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Analytical Methods

Effects of reaction mixture and other components on the determination of the equilibrium and rate constants of the hydration reactions of anthocyanins ☆

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Abstract

The equilibrium and rate constants of the hydration and deprotonation reactions of anthocyanins show how their color intensity changes with pH. In the cases of several anthocyanins, the constants for each obtained by several methods are different. In an effort to resolve these discrepancies, we have examined the effects of several components of the pH-jump experiments on the values of the constants. Storage of the buffers to be used in pH-jump experiments in Pyrex or borosilicate glass bottles results in increasing Al^{3+} concentration in the buffers over several weeks. When these buffers are used, the anthocyanins with two OH groups on the B ring complex with the Al^{3+} which leads to major changes in their spectra, in the equilibrium position, and in the apparent first-order rate constant. Thus, constants determined on the same anthocyanin using the same buffers stored in glass bottles may be different at different times. During the reduction of the experimental data to the rate and equilibrium constants, two divergences from the expected behavior were found. In the calculation $K_a + K_h$ for the anthocyanin acylated with 4-hydroxy-3,5-dimethoxycinnamic acid (6-*O*-(4-hydroxy-3,5-dimethoxycinnamoyl)-β-D-glucopyranosyl-(1 → 6)-[β-D-xylopyranosyl-(1 → 2)]-β-D-galactopyranosyl-(1 → O^3)-cyanidin), the plotted points appear to fit two straight lines, intersecting at an equilibrium pH near pH 4. In the calculation which leads to the individual constants of both anthocyanins examined here, the points below an equilibrium pH of pH 4 curve upward from the line that describes the points from an equilibrium pH above 4. Differences in the composition of solutions used in pH-jump experiments examined here, include (1) the addition of phosphate to the acetate buffer, (2) the presence of 0.5 M NaCl, and (3) the solution of the anthocyanin in either 0.1 N HCl or 0.1 N HOAc. These changes gave differences that were statistically significant in some of the constants for each of the two anthocyanins examined. The constants were both qualitatively and quantitatively different.

Keywords: Acylated anthocyanins; Cyanidin-3-*O*-triglycoside; 4-Hydroxycinnamic acid; 4-Hydroxy-3,5-dimethoxycinnamic acid; 6-*O*-(4-Hydroxycinnamoyl)-β-D-glucopyranosyl-(1 → 6)-[β-D-xylopyranosyl-(1 → 2)]-β-D-galactopyranosyl-(1 → O^3)-cyanidin; 6-*O*-(4-Hydroxy-3,5-dimethoxycinnamoyl)-β-D-glucopyranosyl-(1 → 6)-[β-D-xylopyranosyl-(1 → 2)]-β-D-galactopyranosyl-(1 → O^3)-cyanidin; Hydration reaction; Deprotonation reaction; Equilibrium constant measurement; Rate constant measurement; pH-Jump experiments; Al^{3+} interference; Al^{3+} from glass; Phosphate effects; NaCl effects; Acyl group effects; Equilibrium constants; Rate constants

1. Introduction

Anthocyanins are naturally occurring plant pigments of yellow, red, blue and purple hues. They play a major role in the appeal of many flowers and fruits, and they are indicators of fruit ripening. A number of them are also expected to be nutritionally important as antioxidants. The anthocyanins are structurally based on the flavylium nucleus (see Scheme 1), which in nature is ornamented with hydroxyl groups, sugars and organic acids (Francis, 1989; Goto & Kondo, 1991; Harborne & Williams, 1998; Hari, Patel, & Martin, 1994; Honda & Saito, 2002; Markakis, 1982).

☆ Studies on the stability and conformation of anthocyanins 5. For part 4, (see Whittemore, Welch, Cox, Dougall, and Baker, 2004).

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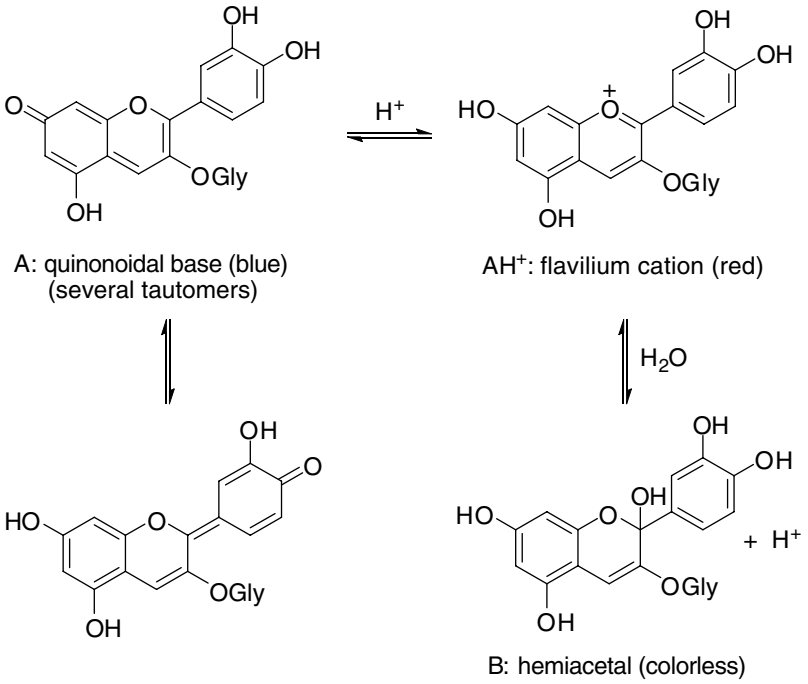

Gly = 6-*O*-Acyl-β-D-glucopyranosyl-(1→6)-[β-D-xylopyranosyl-(1→2)]-β-D-galactopyranosyl-(1→O^3)– where,
Acyl = 4-Hydroxycinnamoyl– or 4-Hydroxy-3,5-dimethoxycinnamoyl–

Scheme 1.

