Chemistry of Anthocyanin Pigments. 3.[1] Relaxation Amplitudes in pH-Jump Experiments

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Abstract: A method for determining the equilibrium constants of the proton transfer, hydration, and tautomeric reactions of the structural transformations of anthocyanins in aqueous acidic media is described. This method is based on the measurement of pH-jump induced relaxation amplitudes. The technique is extremely simple and does not require sophisticated relaxation equipment. For the two pigments studied (malvidin 3-glucoside and malvidin 3,5-diglucoside), there is excellent agreement between the calculated and observed amplitudes. This new method for measuring the equilibrium constants of a complex system whose position is pH dependent is more precise and more direct than the usual methods. Relaxation by pH-jump is a seldom used technique. The most frequently used techniques (temperature, pressure, electric field jumps) are characterized by the shift of a state of chemical equilibrium brought about by modifying values of equilibrium constants. Nothing of the sort is used here; indeed, our experiments have been conducted at a constant temperature and pressure, and the equilibrium of the system is disturbed by increasing or decreasing the acidity of the medium. A significant advantage of this mode of perturbation is that, unlike the previously mentioned techniques, it does not produce any secondary physical effect, thereby avoiding an often present hindrance when determining amplitudes.

From the knowledge of relaxation amplitudes one can obtain the thermodynamic parameters of chemically equilibrated transformations.[2] When the perturbation is a temperature jump, reaction enthalpies are measured directly.[3] In this paper we shall describe a simple and efficient method for measuring the equilibrium constants associated with the structural transformations of anthocyanins in acidic aqueous media. This method is based on the theoretical evaluation of the relaxation amplitudes induced by appropriate pH jumps and on their measurement by means of an absorption spectrophotometer working in the visible range.[4] There is a perfect agreement between theory and experiment.[5]

We have recently shown[6] that Scheme I (eq 1, 2, and 3) expresses the general mechanism involved in the structural transformations of anthocyanins occurring in acidic aqueous media.

Scheme I

$$AH^+ \overset{K'_{12}}{\rightleftharpoons} A + H^+ \quad (1)$$

$$AH^+ \overset{K'_{13}}{\rightleftharpoons} B + H^+ \quad (2)$$

$$B \overset{K_{34}}{\rightleftharpoons} C \quad (3)$$

AH^+, A, B, and C represent respectively the flavylium cation, the quinoidal base, the carbinol, and the chalcone. We have shown[1] that this mechanism is characterized by a relaxation spectrum consisting of three relaxation times: $\tau_1^{-1} \gg \tau_2^{-1} \gg \tau_3^{-1}$. τ_1 is the relaxation time of the proton transfer equilibrium (eq 1), τ_2 of the hydration equilibrium (eq 2), and τ_3 of the tautomeric equilibrium (eq 3). Orders of magnitude for these relaxation times are given in ref 1 and 6. $K'_{12} = ([A]/[AH^+])a_{H^+}$; [A] and [$AH^+$] are the concentrations of the quinoidal base and the flavylium cation, respectively, when the proton transfer reaction is at equilibrium. $K'_{13} = ([B]/[AH^+])a_{H^+}$; [B] and [$AH^+$] are the concentrations of the carbinol and the cation, respectively, when the hydration reaction is at equilibrium. $K_{34} = [C]/[B]$; [C] and [B] are the concentrations for the chalcone and the carbinol, respectively, when the system is completely equilibrated. a_{H^+} is the hydronium ion activity.

Experimental Section

pH-Jump experiments in view of relaxation amplitude studies require only a classical absorption spectrophotometer, a reference cell, a thermostated sample cell fitted with a fast stirring device, and a microsyringe for injecting *very small* quantities of more or less concentrated acidic or basic solutions. pH-Jump relaxation amplitudes for the structural transformations of anthocyanins in aqueous acidic media are measured in the following way.

