

Rate and Equilibrium Constants for the Dehydration and Deprotonation Reactions of Some Monoacylated and Glycosylated Cyanidin Derivatives[†]

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The rate and equilibrium constants for the pH-dependent hydration of a series of monoacylated anthocyanins semi-biosynthesized using a wild carrot suspension culture have been measured. The features of the acyl groups that decrease the hydration and thus decrease the loss of color of these anthocyanins are the presence of the side-chain double bond of the cinnamic acids and the presence of electron-donating substituents at the para-position of the acyl group. Methoxyl groups in the meta-position of the acyl groups have a greater effect than can be explained by their effects on the electron density in the acyl group. The data also suggest that there may be additional features of the acyl groups which contribute to the color retention in anthocyanins.

Keywords: Anthocyanins; color stability; carrot (*Daucus carota* ssp. *carota*); flavylum ion hydration; pH effects

INTRODUCTION

Most of the pigments that give plant parts a color other than green are anthocyanins. Natural anthocyanins consist of 1 of 17 2-phenylbenzopyrylium (flavylum, see Chart 1, Flav) chromophores glycosylated or glycosylated and acylated at various locations on the chromophore (Mazza and Miniati, 1993). Anthocyanins have been investigated extensively because they are natural coloring compounds that may be usable in the food, pharmaceutical, and nutraceutical industries. Most anthocyanins are not suitable for use as colors in these industries because they lose their color and/or are converted into blue quinonoid forms rapidly in aqueous solution in a pH-dependent manner at pH values >3. The loss of color is the result of the addition of water to C-2 of the flavylum cation, converting it into a colorless hemiacetal (Brouillard, 1982; Dangles et al., 1993; Mazza and Miniati, 1993). The hemiacetal undergoes ring opening to give a Z-chalcone, which can rearrange further. The hydration and the ring-opening reactions are reversible, but subsequent reactions are irreversible. The hydration reaction is generally faster than the ring-opening reaction.

Although the majority of anthocyanins lose their color rapidly in a pH-dependent manner, there are a small number in which the color appears to be stable in aqueous solution (Dangles et al., 1993; Goto and Kondo, 1991). These stable anthocyanins are glycosylated, often extensively, and generally are acylated with several

cinnamic acid residues. In 1991, a relatively simple anthocyanin, alatanin C, which is cyanidin with a disaccharide residue monoacylated with 3,5-dimethoxy-4-hydroxycinnamic acid (sinapic acid) attached at C-3, was shown to retain its color at pH 6, whereas the deacylated compound did not (Yoshida et al., 1991). The nature of the sugar linkage between the anthocyanidin and the cinnamoyl residue is important for the color stability because cyanidin 3-[6-*O*-(4-hydroxycinnamoyl)-glucoside]-5-glucoside (shisonin) retains even less color at pH 6 than does the deacylated alatanin C (Yoshida et al., 1991). Thus, some anthocyanins with a single cinnamoyl residue attached do not undergo the rapid pH-dependent hydration reaction and loss of color that is characteristic of nonacylated anthocyanins. This dramatic change in anthocyanin characteristics caused by acylation with aromatic acids increases their potential usefulness. The mechanism by which aromatic acyl groups increase the color retention of anthocyanins is of both theoretical and practical interest, and knowledge of the mechanism may lead to acyl groups that further increase the color retention.

We (Dougall et al., 1998) have prepared and characterized a series of anthocyanins similar to alatanin C, each of which is monoacylated at the same position. These are shown in Chart 1. They differ from alatanin C by the substitution of a galactose residue for the glucose residue attached to the cyanidin in alatanin C. In many of the anthocyanins, the C-2 of the galactose residue carries a 1- β -D-xylose side chain. This series of anthocyanins provides an opportunity to investigate the mechanism by which the acyl group causes resistance to hydration of the anthocyanins.