Some extracted anthocyanins have been used as colorings for food and drink, but because many lose their color in neutral solutions, their use as colorings in foods, pharmaceuticals, etc. has been limited. A few natural anthocyanins lose very little color in neutral solutions, a property which has been associated with these anthocyanins being poly(acyl)ated with cinnamic acids (Goto & Kondo, 1991; Honda & Saito, 2002). In addition to these relatively stable poly(cinnamoyl)ated anthocyanins, the monocinnamoylated anthocyanin alatanin C is relatively stable (Yoshida, Kondo, & Goto, 1991). The stabilities of these unusual anthocyanins suggest that anthocyanins with the color stability required for use in the food industry may exist or may be possible if suitable structural modifications can be identified.

The mechanism that has been proposed (Dangles, Saito, & Brouillard, 1993; Goto & Kondo, 1991; Honda & Saito, 2002) for the resistance of the poly(cinnamoyl)ated anthocyanins to color loss with increased pH, is folding of the molecules so that a cinnamoyl residue covers each side of the flavylium chromophore, thus preventing water molecules from attacking the chromophore. In the case of alatanin C, the single cinnamoyl residue can cover only one side of the chromophore and thus leaves one side exposed to attack by water molecules, which should result in color loss (see Scheme 1). The resistance to color loss by hydration suggests that anthocyanins closely related to alatanin C could provide a simple experimental system in which to investigate the mechanism of the cinnamoyl groups' inhibition of the hydration reaction and color loss. The measurement of the rates and equilibrium of the hydration reaction of anthocyanins that differ only in their acyl group would allow the effects of various features of the acyl groups on the color stability to be unraveled. These might include the need for the exocyclic double bond in the cinnamoyl moiety or the effects of electron-donating substituents on the aromatic ring. We therefore began to investigate the mechanism by which a single acyl group on an anthocyanin could stabilize the molecule against color loss with increasing pH.

The demonstration that carrot cell cultures could produce anthocyanins very similar to alatanin C and that the acyl group on these anthocyanins could be altered by providing specific acids to the cultures (Baker et al., 1994), opened our investigation of the mechanism by which acyl groups on anthocyanins inhibit the loss of color with decreased acidity. As part of this investigation we are measuring the rate and equilibrium constants of the color-loss reactions of a series of otherwise identical anthocyanins that differ only in the acyl group and are correlating these constants with the structures of the acyl groups. To this end, over 25 semibiosynthetic anthocyanins that differ only

in the acyl group have been isolated in our laboratory and are currently being evaluated for their stabilities (Redus, Baker, & Dougall, 1999).

The loss of anthocyanin color is the result of a pH-dependent hydration reaction of the flavylium nucleus that yields a colorless hemiacetal. Anthocyanins also undergo a pH-dependent deprotonation reaction to give blue quinonoidal bases with several tautomers. These reactions (Scheme 1) have been discussed at length by Dangles et al. (1993), Figueiredo, Elhabiri, Saito, and Brouillard (1996a) and others. The equilibrium constant for the deprotonation reaction is denoted by K_a, and the several tautomeric forms are usually treated as a single compound in the numerator of K_a, because they rapidly interconvert. The hydration reaction can be characterized by its rate constants k_1 and k_2 for the forward and reverse reactions, respectively, and by its equilibrium constant $K_h = k_1/k_2$. At constant acidity, a small K_h value shows that the concentration of the colored flavylium ion is high. With a series of anthocyanins acylated with different groups, the influence of the acyl group on K_h, and thus on the retention of anthocyanin color, can be assessed, and the contributions of the acyl groups explored. Studies of this type will lead to a more detailed understanding of the mechanism by which the hydration reaction and resulting color loss is inhibited as well as providing a semiempirical basis for designing anthocyanins with improved properties. Changes in the rate constants k_1 and k_2 with different acyl groups will further the understanding of the acyl-group effects. K_h is also a proxy for the retention of color by an anthocyanin as the pH increases. It is thus of importance for assessing the usefulness of these pigments in coloring foods and pharmaceuticals. The measurement of these constants, their reliability, and the reproducibility of these measurements between laboratories is important so that the data can be widely and reliably used.