Stock Solutions. For each stock solution, the required quantity of the carefully purified pigment[6] (1–2 mg for 100 mL of solvent) is dissolved in a suitably acidified (HCl) aqueous potassium nitrate (Merck suprapur) solution in order to adjust the ionic strength to 0.2 M. Buffered solutions are precluded since this would render the pH-jumps impossible. The initial pH values (pH_0) of the stock solutions ranged from about 2 to 6.

After the pigment is completely dissolved (vigorous magnetic stirring for a few hours), a stock solution is put into a thermostated bath, for a period of at least 10 h, so as to attain perfect equilibrium between the only four species, AH^+, A, B, and C, present at these pH values. A stock solution is then ready for use.

Amplitude and pH Measurements. The UV–visible spectrophotometer (Cary 16 or Cary 118) is fitted with a reference cell and a thermostated sample cell with a magnetic stirring device. At the top of the cell compartment, there is a narrow hole for direct injection of known amounts of acidic or basic solutions. The wavelength is set to a value near the absorption maximum (λ_{max}) of the flavylium cation (515–520 nm); at this wavelength, only the flavylium cation AH^+ and the quinoidal base A absorb. The amplitude is recorded by measuring the appearance or disappearance of both the highly colored forms AH^+ and A, whatever the relaxation amplitude studied.

An aliquot (2.50 mL) of a stock solution is put into the thermostated sample cell and the temperature is carefully controlled throughout the experiment. The initial pH value (pH_0) and the temperature are measured directly in the sample cell. The initial absorbance (D_0) is recorded, and the pH jump is achieved by injecting, into the sample cell, about 0.5–20 μL of a more or less concentrated acidic (HCl), neutral ($NaHCO_3$), or basic (NaOH) aqueous solution. The mixing time is about 0.5 s. The absorbance changes from the initial and no longer equilibrium value D_0 to the final equilibrium value D_f, in three kinetically distinct steps: firstly, D_0 becomes D_1 (the proton transfer equilibrium is then established at pH_f); secondly, at a much slower rate D_1 becomes D_2 (the hydration equilibrium is then reached); and, finally, D_2 very slowly becomes D_f (the overall equilibrium state at pH_f is then attained). Afterwards the final value of the pH (pH_f) is recorded in the same manner as previously.

It should be noted that, in these experiments, there is neither *noticeable dilution* (C_0 *remains constant*) nor, in general, any appreciable change in the ionic strength. When NaOH is used, any jump toward less acidic media does not affect the ionic strength at all, and with $NaHCO_3$, a negligible effect occurs; for jumps toward more acidic media, there is a significant change in the ionic strength of the solution only if $pH_f \lesssim 1$. However, our experiments have led us to consider this effect as insignificant.

Table I. Calculated and Observed Amplitudes

pigment	pH_0	pH_f	amplitude	calcd[a]	obsd
malvidin 3-glucoside (T = 25 °C; λ = 517.5 nm)	3.07	5.20	$D_0 - D_1$	0.08_8[b]	0.08_2[c]
	3.07	5.20	$D_1 - D_2$	0.11_2[b]	0.11_0[c]
	3.90	1.07	$D_2 - D_f$	-0.07_6[d]	-0.07_0[e]
malvidin 3,5-diglucoside (T = 4 °C; λ = 520 nm)	1.76	2.20	$D_1 - D_2$	0.35_5[f]	0.37_5[g]

[a] For malvidin 3-glucoside at 25 °C and 517.5 nm: $K'_{12} = 5.7 \times 10^{-5}$ M, $K'_{13} = 2.5 \times 10^{-3}$ M, $K_{34} = 0.12$, $\epsilon_{AH^+} = 27\,000$ M^{-1} cm^{-1}, and $\epsilon_A = 14\,000$ M^{-1} cm^{-1} (see ref 1). For malvidin 3,5-diglucoside at 4 °C and 520 nm: $K'_{12} = 9 \times 10^{-5}$ M, $K'_{13} = 1.4 \times 10^{-2}$ M, and $K_{34} = 0.03$ (see ref 6); $\epsilon_{AH^+} = 29\,000$ M^{-1} cm^{-1}. [b] $l = 1$ cm; $C_0 = 3.3 \times 10^{-5}$ M (caption, Figure 1 of ref 1). [c] Measured from Figure 1 of ref 1. [d] $l = 1$ cm; $C_0 = 3 \times 10^{-5}$ M (caption, Figure 7 of ref 1). [e] Measured from Figure 7 of ref 1 (in order to obtain $D_2 - D_f$ extrapolation to time $t = 0$ is necessary). [f] $l = 1$ cm; $C_0 = 5.2 \times 10^{-5}$ M (caption, Figure 2 of ref 6). [g] Measured from Figure 2 of ref 6.