We approached an understanding of the mechanism of resistance to hydration by measuring the rate and equilibrium constants for the pH-dependent reactions in this series of anthocyanins in water. Such data allow an evaluation of the roles of various features of the acyl

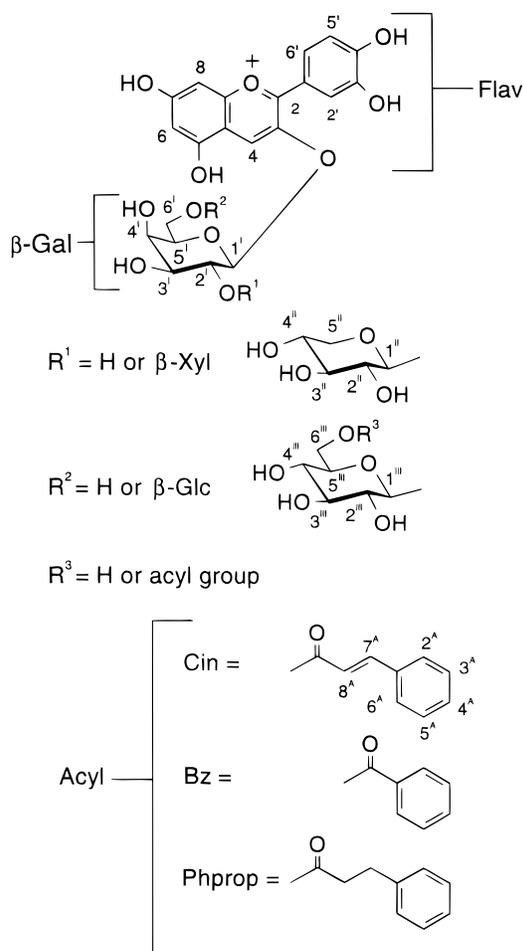
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[†] Part 3 in the series "Studies on the Stability and Conformation of Monoacylated Anthocyanins". For Part 2, see Dougall et al. (1998).

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Chart 1



1	$R^1 = \beta\text{-Xyl}$	$R^2 = \beta\text{-Glc}$	$R^3 = \text{H}$
2	$R^1 = \text{H}$	$R^2 = \beta\text{-Glc}$	$R^3 = \text{H}$
3	$R^1 = \beta\text{-Xyl}$	$R^2 = \text{H}$	
4	$R^1 = \text{H}$	$R^2 = \text{H}$	
5	$R^1 = \beta\text{-Xyl}$	$R^2 = \beta\text{-Glc}$	$R^3 = 4\text{-OH-3,5-(OMe)}_2\text{-Cin}$
6	$R^1 = \text{H}$	$R^2 = \beta\text{-Glc}$	$R^3 = 4\text{-OH-3,5-(OMe)}_2\text{-Cin}$
7	$R^1 = \beta\text{-Xyl}$	$R^2 = \beta\text{-Glc}$	$R^3 = 3,4,5\text{-(OMe)}_3\text{-Cin}$
8	$R^1 = \text{H}$	$R^2 = \beta\text{-Glc}$	$R^3 = 3,4,5\text{-(OMe)}_3\text{-Cin}$
9	$R^1 = \beta\text{-Xyl}$	$R^2 = \beta\text{-Glc}$	$R^3 = 4\text{-OH-Cin}$
10	$R^1 = \text{H}$	$R^2 = \beta\text{-Glc}$	$R^3 = 4\text{-OH-Cin}$
11	$R^1 = \beta\text{-Xyl}$	$R^2 = \beta\text{-Glc}$	$R^3 = 4\text{-OMe-Cin}$
12	$R^1 = \beta\text{-Xyl}$	$R^2 = \beta\text{-Glc}$	$R^3 = 3\text{-OMe-Cin}$
13	$R^1 = \beta\text{-Xyl}$	$R^2 = \beta\text{-Glc}$	$R^3 = 2\text{-OMe-Cin}$
14	$R^1 = \beta\text{-Xyl}$	$R^2 = \beta\text{-Glc}$	$R^3 = \text{Cin}$
15	$R^1 = \beta\text{-Xyl}$	$R^2 = \beta\text{-Glc}$	$R^3 = 4\text{-F-Cin}$
16	$R^1 = \beta\text{-Xyl}$	$R^2 = \beta\text{-Glc}$	$R^3 = 4\text{-Cl-Cin}$
17	$R^1 = \beta\text{-Xyl}$	$R^2 = \beta\text{-Glc}$	$R^3 = 4\text{-CF}_3\text{-Cin}$
18	$R^1 = \beta\text{-Xyl}$	$R^2 = \beta\text{-Glc}$	$R^3 = 4\text{-NO}_2\text{-Cin}$
19	$R^1 = \beta\text{-Xyl}$	$R^2 = \beta\text{-Glc}$	$R^3 = 4\text{-(Me}_2\text{N)-Cin}$
20	$R^1 = \beta\text{-Xyl}$	$R^2 = \beta\text{-Glc}$	$R^3 = 3,4,5\text{-(OMe)}_3\text{-Bz}$
21	$R^1 = \beta\text{-Xyl}$	$R^2 = \beta\text{-Glc}$	$R^3 = 4\text{-OH-Bz}$
22	$R^1 = \beta\text{-Xyl}$	$R^2 = \beta\text{-Glc}$	$R^3 = 4\text{-Cl-Bz}$
23	$R^1 = \beta\text{-Xyl}$	$R^2 = \beta\text{-Glc}$	$R^3 = 4\text{-CF}_3\text{-Bz}$
24	$R^1 = \beta\text{-Xyl}$	$R^2 = \beta\text{-Glc}$	$R^3 = 4\text{-OH-Phprop}$