Dangles et al. (1993) and Figueiredo et al. (1996a) have described "pH-jump" methods for determining these constants, and Stintzing, Stintzing, Carle, Frei, and Wrolstad (2002) have used an equilibrium method to estimate the equilibrium constant K_h. Redus et al. (1999) have published the equilibrium and rate constants for a series of cyanidin-based anthocyanins that differ only in the acyl substituent on the sugar chain attached to O-3 of the cyanidin (3,3′,4′,5,7-pentahydroxyflavylium) moiety (Scheme 1). In this paper (Redus et al., 1999), the constants were determined using the method described by Dangles et al. (1993). Stintzing et al. (2002) noted that the value for the equilibrium constant K_h for the non-acylated cyanidin trisaccharide determined by them was different from that published by Redus et al. (1999). The difference in the methods used in these two studies may account for the observed difference. The measurements of the constants for the reactions of cyanidin-based anthocyanins have been continuing in our laboratory, with the pH-jump measurements being performed with the reaction mixture composition given by Figueiredo et al. (1996a, 1996b) and Elhabiri et al. (1997). We therefore compared the values obtained recently with those given by Redus et al. (1999) to determine whether or not the different reaction mixtures for the pH-jump experiments gave the same values for the constants. Many differences between the two sets were found (data not shown). As a result, this study was undertaken to identify the factors that led to the different values of these constants of an anthocyanin. In this study, we have chosen to examine the anthocyanins acylated with 4-hydroxycinnamic acid [6-*O*-(4-hydroxycinnamoyl)-β-D-glucopyranosyl-(1 → 6)-[β-D-xylopyranosyl-(1 → 2)]-β-D-galactopyranosyl-(1 → O^3)-cyanidin] and with 4-hydroxy-3,5-dimethoxycinnamic acid [6-*O*-(4-hydroxy-3,5-dimethoxycinnamoyl)-β-D-glucopyranosyl-(1 → 6)-[β-D-xylopyranosyl-(1 → 2)]-β-D-galactopyranosyl-(1 → O^3)-cyanidin] because their measured hydration equilibrium constants differed by 40% or more from those given by Redus et al. (1999) and were in opposite directions. We have examined the effects of the components of the reaction mixture on the values of the equilibrium and rate constants for these two compounds. Throughout this paper the anthocyanins used differ only in the acyl group on the 6-position of the terminal glucose unit (see Scheme 1), and they are referred to in this paper by the acyl group present for simplicity.

The differences in the reaction mixtures used by Redus et al. (1999) and Figueiredo et al. (1996a, 1996b) or Elhabiri et al. (1997) are as follows: (1) the use of HCl or HOAc to dissolve the anthocyanins, (2) the use of acetate or phosphate as buffers, (3) presence or absence of 0.5 M NaCl, and (4) determination of $K_a + K_h$ separately from the rate determinations or from the final absorbance values of the rate measurements. The effects of these differences on the constants for the two anthocyanins were investigated. In addition, we investigated (1) the consequences of storing buffer solutions in borosilicate glass versus plastic containers and (2) the use of curve fitting to the pH-jump experiment data to obtain both the rate of the reaction and the equilibrium value of the reaction at each pH.

2. Results and discussion[1]

2.1. Use of glass containers

The pH-jump experiments involve rapidly adjusting the pH of a solution of anthocyanin in acid to a new pH. This is done with alkaline buffers or with solutions of NaOH of different concentrations. We experienced changes in the measured constants when the alkaline buffers for pH-jump experiments were stored in Pyrex or borosilicate glass bottles over long periods of time before use (data not shown). When a solution of 4-hydroxy-3,5-dimethoxycinnamoyl

[1] In this paper, the words significant, significantly, and significance are used to describe differences which are ***statistically significant*** as demonstrated by Student's ***t***-test.

anthocyanin was adjusted with buffer stored in glass, but not when adjusted with buffer stored in a polypropylene container, the visible absorbance maximum shifted to a longer wavelength by approximately 25 nm, and the absorbance doubled. (For details see Figure S1 in the Supplementary data section.) This led us to hypothesize that the buffer solution was extracting one or more components from the glass that affected the measurements. A search of the literature revealed that the approximate composition of Pyrex or borosilicate glass is SiO_2 70–90%, Na_2O 4–9%, B_2O_3 10–13%, and Al_2O_3 2–6% (Wheaton Scientific International, From: Physical Properties of Glass, Cole–Parmer Technical Information. http://www.coleparmer.com/techinfo/techinfo.asp?htmlfile=Properties_Glass.htm&ID=608; accessed:06/27/2007.). The addition of $Na_2SiO_3{\cdot}5H_2O$ or $Na_2B_4O_7{\cdot}10H_2O$ to buffered anthocyanin solutions gave no observable change, but the addition of small amounts of $AlCl_3$ resulted in significant changes in the visible spectrum. Some of the effects of Al^{3+} on 4-hydroxy-3,5-dimethoxycinnamoyl anthocyanin are shown in Table 1, and the effects of Al^{3+} on the 4-hydroxycinnamoyl were strictly comparable. (For details see Table S1 in the Supplementary data section). First, the presence of phosphate ion in the reaction mixtures negated most of the effects of Al^{3+} on the hydration reaction and the visible spectrum of both anthocyanins presumably because the Al^{3+} was complexed by the phosphate and perhaps other ligands such as OH^- (Akitt, Greenwood, & Lester, 1971; Daydé, Filella, & Berthon, 1990; Salmon & Wall, 1958; White, Tiffin, & Taylor, 1976). Second, in the absence of phosphate, increasing the Al^{3+} concentration (1) increased the visible absorbance maximum and the absorbance at that wavelength, with greater effect at pH 5.2 than at pH 4.2, and (2) increased the absorbance at 500 nm, the calculated absorbance at equilibrium of the reaction mixture (Y_0), and the first-order apparent rate constant at pH 5.2 and decreased these values at pH 4.2. Third, the responses of one anthocyanin were very similar to those of the other.