Examples of amplitudes measured in this way are found in Table I, and detailed figures are given in ref 1 and 6.

Results and Discussion

Given the complexity of the system studied (Scheme I), a temperature-jump study of the amplitudes would be both difficult and lacking in precision. In contrast, the theoretical treatment of pH-jump studies is easier and more rigorous and, moreover, experimental accuracy is satisfactory, as indicated by the results shown in Table I.

Concentration Changes of AH^+, A, B, and C for the Different Equilibration Steps (τ_1, τ_2, τ_3) after a pH Jump from pH_0 ($a^0_{H^+}$) to pH_f ($a^f_{H^+}$). After the pH jump has occurred the evolution of the system from its initial state ($[AH^+]_0$, $[A]_0$, $[B]_0$, and $[C]_0$)[7] to its final state ($[AH^+]_f$, $[A]_f$, $[B]_f$ and $[C]_f$) takes place in three kinetically distinct steps. In the first step $[AH^+]_0$ and $[A]_0$ become $[AH^+]_1$ and $[A]_1$ (the proton transfer reaction is then equilibrated) with $[B]_1 = [B]_0$ and $[C]_1 = [C]_0$. In the second step, $[AH^+]_1$, $[A]_1$, and $[B]_0$ become $[AH^+]_2$, $[A]_2$, and $[B]_2$ (the hydration equilibrium is established) with $[C]_2 = [C]_1 = [C]_0$. Finally in the last step $[AH^+]_2$, $[A]_2$, $[B]_2$, and $[C]_0$ become $[AH^+]_f$, $[A]_f$, $[B]_f$, and $[C]_f$. The expression of $[AH^+]_0$ as a function of the initial acidity $a^0_{H^+}$ and the equilibrium constants is given by

$$[AH^+]_0 = (a^0_{H^+}C_0)/\delta_0 \tag{4}$$

with

$$\begin{aligned} C_0 &= [AH^+]_0 + [A]_0 + [B]_0 + [C]_0 \\ &= [AH^+]_1 + [A]_1 + [B]_0 + [C]_0 \\ &= [AH^+]_2 + [A]_2 + [B]_2 + [C]_0 \\ &= [AH^+]_f + [A]_f + [B]_f + [C]_f \end{aligned}$$

$$\delta_0 = K'_{12} + K'_{13}(1 + K_{34}) + a^0_{H^+}$$

$[A]_0$, $[B]_0$, and $[C]_0$ are obtained from eq 4 and from the definitions of K'_{12}, K'_{13}, and K_{34}, a_{H^+} being equal to $a^0_{H^+}$. In a similar manner one calculates $[AH^+]_f$, $[A]_f$, $[B]_f$, and $[C]_f$, a_{H^+} being equal to $a^f_{H^+}$. $[AH^+]_1$ is given by eq 5 and $[A]_1$ by eq 6:

$$[AH^+]_1 = \frac{(K'_{12} + a^0_{H^+})a^f_{H^+}}{\delta_0(K'_{12} + a^f_{H^+})} C_0 \tag{5}$$

$$[A]_1 = \frac{K'_{12}}{a^f_{H^+}} [AH^+]_1 \tag{6}$$

$[AH^+]_2$, $[A]_2$, and $[B]_2$ are given by eq 7, 8, and 9, respectively:

$$[AH^+]_2 = \frac{(K'_{12} + K'_{13} + a^0_{H^+})a^f_{H^+}}{\delta_0(K'_{12} + K'_{13} + a^f_{H^+})} C_0 \tag{7}$$

$$[A]_2 = \frac{K'_{12}}{a^f_{H^+}} [AH^+]_2 \tag{8}$$

$$[B]_2 = \frac{K'_{13}}{a^f_{H^+}} [AH^+]_2 \tag{9}$$

Relaxation Amplitudes Associated with the Structural Transformations of Anthocyanins in Aqueous Acidic Media after a pH Jump. A_1, A_2, and A_3 are the amplitudes of the relaxation modes corresponding to the proton transfer reaction (eq 1), to the hydration reaction (eq 2), and to the tautomeric reaction (eq 3), respectively. Since the three relaxation times are well separated, the amplitudes will be independent of the relaxation times.[8] By definition we have

$$A_1 = [A]_0 - [A]_1 = -\{[AH^+]_0 - [AH^+]_1\}$$

$$A_2 = [B]_0 - [B]_2 = -\{([AH^+]_1 + [A]_1) - ([AH^+]_2 + [A]_2)\}$$

$$A_3 = [C]_0 - [C]_f = -\{([AH^+]_2 + [A]_2 + [B]_2) - ([AH^+]_f + [A]_f + [B]_f)\}$$

It is easy to show that

$$A_1 = \frac{K'_{12}(a^f_{H^+} - a^0_{H^+})C_0}{\delta_0(K'_{12} + a^f_{H^+})} \tag{10}$$

$$A_2 = \frac{K'_{13}(a^f_{H^+} - a^0_{H^+})C_0}{\delta_0(K'_{12} + K'_{13} + a^f_{H^+})} \tag{11}$$

$$A_3 = \frac{K'_{13}K_{34}(a^f_{H^+} - a^0_{H^+})C_0}{\delta_0\delta_f} \tag{12}$$

with $\delta_f = K'_{12} + K'_{13}(1 + K_{34}) + a^f_{H^+}$.

The proton transfer equilibrium being the fastest one and the hydration equilibrium being much faster than the tautomeric equilibrium, it is possible to record the complete relaxation spectrum by measuring the successive variations of the concentrations of the species AH^+ and/or A.[9] Of the four structures AH^+, A, B, and C, only AH^+ and A absorb in the visible range;[1,6] thus the measurement of relaxation amplitudes can be performed by means of visible absorption spectroscopy. The *observable spectroscopic amplitudes* corresponding to amplitudes A_1, A_2, and A_3 are given by eq 13, 14, and 15, respectively:

$$D_0 - D_1 = -(\epsilon_{AH^+} - \epsilon_A)lA_1 \tag{13}$$

$$D_1 - D_2 = -\frac{(\epsilon_{AH^+}a^f_{H^+} + \epsilon_A K'_{12})l}{K'_{12} + a^f_{H^+}} A_2 \tag{14}$$

$$D_2 - D_f = -\frac{(\epsilon_{AH^+}a^f_{H^+} + \epsilon_A K'_{12})l}{K'_{12} + K'_{13} + a^f_{H^+}} A_3 \tag{15}$$

The total observable spectroscopic amplitude $D_0 - D_f$ corresponding to the sum of the partial amplitudes (eq 13, 14, and 15) is given by

$$D_0 - D_f = \frac{a^0_{H^+} - a^f_{H^+}}{\delta_0\delta_f}\{\epsilon_{AH^+}[K'_{12} + K'_{13}(1 + K_{34})] - \epsilon_A K'_{12}\}lC_0 \tag{16}$$

ϵ_{AH^+} and ϵ_A are the molecular extinction coefficients of the flavylium cation and the quinoidal base, respectively, in the visible range 450–650 nm; l is the optical path length; D_0 is the initial absorbance for equilibrium at pH_0; D_1, D_2, and D_f are the absorbances when (1) the acid-base equilibrium is reached, (2) the hydration equilibrium is attained, and (3) the entire

