at all. This can be done by examining the second extreme case.

For $[H^+] \ll K_{12}$ (Figure 5c),[18] eq 18 reduces to 26 and eq 22 to eq 27:

$$\tau_2^{-1} = k_{32} + k_{23} + \left(\frac{k_{13}}{K_{12}} + \beta k_{31}\right)[H^+] \tag{26}$$

$$\tau_2^{-1} = \left(\frac{k_{13}}{K_{12}} + \beta k_{31}\right)[H^+] \tag{27}$$

where:

$$\beta = \frac{K_{12} + [B]}{K_{12}} \simeq 1 \tag{28}$$

Both expressions are linear in $[H^+]$ with a slope practically equal to k_{13}/K_{12} (the term βk_{31} is negligible) and the intercepts are respectively $k_{32} + k_{23}$ for eq 26 and zero for eq 27. Inspection of Figure 5c reveals that in the least acidic range eq 27 is in agreement with the experimental relationship 5.[19] By identification one obtains $\alpha = k_{13}/K_{12} = 4.5\ (\pm\ 0.5) \times 10^2\ M^{-1}\ s^{-1}$. From the values of α and k_{13}, we obtain a second estimate of the K_{12} value: $K_{12} = 1.1\ (\pm\ 0.2) \times 10^{-4}$ M at 4 (± 0.5) °C. This latter value is in good agreement with that measured by the T-jump technique.

The case for which $[H^+] \simeq K_{12}$ (Figure 5b) remains to be examined. The variation of τ_2^{-1} as a function of hydronium ion concentration is complex and corresponds to unsimplified eq 22. When the acidity is progressively lowered, the second term of eq 22 rapidly vanishes and τ_2^{-1} thus reduces to $k_{13}[H^+]/(K_{12} + [H^+])$.

(c) Anthocyanins Structural Transformations in Acidic Media Follow Mechanism III. Taking malvin as an example, we have demonstrated that the flavylium cation-carbinol equilibrium occurs directly without going through the anhydro base and that it is even erroneous to label it as "anhydro" insofar as this implies the addition of water in a reaction which we have shown does not occur. We wish to point out that when a suitable pH-jump occurs, the readily formed quinonoidal base is the *kinetic reaction product* and that this kinetic product turns, through the cation yielding it, to the carbinol which is the *thermodynamic reaction product*. Rate and equilibrium constants associated with this mechanism are reported in Table I.

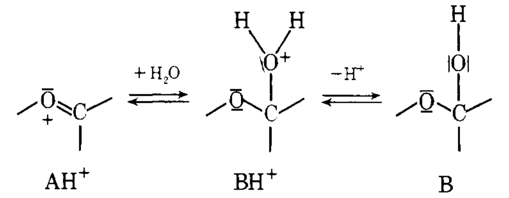
Table I. Rate and Equilibrium Constants[a] for the Structural Transformations of Malvin in Aqueous Acidic Medium at Ionic Strength 0.2 M (KNO_3)

$k_{12} = 1.8\ (\pm\ 0.1) \times 10^4\ s^{-1}$ [b]	
$k_{21} = 1.4\ (\pm\ 0.1) \times 10^8\ M^{-1}\ s^{-1}$ [b]	$K_{12} = 1.3\ (\pm\ 0.2) \times 10^{-4}$ M [b]
$k_{13} = 4.7\ (\pm\ 0.2) \times 10^{-2}\ s^{-1}$ [c]	
$k_{31} = 2.6\ (\pm\ 0.1)\ M^{-1}\ s^{-1}$ [c]	$K'_{13} = 1.4\ (\pm\ 0.3) \times 10^{-2}$ M [c]
$K_{23} = [B]/[A] = 1.6\ (\pm\ 0.5) \times 10^2$ [c,d]	

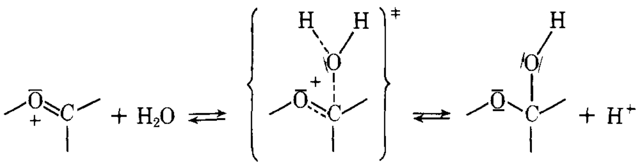
[a] Cf. text for definitions. [b] $T = 6.5$ °C. [c] $T = 4$ °C. [d] The stability of the quinonoidal base A depends only on the ratio K_{12}/K'_{13}.