group in the resistance to pH-dependent hydration and loss of color of these anthocyanins. This paper describes those measurements, provides the rate and equilibrium constants, and identifies some features of the acyl groups that are important in the retention of color of monoacylated anthocyanins.

EXPERIMENTAL PROCEDURES

The anthocyanins used here have been described by Dougall et al. (1998). Each compound gave a single major peak on HPLC (Dougall et al., 1998) containing >98% of the material absorbing at 540 nm, and the ^1H NMR showed no evidence of additional compounds. The constants of the reactions were measured in pH-jump experiments as described by Dangles et al. (1993) using a Lambda 3B UV-vis spectrometer (Perkin-Elmer, Norwalk, CT) with a Haake (Berlin, Germany) A80 circulating water bath. The reactions were monitored at 500 nm, a wavelength shorter than that of the visible maximum at pH ~ 1 , chosen because it is the maximum wavelength at which the quinonoid forms had no measurable absorbance. This precluded a contribution to the absorbance measurements by the quinonoid forms. For each compound, the initial absorbance of all samples in a set of pH-jump experiments was the same. Each set of pH-jump experiments consisted of triplicate determinations at seven pH values with different pH ranges for different compounds. All experiments were performed in 0.5 M NaCl/0.05 M acetic acid and various amounts of NaOH at 25 °C. The initial absorbance of the solutions in the sets of pH-jump experiments within and between compounds was 0.8 ± 0.1 , corresponding to initial concentrations of $\sim 25 \mu\text{M}$. The absorbance of each sample was recorded at 1 s intervals and followed until no further change

was observable. This required 2 min for all compounds except **6**, which required 5 min. The first-order rate constant was calculated for each pH-jump experiment, and the rate and equilibrium constants for each compound were calculated as given by Dangles et al. (1993). Statistical analysis of the data was performed according to the methods given by Bailey (1959).