Table II. Simplified Expressions for the Relaxation Amplitudes A_1, A_2, and A_3 and the Corresponding Absorbance Variations $D_0 - D_1$, $D_1 - D_2$, and $D_2 - D_f$ for Some Characteristic Values of the Initial (a^0_{H+}) and Final (a^f_{H+}) Acidities[a]

a^0_{H+}	a^f_{H+}	A_1/C_0	A_2/C_0	$\frac{D_1 - D_2}{lC_0}$	A_3/C_0	$\frac{D_2 - D_f}{lC_0}$
	$\simeq K'_{13}$	$\frac{K'_{12}(a^f_{H+} - a^0_{H+})}{a^0_{H+}(K'_{12} + a^f_{H+})}$	$-\frac{K'_{13}}{K'_{13} + a^f_{H+}}$	$\frac{\epsilon_{AH+}K'_{13}}{K'_{13} + a^f_{H+}}$	$-\frac{K'_{13}K_{34}}{K'_{13}(1 + K_{34}) + a^f_{H+}}$	$\frac{\epsilon_{AH+}a^1_{H+}K'_{13}K_{34}}{(K'_{13}[1 + K_{34}] + a^f_{H+})(K'_{13} + a^f_{H+})}$
>0.2 N	$\simeq K'_{12}$	$-\frac{K'_{12}}{K'_{12} + a^f_{H+}}$	-1	$\frac{(\epsilon_{AH+}a^f_{H+} + \epsilon_A K'_{12})}{K'_{12} + a^f_{H+}}$	$-\frac{K_{34}}{1 + K_{34}}$	$\frac{(\epsilon_{AH+}a^f_{H+} + \epsilon_A K'_{12})K_{34}}{K'_{13}(1 + K_{34})}$
	$\simeq 10^{-6}$ N	-1	-1	ϵ_A	$-\frac{K_{34}}{1 + K_{34}}$	$\frac{\epsilon_A K'_{12}K_{34}}{K'_{13}(1 + K_{34})}$
	>0.2 N	$\frac{K'_{12}}{K'_{13}(1 + K_{34}) + a^0_{H+}}$	$\frac{K'_{13}}{K'_{13}(1 + K_{34}) + a^0_{H+}}$	$-\frac{\epsilon_{AH+}K'_{13}}{K'_{13}(1 + K_{34}) + a^0_{H+}}$	$\frac{K'_{13}K_{34}}{K'_{13}(1 + K_{34}) + a^0_{H+}}$	$-\frac{\epsilon_{AH+}K'_{13}K_{34}}{K'_{13}(1 + K_{34}) + a^0_{H+}}$
$\simeq K'_{13}$	$\simeq K'_{12}$	$\frac{-K'_{12}a^0_{H+}}{(K'_{13}[1 + K_{34}] + a^0_{H+})(K'_{12} + a^f_{H+})}$	$\frac{-a^0_{H+}}{K'_{13}(1 + K_{34}) + a^0_{H+}}$	$\frac{(\epsilon_{AH+}a^f_{H+} + \epsilon_A K'_{12})a^0_{H+}}{(K'_{12} + a^f_{H+})(K'_{13}[1 + K_{34}] + a^0_{H+})}$	$\frac{-K_{34}a^0_{H+}}{(K'_{13}[1 + K_{34}] + a^0_{H+})(1 + K_{34})}$	$\frac{(\epsilon_{AH+}a^f_{H+} + \epsilon_A K'_{12})K_{34}a^0_{H+}}{K'_{13}(K'_{13}[1 + K_{34}] + a^0_{H+})(1 + K_{34})}$
	$\simeq 10^{-6}$ N	$\frac{-a^0_{H+}}{K'_{13}[1 + K_{34}] + a^0_{H+}}$	$\frac{-a^0_{H+}}{K'_{13}[1 + K_{34}] + a^0_{H+}}$	$\frac{\epsilon_A a^0_{H+}}{K'_{13}(1 + K_{34}) + a^0_{H+}}$	$\frac{K_{34}a^0_{H+}}{(K'_{13}[1 + K_{34}] + a^0_{H+})(1 + K_{34})}$	$\frac{\epsilon_A K'_{12}K_{34}a^0_{H+}}{K'_{13}(K'_{13}[1 + K_{34}] + a^0_{H+})(1 + K_{34})}$
	>0.2 N	$\frac{K'_{12}}{K'_{13}(1 + K_{34})}$	$\frac{1}{1 + K_{34}}$	$-\frac{\epsilon_{AH+}}{1 + K_{34}}$	$\frac{K_{34}}{1 + K_{34}}$	$-\frac{\epsilon_{AH+}K_{34}}{1 + K_{34}}$
$\simeq 10^{-6}$ N	$\simeq K'_{13}$	$\frac{K'_{12}}{K'_{13}(1 + K_{34})}$	$\frac{a^f_{H+}}{(K'_{13} + a^f_{H+})(1 + K_{34})}$	$-\frac{\epsilon_{AH+}a^f_{H+}}{(K'_{13} + a^f_{H+})(1 + K_{34})}$	$\frac{K_{34}a^f_{H+}}{(K'_{13}[1 + K_{34}] + a^f_{H+})(1 + K_{34})}$	$\frac{-\epsilon_{AH+}K_{34}(a^f_{H+})^2}{(K'_{13}[1 + K_{34}] + a^f_{H+})(K'_{13} + a^f_{H+})(1 + K_{34})}$
	$\simeq K'_{12}$	$\frac{K'_{12}a^f_{H+}}{K'_{13}(K_{12'} + a^f_{H+})(1 + K_{34})}$	$\frac{a^f_{H+}}{K'_{13}(1 + K_{34})}$	$-\frac{(\epsilon_{AH+}a^f_{H+} + \epsilon_A K'_{12})a^f_{H+}}{K'_{13}(K'_{12} + a^f_{H+})(1 + K_{34})}$	$\frac{K_{34}a^f_{H+}}{K'_{13}(1 + K_{34})^2}$	$-\frac{(\epsilon_{AH+}a^f_{H+} + \epsilon_A K'_{12})K_{34}a^f_{H+}}{(K'_{13}[1 + K_{34}])^2}$