The slowing down of the disappearance of the quinonoidal base with pH increase has often been rationalized on the basis of the formation of anion A^- which is reputed to be more stable than A.[6a] This interpretation can no longer be considered as valid; it is the kinetic nature (see τ_2^{-1}) of both coupled reactions which accounts for this slowing down. In the light of our results some conclusions should be revised, in particular those of Sperling, Werner, and Kuhn[7] who use base A as the cornerstone of their reaction scheme while ignoring the flavylium cation hydration reaction.

(C) Elementary Steps for the Flavylium Cation–Carbinol Equilibrium. Equilibrium 1 looks like an acid–base equilibrium. However, it is not a simple proton transfer reaction. Two closely linked mechanisms are possible. First, it might be thought that there is nucleophilic addition of the solvent at position 2, thus leading to protonated carbinol BH^+. This protonated species would be highly unstable and would yield carbinol B by transfer of a proton to the solvent molecule (eq 29). The essential characteristic of this two-step mechanism is the separation in time of the proton transfer from the breaking (or making) of the hydroxyl group C–O bond. We must recognize that we have no evidence at all for the existence of BH^+. An alternative to this mechanism is given by a mechanism (eq 30) in which the two elementary acts, i.e., proton transfer and C–O bond breaking (or making), are synchronized. This is a concerted process which takes place without an intermediate state.



(29)



(30)

Therefore general acid–base catalysis could be effective for the flavylium cation–carbinol interconversion. As we have already pointed out, buffer effects on the equilibration kinetics can be important, especially for the least acidic media.[20]

This mechanism is clearly analogous with that of the hydrolysis of hemiacetals, acetals, ketals, and ortho esters,[21] as well as with the hydrolysis of the *N,O*-trimethylenephthalimidium cation.[22]

Conclusion

Chemical relaxation appears to be an efficient method for investigating the kinetics of the structural transformations of anthocyanins and of flavylium cations in general. In acidic medium the experimental results have usually been interpreted in terms of the following reactions: an acid-base equilibrium between the flavylium cation and the quinonoidal base and a hydration equilibrium of the latter to give the carbinol. Not having been based on quantitative kinetic results, the validity of this mechanism has never been demonstrated. The analysis of the chemical relaxation spectrum of malvin in acidic medium (one relaxation time in the microsecond range and another in the second range) confirms the existence of the fast proton transfer equilibrium. On the other hand, the hydration step does not take place via the quinonoidal base but rather the flavylium cation.

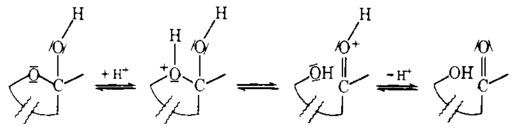
Consequently, the currently held mechanism must be considered in error and replaced by the only mechanism which we have shown to be consistent with the relaxation kinetics and results obtained prior to this work. The equilibrium between the two neutral forms, the quinonoidal base and the carbinol, is, according to the new mechanism, established exclusively by the flavylium cationic intermediate. The hydration of this cation is specific to flavylium cations; it leads directly to the carbinol which possesses a hemiacetal function. The proton transfer reaction is specific to anthocyanins as well as to flavylium cations bearing an acidic hydroxyl group.

Note Added in Proof. We have just succeeded in demonstrating the existence of chalcone form (C) by increasing the temperature so as to favor the chalcone at the expense of the carbinol. For malvin, the amount of chalcone at equilibrium at 4 °C is about 3% relative to carbinol. A paper giving full details of this exciting finding will be submitted for publication to this journal.

Acknowledgments. We would like to express our appreciation to M. Dreyfus, P. Fellmann, and Dr. J. S. Lomas for helpful discussions. The authors are grateful to J. Y. Dugast for technical assistance.