Alteration of the spectrum and color of anthocyanins with 3,4-dihydroxy substitution of the B ring (see Scheme 1) upon the addition of Al^{3+} is well-known (Markham, 1982). In addition, Dangles, Elhabiri, and Brouillard

Table 1
Effects of Al^{3+} on the equilibrium absorbance (Y_0), the apparent rate of the hydration reaction and the visible spectrum of anthocyanin acylated with 4-hydroxy-3,5-dimethoxycinnamic acid at two pH's in the presence and absence of phosphate[a]

Al^{3+} (mM)	pH	Y_0	k (s^{-1})	Vis. Abs. Max. (nm)	Vis. Abs. Max. OD	OD 500 nm
	PO_4^{3-} absent					
0	5.2	0.2542	0.0138	545	0.388	0.216
0.025	5.19	0.2959	0.0321	562	0.713	0.251
0.05	5.19	0.3176	0.0439	564	0.803	0.272
0.075	5.19	0.3170	0.0513	564	0.844	0.273
0.10	5.21	0.3185	0.0543	564	0.866	0.274
	PO_4^{3-} present					
0	5.08	0.2604	0.0156	539	0.369	0.227
0.025	5.08	0.2604	0.0154	540	0.376	0.227
0.05	5.07	0.2638	0.0155	541	0.382	0.231
0.075	5.06	0.2662	0.0156	539	0.385	0.234
0.10	5.06	0.2693	0.0152	540	0.390	0.239
	PO_4^{3-} absent					
0	4.22	0.4035	0.0611	533	0.509	0.365
0.025	4.21	0.3971	0.0523	538	0.551	0.353
0.05	4.21	0.3851	0.0488	540	0.578	0.340
0.075	4.25	0.3776	0.0450	550	0.663	0.327
0.10	4.24	0.3760	0.0429	547	0.652	0.329
	PO_4^{3-} present					
0	4.24	0.4079	0.0627	530	0.512	0.370
0.025	4.24	0.4044	0.0627	530	0.508	0.366
0.05	4.25	0.4132	0.0628	533	0.518	0.375
0.075	4.24	0.4092	0.0620	530	0.515	0.370
0.10	4.25	0.4041	0.0601	533	0.511	0.366
HCl[b]		0.6712	0	526	0.797	0.632
		0.6766	0	524	0.803	0.639

[a] See Section 3 for details. The spectra were determined on the samples used for the measurements of the apparent rate constant (k) of the flavylium ion hydration approx. 5 min after the measurements were initiated. Each row has the observations on a single sample.
[b] 0.1 N HCl with no Al^{3+} was used in the HCl treatments.

(1994) showed that, with 3′,4′,7-trihydroxyflavylium chloride in the presence of Al^{3+}, the first-order apparent rate constant decreased with decreasing pH, and that in the absence of Al^{3+} the rate constant increased with decreasing pH. Further, Dangles et al. (1994) and Elhabiri et al. (1997) concluded that Al^{3+} forms complexes with both the anionic and the colorless forms of anthocyanins and synthetic flavylium chlorides containing a catechol-type substitution in the B ring, the former complex being preponderant at lower pHs.

The possibility that glass vessels were a source of Al^{3+} in the reagents, and hence, the cause of the changes in rate constants, was examined. 2′,3,4′,5,7-Pentahydroxyflavone (Morin), shown by Ahmed and Hossan (1995) to be a highly sensitive and specific reagent for Al^{3+}, was used to determine the ability of alkaline buffers or 0.1 N HCl to extract Al^{3+} from Pyrex and borosilicate glass bottles. The results are shown in Table 2. The amounts of Al^{3+} in the extracts of both types of glass increased with time. The amounts of Al^{3+} were higher in the alkaline extracts compared to the HCl extracts. They quickly reached levels which alter the behavior of these anthocyanins as shown in Table 1 (see also Table S1 in the Supplementary data section). Consequently, for any anthocyanin with 3,4-substitution on the B ring, the constants determined at different times after the solutions were prepared and stored in glass, will be different because of the different concentrations of Al^{3+}. We have therefore stored all solutions for the determination of the constants in polypropylene containers. Further, these data suggest that any measurements of constants of anthocyanins with adjacent phenolic hydroxyl groups on the B-ring should be discarded if the alkaline reagents are not explicitly freshly made or stored in plastic vessels to prevent Al^{3+} interference.

Table 2
Time course of Al^{3+} extraction from bottles by alkaline buffer or HCl at 25 °C

Days	Al^{3+} (μM)			
	Pyrex [a] (Wheaton)		Borosilicate[b](Gibco)	
	Buffer[c]	0.1 N HCl	Buffer[c]	0.1 N HCl
0	−4 ± 6	−7 ± 5	−1 ± 11	−14 ± 6
4	6 ± 5	11 ± 9	22 ± 7	−7 ± 6
7	3 ± 2	12 ± 12	20 ± 2	−8 ± 6
13	21 ± 4	29 ± 5	46 ± 5	−5 ± 2
30	57 ± 4	29 ± 4	106 ± 7	−7 ± 5
37	46 ± 4	29 ± 2	112 ± 8	−9 ± 4
44	53 ± 6	35 ± 2	128 ± 8	4 ± 12
51	56 ± 8	34 ± 1	152 ± 8	−3 ± 4
58	59 ± 9	35 ± 2	139 ± 14	−1 ± 3
66	59 ± 6	36 ± 2	164 ± 14	−3 ± 3

[a] Wheaton. 8-oz square Pyrex Wheaton bottles were used.
[b] Gibco. 100-mL borosilicate bottles were used.
[c] Buffer was 0.1 M NaOAc + NaOH and gave pH of 4.13 with an equal volume of 0.1 N HCl. Data are the means and standard deviation ($n = 4$) of duplicate samples from two bottles in each case.