[a] Whatever the values of a^0_{H+} and a^f_{H+} we always have $D_0 - D_1 = -(\epsilon_{AH+} - \epsilon_A)lA_1$. We have only considered here the case where $\epsilon_{AH+} \simeq \epsilon_A$. The terms $(\epsilon_{AH+}a^f_{H+} + \epsilon_A K'_{12})/(K'_{12} + a^f_{H+})$ and $(\epsilon_{AH+}a^f_{H+} + \epsilon_A K'_{12})/(K'_{12} + K'_{13} + a^f_{H+})$ represent the sum of the contributions of AH^+ and A, respectively, to the amplitudes $D_1 - D_2$ and $D_2 - D_f$. For either thermodynamic ($a^f_{H+} \gg K'_{12}$ or $a^f_{H+} \ll K'_{12}$) or spectroscopic ($\epsilon_{AH+} \gg \epsilon_A$ or $\epsilon_A \gg \epsilon_{AH+}$) reasons, one of the two terms $\epsilon_{AH+}a^f_{H+}$ and $\epsilon_A K'_{12}$ can be neglected.

system is at equilibrium at pH_f.[10] Calculated and observed amplitudes are given in Table I.

Determination of K'_{12}, K'_{13}, and K_{34} and the Absorption Spectra of AH^+, A, B, and C. The measurement of the amplitudes $D_0 - D_1$, $D_1 - D_2$, and $D_2 - D_f$ is remarkably simple and does not require a chemical relaxation apparatus. From eq 13–16 and the amplitude measurements, one can deduce very easily and with good precision not only the values of constants K'_{12}, K'_{13} and K_{34},[11] but also the absorption spectra characteristic of each structure AH^+, A, B, and C.[12] Table II shows the simplified expressions[13] for A_1, A_2, A_3, $D_0 - D_1$, $D_1 - D_2$, and $D_2 - D_f$ obtained for $K'_{12} \simeq 10^{-4}$ M, $K'_{13} \simeq 10^{-2}$ M, and for some characteristic values of the initial and final acidities.[14] For some pairs of a^0_{H+} and a^f_{H+} values, we can immediately obtain the three equilibrium constants, as shown in eq 17–25.