RESULTS

The structures of the anthocyanins studied here and their identification numbers are shown in Chart 1. The numbering of the anthocyanins here is identical with that in Dougall et al. (1998). The rate constants for the hydration reaction (k_1) and for the dehydration reaction (k_2), equilibrium constant for the hydration reaction (K_h) and the equilibrium constant for the deprotonation to give quinonoid forms (K_a) are given in Table 1. The anthocyanin acylated with 4-(dimethylamino)cinnamic acid (**19**) was also examined. For **19**, the plots of $D_0/(D_0 - D)$ against $10^{-\text{pH}}$ were not linear as required in the treatment by Dangles et al. (1993), and consequently the calculations of the constants could not be completed. This anomaly may be due to the facts that the molecule contains a second ionizable group which responds to the pH changes used and that the electron-donating capacity of the dimethylamino group in the ionized form is very different from that of the un-ionized form (March, 1992).

Comparison of K_h and K_a in Table 1 shows that K_h is 4–10 times larger than K_a , showing that at equilibrium,

Table 1. Rate Constants of the Hydration Reaction and the Equilibrium Constants of the Hydration Reaction (K_h) and Quinonoid Formation (K_a) of Some Monoacylated and Nonacylated Anthocyanins^a

compd ^b	$k_1 \times 10^2$ (s ⁻¹)	k_2 (s ⁻¹ M ⁻¹)	$K_h \times 10^5$ (M)	$K_a \times 10^5$ (M)
1	6 ± 1	139 ± 1	43 ± 6	4.3 ± 0.2
3	8.1 ± 0.3	208 ± 1	39 ± 1	4.2 ± 1.9
5	2.5 ± 0.4	633 ± 52	3.9 ± 0.5	0.9 ± 0.4
6	2.0 ± 0.1	394 ± 20	5.1 ± 0.0	0.35 ± 0.01
7	3.57 ± 0.04	684 ± 42	5.2 ± 0.3	1.65 ± 0.08
8	2.78 ± 0.08	519 ± 20	5.38 ± 0.08	1.6 ± 0.3
9	4.8 ± 0.9	716 ± 56	6.6 ± 0.8	1.6 ± 0.5
11	4.7 ± 0.5	588 ± 17	7.9 ± 0.7	1.2 ± 0.5
12	3.7 ± 0.1	626 ± 12	5.9 ± 0.3	0.19 ± 0.02
13	3.61 ± 0.02	732 ± 18	5.0 ± 0.2	2.02 ± 0.02
14	4.6 ± 0.2	636 ± 8	7.3 ± 0.1	1.6 ± 0.1
15	5.34 ± 0.03	577 ± 1	9.27 ± 0.06	2.6 ± 0.1
16	4.82 ± 0.07	590 ± 9	8.16 ± 0.02	2.0 ± 0.1
17	6.7 ± 0.3	601 ± 106	11 ± 2	2.3 ± 0.4
18	7.88 ± 0.03	409 ± 1	19.25 ± 0.02	2.52 ± 0.01
20	8.7 ± 0.6	586 ± 6	14.9 ± 0.8	1.33 ± 0.03
21	6.9 ± 0.2	629 ± 1	10.9 ± 0.4	2.8 ± 0.3
22	8.1 ± 0.1	515 ± 4	15.8 ± 0.1	2.2 ± 0.2
23	10.8 ± 0.6	455 ± 2	24 ± 1	2.4 ± 0.1
24	6.0 ± 0.2	213 ± 1	28.2 ± 0.9	3.58 ± 0.05

^a Unless otherwise noted, all of the anthocyanins are 6-*O*-(acyl)-β-D-glucopyranosyl-(1→6)-[β-D-xylopyranosyl-(1→2)]-β-D-galactopyranosyl-(1→O³)-cyanidins. The data are the mean and range of two independent sets of measurements except for those for compounds 5 and 17, for which three and four sets of measurements, respectively, give means and standard deviations. ^b The compound numbers here are identical to those used in Dougall et al. (1998) and in Chart 1.

the hemiacetal was at higher concentration than the quinonoid forms. K_h and K_a were correlated with each other, and the correlation coefficient over the 20 observations was 0.844, which was very highly significant ($P < 0.01$).