2.2. Determination of $K_a + K_h$

In the calculation of the constants for a particular anthocyanin, the sum of the two equilibrium constants $K_a + K_h$ is first determined from the equilibrium absorbance at several pHs, and then the individual constants are determined using the apparent first-order rate constants at several pHs. Dangles et al. (1993) and others determined $K_a + K_h$ from a separate set of data using a 30-min equilibration period. Redus et al. (1999) used the lowest absorbance values reached in the time courses of the pH-jump experiments for this calculation based on the assumption that equilibrium was reached in the 2 or 3 min that the reaction was followed. Dangles et al. (1994) used the calculated equilibrium value (Y_0) obtained during the calculation of the apparent first-order rate constant for each pH-jump experiment. Comparison of $K_a + K_h$ obtained from the Y_0 values with the other values (by Student's t-test) showed that only the $K_a + K_h$ from the equilibrium method for the 4-hydroxycinnamoyl anthocyanin differed significantly ($P < 0.05$) from that obtained from Y_0. These data suggest that there is little difference between the $K_a + K_h$ determined by the three methods. It is to be noted that the slow Z–E isomerization of the chalcone subsequent to the hemiacetal is omitted from Scheme 1, and that over 30 min this process will remove some of the flavylium ion and thus increase the value of $K_a + K_h$. The use of the lowest value of the absorbance in the pH-jump experiments assumes that equilibrium has been reached, which may not be true especially when the rate of the reaction is as low as it is at the higher pHs used in the pH-jump experiments. We have chosen to use the value for the equilibrium position (Y_0) of each pH-jump experiment obtained by fitting an exponential decay curve with three parameters to each pH-jump experiment because this is the model obtained from the theory of first-order equilibrium reactions and because the two needed values are obtained from each pH jump experiment.

2.3. Effects of PO_4^{3-}, NaCl, and acid used to dissolve anthocyanins on the constants

The effects of phosphate and NaCl on the equilibrium and rate constants for the hydration and deprotonation reactions of the 4-hydroxycinnamoyl anthocyanin and of the 4-hydroxy-3,5-dimethoxycinnamoyl anthocyanin are shown in Tables 3 and 4, respectively.

2.3.1. 6-O-(4-Hydroxycinnamoyl)-β-D-glucopyranosyl-(1 → 6)-[β-D-xylopyranosyl-(1 → 2)]-β-D-galactopyranosyl-(1 → O^3)-cyanidin ("4-hydroxycinnamoyl anthocyanin")

When the first calculation was applied to the data obtained with the 4-hydroxycinnamoyl anthocyanin dissolved in HOAc with NaCl present, a straight line resulted and $K_a + K_h$ was obtained. When the second calculation was applied to the data, the points from the pH-jump

Table 3
Equilibrium and rate constants of the anthocyanin acylated with 4-hydroxycinnamic acid determined in the presence or absence of phosphate or NaCl[a]

	Composition of the reaction mixtures						
Phosphate	–	+		–		–	
NaCl	–	–		+		+	
Acid	HCl	HCl		HCl		HOAc	
$K_a + K_h \times 10^5$							
Mean (M)	8.06	7.37	*	7.72	ns	7.73	ns	ns
SD	0.37	0.24		0.54		0.11		
$K_h \times 10^5$							
Mean (M)	5.69	4.58	***	5.43	ns	5.41	ns	ns
SD	0.29	0.84		0.41		0.70		
$k_1 \times 10^2$							
Mean (s^{-1})	3.92	3.88	ns	4.09	ns	3.87	ns	ns
SD	0.11	0.09		0.12		0.15		
k_2							
Mean ($M^{-1}\ s^{-1}$)	690	847	***	753	*	716	ns	*
SD	42	23		23		20		
$K_a \times 10^5$							
Mean (M)	2.36	2.79	*	2.29	ns	2.32	ns	ns
SD	0.29	0.08		0.04		0.07		

Significance at $P < 0.05$ is shown by *, at $P < 0.01$ by **, and at $P < 0.001$ by ***.
[a] Statistical analysis was by Student's *t*-test comparing each treatment with the first, except in the last column where NaCl + HCl is compared with NaCl + HOAc. There were four separately determined values for each mean. If $P > 0.05$ the effect was treated as not significant (ns).