(A) $a^0_{H+} > 0.2$ N

(a) $a^f_{H+} \simeq K'_{13}$

$$\log \frac{D_1 - D_2}{D_0 - (D_1 - D_2)} + pK'_{13} = pH_f \qquad (17)$$

(b) $a^f_{H+} \simeq K'_{12}$

$$\log \frac{D_0 - D_1}{(\epsilon_{AH^+} - \epsilon_A)lC_0 - (D_0 - D_1)} + pK'_{12} = pH_f \qquad (18)$$

$(\epsilon_{AH^+} - \epsilon_A)lC_0$ being the amplitude $D_0 - D_1$ for the same initial pH, and for a final pH of about 6.

(c) $a^f_{H+} \simeq 10^{-6}$ N

$$\frac{D_2 - D_f}{D_2} = \frac{K_{34}}{1 + K_{34}} \qquad (19)$$

(B) $a^0_{H+} \ll 0.2$ N and $a^f_{H+} > 0.2$ N

$$\frac{D_2 - D_f}{D_1 - D_2} = K_{34} \qquad (20)$$

$$\frac{D_0 - D_f}{D_2 - D_f} = 1 + \frac{1}{K_{34}} \qquad (21)$$

$$\frac{D_0 - D_f}{D_0 - D_1} = \frac{\epsilon_{AH^+}}{\epsilon_{AH^+} - \epsilon_A} \frac{K'_{13}(1 + K_{34})}{K'_{12}} \qquad (22)$$

$$\frac{D_1 - D_2}{D_0 - D_1} = \frac{\epsilon_{AH^+}}{\epsilon_{AH^+} - \epsilon_A} \frac{K'_{13}}{K'_{12}} \qquad (23)$$

(C) $a^0_{H+} \simeq 10^{-6}$ N and $a^f_{H+} > 0.2$ N

$$\frac{D_2 - D_f}{D_2} = -K_{34} \qquad (24)$$

$$\frac{D_1 - D_2}{D_1} = -\frac{K'_{13}}{K'_{12}} \qquad (25)$$

To obtain the highest precision by this method, it is necessary to choose carefully among eq 17–25 (and any other expression deduced from those in Table II), because for certain values of a^0_{H+} and/or a^f_{H+}, some amplitudes will be very small. For instance, this will be the case for $D_0 - D_1$ when $a^0_{H+} < 10^{-4}$ N or $a^f_{H+} > 10^{-3}$ N. If we still wish to measure these amplitudes with precision, we have to increase either l or C_0, or both. On the other hand, the value of the amplitude $D_2 - D_f$ is closely related to the K_{34} value; the higher K_{34}, the higher $D_2 - D_f$.

Appendix

Complete Solution for the Linearized Rate Equations Associated with Scheme I. After a suitable pH jump has occurred, the instantaneous values of $\Delta[A]$, $\Delta[B]$, and $\Delta[C]$ are given by

$$\Delta[A] = A_1 e^{-t/\tau_1} + ([A]_1 - [A]_2)e^{-t/\tau_2} + ([A]_2 - [A]_f)e^{-t/\tau_3}$$

$$\Delta[B] = A_2 e^{-t/\tau_2} + ([B]_2 - [B]_f)e^{-t/\tau_3} \qquad (I)$$

$$\Delta[C] = A_3 e^{-t/\tau_3}$$

with $\Delta[A] = [A]_t - [A]_f$; $\Delta[B] = [B]_t - [B]_f$; $\Delta[C] = [C]_t - [C]_f$

$$[A]_2 - [A]_f = \frac{K'_{12}K'_{13}K_{34}(a^0_{H+} - a^f_{H+})C_0}{\delta_0\delta_f(K'_{12} + K'_{13} + a^f_{H+})} \qquad (II)$$

$$[A]_1 - [A]_2 = \frac{([A]_2 - [A]_f)\delta_f}{K_{34}(K'_{12} + a^f_{H+})} \qquad (III)$$

$$[B]_2 - [B]_f = \frac{([A]_2 - [A]_f)K'_{13}}{K'_{12}} \qquad (IV)$$

The expressions for τ_1^{-1}, τ_2^{-1}, and τ_3^{-1} may be found in ref 1.