References and Notes

(1) R. Willstätter and A. E. Everest, *Justus Liebigs Ann. Chem.*, **401**, 189 (1913).
(2) D. D. Pratt and R. Robinson, *J. Chem. Soc.*, 1577 (1922).
(3) F. M. Dean, "Naturally Occurring Oxygen Ring Compounds", Butterworths, London, 1963, p 388.
(4) E. Sondheimer, *J. Am. Chem. Soc.*, **75**, 1507 (1953).
(5) L. Jurd, *J. Org. Chem.*, **28**, 987 (1963).
(6) (a) L. Jurd and T. A. Geissman, *J. Org. Chem.*, **28**, 2394 (1963); (b) L. Jurd and S. Asen, *Phytochemistry*, **5**, 1263 (1966); (c) C. F. Timberlake and P. Bridle, *J. Sci. Food Agric.*, **18**, 473 (1967); (d) K. A. Harper and B. V. Chandler, *Aust. J. Chem.*, **20**, 745 (1967); (e) L. Jurd, "The Chemistry of Plant Pigments", C. O. Chichester, Ed., Academic Press, New York, N.Y., 1972, p 123.
(7) W. Sperling, F. C. Werner, and H. Kuhn, *Ber. Bunseges. Phys. Chem.*, **70**, 530 (1966).
(8) M. Eigen and L. De Maeyer, "Technique of Organic Chemistry", Vol. 8, A. Weissberger, Ed., Interscience, New York, N.Y., 1963.
(9) E. C. Bate-Smith and R. G. Westall, *Biochem. Biophys. Acta*, **4**, 427 (1950).
(10) M. Dreyfus, G. Dodin, O. Bensaude, and J. E. Dubois, *J. Am. Chem. Soc.*, **97**, 2369 (1975).
(11) Kinetic and thermodynamic results of Sperling, Werner, and Kuhn[7] concerned synthetic flavylium cations and not natural products. The classical mechanism (eq 2) accepted by these authors is refuted by our work.
(12) C. F. Timberlake and P. Bridle, *Nature (London)*, **212**, 158 (1966); S. Asen, K. H. Norris, and R. N. Stewart, *Phytochemistry*, **8**, 653 (1969); T. C. Somers, *ibid.*, **10**, 2175 (1971).
(13) The rapid phenomenon can only be measured by T-Jump; the slow phenomenon can be measured by a mixing technique.
(14) For the sake of clarity, we have decided to represent the solvated proton by H^+ and to omit the water molecule in the transformations where it intervenes. In the kinetic equations [A], [B], and $[H^+]$ refer to the equilibrium state.
(15) M. Eigen, *Q. Rev. Biophys.*, 1, 3 (1968).
(16) Jurd and Geissman[6a] have shown that only the quinonoidal base related to the ionization of the hydroxyl group at position 7 was formed. Our spectroscopic measurements agree well with this result; we have therefore not considered the existence of a second quinonoidal base resulting from the ionization of the hydroxyl group at position 4′.
(17) M. Eigen, *Angew. Chem., Int. Ed. Engl.*, **3**, 1 (1964).
(18) $[H^+] \ll K_{12}$ falls within the acidity limits pH 1-6.
(19) For such low values of the medium acidity, it would have seemed preferable to use buffered solutions. Indeed the acidity could have been precisely controlled, thereby avoiding the fluctuations appearing in Figure 5c. However, as we shall see further on, the flavylium cation-carbinol equilibrium is likely to be general acid-base catalyzed. If this is the case, then for a given hydronium ion concentration there will be buffer-dependent rate increase and the values obtained for τ_2^{-1} will be abnormally high.
(20) It is well known that some flavylium cations, particularly those not substituted at position 3, give rise to an equilibrium similar to 1. However, as a result of the pyrylium ring opening, a chalcone (C) instead of a carbinol is formed.[5] Since the carbinol is at the origin of the chalcone formation, it can be reasonably supposed that a mechanism similar to 29 or 30 governs this formation. This mechanism is in every way like the one for the acidocatalyzed formation of a hemiacetal from a ketone.[21] In our case it appears as an intramolecular reaction giving rise to a prototropic ring-chain tautomerism. We can thus plausibly explain why anthocyanins do not form an appreciable quantity of chalcone product. The O-glucose group at position 3 would strongly stabilize the carbinol at the expense of the chalcone. Consequently, there can be traces of chalcone in acidic anthocyanin solutions and this same form, although present in very small amounts, can be under certain conditions the form in which the compound will react. This is surely what occurs with anthocyanidins (OH at position 3) whose chalcone form is an unstable α-diketone which finally leads to decomposition of the molecule. It is noteworthy that prior synthesis of a chalcone form is a general pathway to flavylium cations.



B

C

(21) E. H. Cordes and H. G. Bull, *Chem. Rev.*, **74**, 581 (1974).
(22) N. Gravitz and W. P. Jencks, *J. Am. Chem. Soc.*, **96**, 489 (1974).