Table 4
Equilibrium and rate constants of the anthocyanin acylated with 4-hydroxy-3,5-dimethoxycinnamic acid determined in the presence or absence of phosphate or NaCl[a]

	Composition of the reaction mixtures						
Phosphate	–	+		–		–	
NaCl	–	–		+		+	
Acid	HCl	HCl		HCl		HOAc	
$K_a + K_h \times 10^5$							
Mean (M)	9.11	9.68	*	9.39	ns	8.68	ns	ns
S.D.	0.37	0.19		0.13		0.93		
$K_h \times 10^5$							
Mean (M)	6.76	7.33	***	7.40	***	6.47	*	***
S.D.	0.11	0.27		0.18		0.24		
$k_1 \times 10^2$							
Mean (s^{-1})	4.05	4.14	ns	3.47	***	3.49	***	ns
S.D.	0.12	0.18		0.11		0.04		
k_2							
Mean ($M^{-1}\ s^{-1}$)	600	565	ns	469	***	541	***	***
S.D.	18	45		11		20		
$K_a \times 10^5$							
Mean (M)	2.35	2.34	ns	2.00	**	2.21	ns	ns
S.D.	0.11	0.27		0.18		0.24		

Significance at $P < 0.05$ is shown by *, at $P < 0.01$ by **, and at $P < 0.001$ by ***.
[a] Statistical analysis was by Student's *t*-test comparing each treatment to the first one, except in the last column where NaCl + HCl is compared with NaCl + HOAc. The treatment without phosphate or NaCl had eight separately determined values for each mean, and the other means had four separately determined values. If $P > 0.05$, the effect was treated as not significant (ns).

experiments where the final pH was less than 3.9 curved upwards and did not lie on the straight line that described all the other points that covered the pH range from 3.9 to 5.2 (data not shown). We calculated the constants using the data from experiments where the equilibrium pH was above 3.9. Data for the other treatments were obtained in all cases by limiting the range of pHs used to pH 4.0–5.4 and gave straight lines. At this time we do not understand the data from below pH 3.9.

Phosphate significantly increased k_2 for the 4-hydroxycinnamoyl anthocyanin (Table 3), and as a result K_h (which is k_1/k_2) was significantly decreased. The equilibrium constant (K_a) for the deprotonation reaction was increased significantly. NaCl in the HCl solution of the anthocyanin

had only a small effect on the constants, giving a small but significant increase in k_2, which did not lead to a statistically significant decrease in K_h. The constants determined with NaCl in the HOAc solution of the anthocyanin were not significantly different from the values determined for the solutions in HCl in the absence or the presence of NaCl, except for the values of k_2 measured in the presence of NaCl.

2.3.2. 6-O-(4-Hydroxy-3,5-dimethoxycinnamoyl)-β-D-glucopyranosyl-(1 → 6)-[β-D-xylopyranosyl-(1 → 2)]-β-D-galactopyranosyl-(1 → O^3)-cyanidin ("4-hydroxy-3,5-dimethoxycinnamoyl anthocyanin")

The 4-hydroxy-3,5-dimethoxycinnamoyl anthocyanin gave a different set of responses compared to those obtained with the 4-hydroxycinnamoyl analogue (Table 4). For the data from this anthocyanin dissolved in HOAc with NaCl present, the plot of $D_0/(D_0 - D)$ against 10^{-pH} to determine $K_a + K_h$ did not give a single straight line. The data could be fitted to two lines with a break point at pH 4.1. (For details see Figure S2 in the Supplementary data section.) The $K_a + K_h$ calculated for the data from experiments where the final pH was above pH 4.11 was $8.7 \times 10^{-5} \pm 9 \times 10^{-6}$, and for data from below pH 4.11 the value was $1.70 \times 10^{-4} \pm 4 \times 10^{-6}$. In the second calculation the data from above pH 4.11 fitted a straight line with a positive slope, while the points from pHs below 4.1 curved upwards as found for the 4-hydroxycinnamoyl anthocyanin (see previous section). (For details see Figure S3 in the Supplementary data section.) The data from pHs above 4.11 were used to calculate the constants shown in Table 4. A straight line can be fitted to the data from below pH 4.11, but it has a negative slope. For the experiments with this anthocyanin dissolved in HCl, the experimental values used to calculate the constants were limited to the range of pH 4.1–5.3. Points from below this range were not on the straight line that described those above pH 4.1 in the first calculation and gave a negative slope in the second calculation as found for the HOAc plus NaCl solutions. While the data for the HCl solutions are not as extensive as those for the HOAc solutions, they show the same characteristics when the two calculations are performed on them.

A possibility to explain the fitting to two straight lines in the first calculation was given by Figueiredo et al. (1996a). These workers developed a mathematical model based on two conformations of anthocyanins, one of which was an intramolecular complex. The characteristics of their anthocyanins that led to their model was that the visible absorbance maximum decreased when the pH of the anthocyanin solution was increased from pH 0.7–0.8 to 1.3–1.6. With 4-hydroxy-3,5-dimethoxycinnamoyl anthocyanin, the absorbance maximum and the maximum absorbance was the same at pHs 0.79, 1.23, 1.81, and 2.15. (For details see Figure S4 in the Supplementary data section.) Above pH 2.15, the absorbance maximum declined progressively, and the position of the maximum moved to longer wavelengths. The behavior of this anthocyanin at pHs below 2.15 and the similar behavior of the 4-hydroxycinnamoyl anthocyanin (data not shown) indicated that Figueiredo et al.'s (1996a) model is not appropriate for the anthocyanins acylated with a 4-hydroxy-3,5-dimethoxycinnamoyl or a 4-hydroxycinnamoyl group used here.