Supplementary Material Available: The detailed calculations of eq 10–25 and I–IV (11 pages). Ordering information is given on any current masthead page.

References and Notes

(1) Part 2: R. Brouillard and B. Delaporte, *J. Am. Chem. Soc.*, **99**, 8461 (1977).
(2) (a) D. Thusius in "Chemical and Biological Applications of Relaxation Spectrometry", E. Wyn-Jones, Ed., Reidel, Dordrecht-Holland, 1974; (b) C. F. Bernasconi, "Relaxation Kinetics", Academic Press, New York, N.Y., 1976.
(3) Although this type of perturbation is the most frequently used, there are few reports of amplitudes measured in this way.[2]
(4) In the Appendix, the complete solution for the linearized rate equations associated with Scheme I is given.
(5) Our method can be applied to any pH-dependent equilibrated system for which only one relaxation time is shorter than the time necessary for changing the acidity of the medium. The fastest pH jump produced by any mixing technique takes about 10^{-3} s (stopped-flow). The pH jump technique is a particular case of the more general concentration-jump technique: Z. A. Schelly in ref 2a; D. Y. Chao and Z. A. Schelly, *J. Phys. Chem.*, **79**, 2734 (1975).
(6) R. Brouillard and J. E. Dubois, *J. Am. Chem. Soc.*, **99**, 1359 (1977).
(7) In this paper it is assumed that the solution submitted to a pH jump is initially totally equilibrated. The other case (the solution initially not equilibrated) is of little interest.
(8) D. Thusius, *J. Am. Chem. Soc.*, **94**, 356 (1972).
(9) It is worth noticing that in the case where $\tau_1^{-1} \gg \tau_3^{-1} \gg \tau_2^{-1}$, the relaxation process characteristic of the tautomeric equilibrium is not observable by a pH jump. Another type of perturbation must then be used for studying the kinetics of this equilibrium.
(10) We previously showed[6] that there were three possible mechanisms for the structural transformations of anthocyanins in acidic aqueous media. We demonstrated kinetically that the true mechanism was that given in the present article. It is important to realize that the pH-jump relaxation amplitudes (eq 10–16) are the same for all three mechanisms. Thus it is not possible to distinguish between the three postulated mechanisms by means of the relaxation amplitudes. Generally speaking, the thermodynamically stable chemical states can be identified by relaxation amplitudes. When these stable states are known, it is possible to choose between the different interconversion mechanisms consistent with their existence on the basis of kinetic studies.
(11) For each equilibrium, enthalpy and entropy variations are readily obtained by performing experiments at different temperatures.
(12) In the spectral range where the four structures absorb, eq 13 will remain unchanged, eq 14 will contain term ϵ_B, and eq 15 will contain terms ϵ_B and ϵ_C. As an example we give the expression corresponding to $D_2 - D_f$:
$$D_2 - D_f = \frac{K'_{13}K_{34}(a^0_{H+} - a^f_{H+})\{a^f_{H+}(\epsilon_{AH^+} - \epsilon_C) + K'_{12}(\epsilon_A - \epsilon_C) + K'_{13}(\epsilon_B - \epsilon_C)\}lC_0}{\delta_0\delta_f(K'_{12} + K'_{13} + a^f_{H+})}$$
(13) The approximations involved are smaller than or similar to the experimental uncertainty.
(14) This is the case for most of the anthocyanins.[1,6] Up to this point no assumptions regarding the respective values of K'_{12} and K'_{13} have been made.