Phosphate significantly increased K_h but not k_1 or k_2. This was the result of phosphate giving a small numerical increase in k_1 and a small numerical decrease in k_2 so that the ratio $K_h = k_1/k_2$ increased enough to become statistically significant. NaCl in HCl solutions of the anthocyanin significantly decreased both k_1 and k_2 but differentially, which resulted in a significant increase in K_h. NaCl also significantly decreased K_a. NaCl in an HOAc solution of the anthocyanin significantly decreased k_1 and k_2, which resulted in a small but significant decrease in K_h.

2.4. Comparison of the two anthocyanins

2.4.1. Calculation of constants

During the calculation of the constants from the data, the two anthocyanins behaved differently. For the 4-hydroxycinnamoyl anthocyanin, the plot for the calculation of $K_a + K_h$ gave a single straight line, whereas the same plot for the 4-hydroxy-3,5-dimethoxycinnamoyl analogue could be fitted to two straight lines. In both cases, the plots from which the individual constants were obtained, contain points from below an equilibrium pH of pH 4 which curved upwards. As far as we can determine, these behaviors have not been reported in the literature, and we have no explanation for these observations.

2.4.2. The constants for the two anthocyanins respond differently to changes in the reaction mixtures

The data shown in Tables 3 and 4 and those for the calculations of the constants show that these two anthocyanins behave differently under different reaction conditions. Examples for these differences are as follows:

(1) For the 4-hydroxycinnamoyl anthocyanin there was little difference between the constants determined with the anthocyanin dissolved in HOAc + NaCl or HCl + NaCl, with only k_2 being significantly decreased at $p < 0.05$ (Table 3). In contrast, for the 4-hydroxy-3,5-dimethoxycinnamoyl anthocyanin, k_2 was significantly increased ($P < 0.001$), and K_h was significantly decreased ($P < 0.001$) in HOAc + NaCl compared to the values obtained in HCl + NaCl.
(2) The K_h of the 4-hydroxycinnamoyl analogue is significantly lower in the presence of than in the absence of phosphate (Table 3), whereas the reverse is true for the 4-hydroxy-3,5-dimethoxycinnamoyl compound (Table 4). This difference arises for the 4-hydroxycinnamoyl analogue because k_2 is increased and k_1 is unchanged in the presence of phosphate, leading to a significantly smaller K_h, whereas with the 4-hydroxy-3,5-dimethoxycinnamoyl compound, k_2 is

numerically but not significantly smaller in the presence of phosphate, k_1 is numerically but not significantly larger, and K_h is significantly larger in the presence of phosphate.

(3) When compared to the values of the constants measured in its absence, NaCl significantly altered the constants for the 4-hydroxy-3,5-dimethoxycinnamoyl anthocyanin but had little effect on the constants for the 4-hydroxycinnamoyl analogue. The question of why and how the increase in ionic strength gave these differential effects on the constants of the two compounds is outside the scope of this study.

(4) Other differences can be seen on inspection of Tables 3 and 4.

From these data, it is clear that changes in the compositions of the solutions used in pH-jump experiments can lead to different values for the constants of the hydration and deprotonation reactions of anthocyanins. Consequently, only when the compositions of the solutions used in pH-jump experiments with anthocyanins are identical, can valid conclusions be drawn when comparing the constants of one anthocyanin with those of another. The mechanism(s) by which components of the reaction mixture alter the constants of these two anthocyanins is not clear. How other anthocyanins will respond to the acid used, to phosphate and to NaCl remains to be determined.

2.4.3. Comparison with published data

The constant determined with the anthocyanins dissolved in HOAc plus NaCl shown in Tables 3 and 4 are different from those given in Redus et al. (1999). For example, here K_h for the 4-hydroxy-3,5-dimethoxycinnamoyl anthocyanin is larger and that for the 4-hydroxycinnamoyl anthocyanin is smaller than those given by Redus et al. (1999). In the study of Redus et al. (1999), these differences are due, at least in part, to differences in the Al^{3+} concentration in the reaction mixtures resulting from extraction of Al^{3+} from the glass bottles by the alkaline solutions stored in them. No systematic study of the effects of Al^{3+} concentration on the rates and equilibrium constants of these anthocyanins has been performed in this work because such a study is beyond that needed to show the source of Al^{3+} contamination of the reagents and to confirm that Al^{3+} alters the rates of the reactions.

3. Materials and methods

3.1. Data manipulation

The data were stored and manipulated in Microsoft Excel Office 2000. Statistical analysis and the fitting of rate data to an exponential decay curve with three parameters were performed using SigmaPlot versions 5.0 and 6.0.

3.2. Chemicals

Anthocyanins used here were 6-*O*-(4-hydroxycinnamoyl)-β-D-glucopyranosyl-(1 → 6)-[β-D-xylopyranosyl-(1 → 2)]-β-D-galactopyranosyl-(1 → O^3)-cyanidin and 6-*O*-(4-hydroxy-3,5-dimethoxycinnamoyl)-β-D-glucopyranosyl-(1 → 6)-[β-D-xylopyranosyl-(1 → 2)]-β-D-galactopyranosyl-(1 → O^3)-cyanidin. They were prepared as described in Dougall, Baker, Gakh, Redus, and Whittemore (1998) and Redus et al. (1999). Concentrated solutions of purified anthocyanins in 0.25–1% HCO_2H were stored at −15 °C. Isocratic and gradient HPLC on a C_{18} column with monitoring at both 525 nm and 280 nm showed that each compound was greater than 97% pure after the measurements were completed. Morin (2′,3,4′,5,7-pentahydroxyflavone) was obtained from Sigma–Aldrich Chemical Co. (lot 114K2617). Reagent grade chemicals were used without further purification.

3.3. Measurement of constants

The pH-jump experiments were performed at 25 °C as described by Redus et al. (1999) except that a Shimadzu UV-2101PC scanning spectrophotometer with a Haake F.J. circulating water bath was used. For each set of samples for a pH-jump experiment, a dilute (74–83 μM) solution of anthocyanin was prepared. It was dispensed in 1.0-mL portions, and equilibrated in the dark at 25 °C for 1.5–2.5 h, immediately before each series of pH-jump experiments was performed. The anthocyanins were dissolved in 0.1 N HCl or 0.1 N HOAc. Each series of measurements consisted of 14 or 15 determinations at pH 3.6–5.2, achieved by adding an equal volume 0.1 M NaOAc buffer containing different amounts of 1 M NaOH to identical samples dissolved in HCl, or by adding an equal volume of NaOH solution ranging from 0.015 N to 0.090 N at 0.005-N intervals when the anthocyanins were dissolved in HOAc. Included in each set were five samples that were treated with an equal volume of 0.1 N HCl giving the initial absorbance value (D_0) for each set of data. When phosphate was added to the reaction mixtures, its final concentration was 0.05 M. Buffers were stored in either Falcon or Corning Costar 50-mL polypropylene conical centrifuge tubes. When NaCl was added to the reaction mixtures, it was dissolved in the solution of anthocyanin in acid to give a 1 M solution so that the final concentration in the reaction mixture was 0.5 M. The pH of each buffered solution was measured after the absorbance measurements using a Fisher Accumet AR15 pH meter and a AccuTupH model 13-620-185 pH electrode.

3.4. Calculation of constants

The calculations of the constants from pH-jump measurements are described by Dangles et al. (1993). First is the calculation of $K_a + K_h$, which is obtained from a plot of the equilibrium optical densities (D) in the form of $D_0/$

$(D_0 - D)$ against 10^{-pH}, where D_0 is the absorbance of the solution of anthocyanin when diluted with an equal volume of 0.1 N HCl, and where pH is the pH of the reaction mixtures at the end of the absorbance measurements. The use of D or D_0 for the equilibrium optical densities follows the notation of Dangles et al. (1993). D and D_0 are identical to Y_0 obtained by curve fitting to the data of each experiment. Y_0 is the notation used by SigmaPlot. $K_a + K_h$ is given by the ratio of the intercept to the slope of the plot. The second calculation gives the constants K_a, K_h, k_1, and k_2, which are obtained from a plot of $(K_a + K_h + 10^{-pH})/k$ against 10^{pH} (where k is the apparent first-order rate constant of the flavylium ion color loss of a sample), which gives a straight line with a slope equal to K_a/k_2 and an intercept equal to $1/k_2$.

Constants for each anthocyanin were calculated from four sets of data each containing at least 14 pH-jump determinations and five determinations where HCl was added to measure the absorbance of the anthocyanin solution without pH jump. The four values of $K_a + K_h$ obtained were averaged, and the average was used in the calculations of the individual constants from each of the four sets of pH-jump experiments

3.5. Measurement of Al^{3+} concentration

Aluminum ion concentration was estimated using Morin (2′,3,4′,5,7-pentahydroxyflavone) as the reagent in a method adapted from that of Ahmed and Hossan (1995). Samples were buffered to pH 4.13 using 0.5 mL of 0.1 N HCl or 0.5 mL of 0.1 M NaOAc with sufficient 1 M NaOH added to it so that when combined the pH was 4.13. Standards were in 0.1 mL of 0.01 N HCl, and 0.1 mL of 0.01 N HCl was added to all samples. The standards were from 0 to 1.0 μmol of Al^{3+} in 1.1 mL total volume. To these solutions was added 1.0 mL 0.2 M NaOAc buffer pH 4.13 and 5.0 mL of a solution of Morin (0.046 mg/mL) in 35:65 H_2O–EtOH. The absorbances of the mixed solutions were measured at 422 nm after standing at room temperature for approximately 1 h. The measurement at 422 nm gave the greatest difference between the absence and presence of Al^{3+}.

3.6. Al^{3+} extraction from glass

The extraction of Al^{3+} from glass bottles was performed using Wheaton 8-oz square Pyrex bottles (lot 76290-01) that had previously been used to store alkaline solutions and Gibco 100-mL borosilicate bottles that had not been used to store alkaline solutions. Two of each type of bottle contained approximately 80 mL of 0.1 M NaOAc containing sufficient 1 M NaOH so that when mixed with an equal volume of 0.1 N HCl, the pH was 4.13. In addition, two of each type of bottle contained approximately 80 mL of 0.1 N HCl. These bottles were maintained at 25 °C, and duplicate 0.5-mL samples were taken from each at intervals for Al^{3+} measurement.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2007.07.035.

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