The nonacylated compounds 1 and 3 have the biggest K_h and K_a values, indicating that, at equilibrium, they have the lowest proportion of red form and the highest proportion of hemiacetal and quinonoid forms of all the compounds examined here. Their k_2 values are smaller than those of all other compounds, and their k_1 values are among the largest measured here, showing that they display a combination of rapid formation of the hemiacetal and slow conversion of the hemiacetal back into the flavylium ion; that is, they retain less color than do the acylated anthocyanins.

The equilibrium constants for 24—the anthocyanin acylated with 4-hydroxyphenylpropionic acid—approach those of 1 and 3 and are larger than those of any other monoacylated anthocyanin. The rate constants for 24 are similar to those of 1 and 3 and at the extreme for all of the compounds measured. Thus, 24 is very similar to 1 and 3 and different from the other acylated compounds. Comparison of the values for 24 (side-chain saturated) with those for 9 (with the side-chain double bond) and 21 (without the side chain) shows that removal of the double bond leads to major increases in the equilibrium constants and reduces k_2 by a factor of ≥ 3 . The double bond of the cinnamic acids is therefore a structural component of the acyl groups, which leads to a major increase in the retention of anthocyanin color.

The acyl groups examined here allow two general groups of comparisons to be made. These are the effects of (1) cinnamoyl versus benzoyl groups and (2) electron-withdrawing or -donating substituents on the aromatic rings on the rate and equilibrium constants. The Ham-

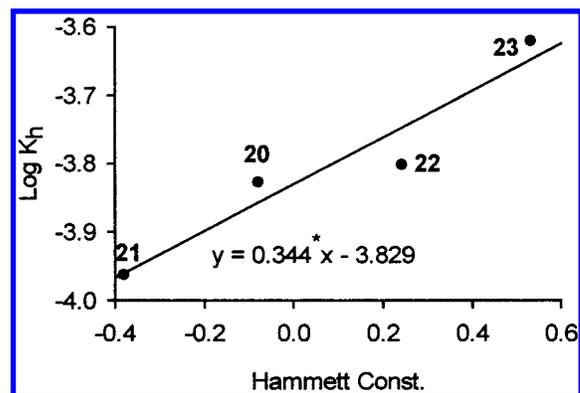


Figure 1. Relationship between log average equilibrium constants of the hydration reactions of some benzoylated anthocyanins and the electron-donating capacity of the substituents on the acyl group. The asterisk indicates that the coefficient was statistically significant with $0.01 < P < 0.05$. 21 on the graph refers to compound 21, etc.

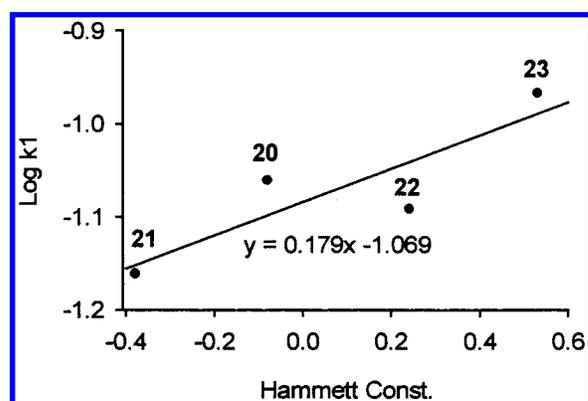


Figure 2. Relationship between log average rates of the hydration reactions of some benzoylated anthocyanins and the electron-donating capacity of the substituents on the acyl group. The absence of the asterisk indicates that the coefficient was not statistically significant ($0.05 < P$). 21 on the graph refers to compound 21, etc.

mett constant for para- and meta-substituents is a quantitative measure of their capacity to increase or decrease the electron density of aromatic ring systems, so that the second group of comparisons can be easily performed by comparing the log of the rate or equilibrium constants to the Hammett constants for the substituents on the cinnamoyl groups and on the benzoyl groups. When multiple substituents were present, the Hammett constants for the individual substituents were summed algebraically to give a combined constant (Jaffé, 1953).

The relationship between log K_h and the Hammett constants for the benzoylated compounds is linear and increases with increasing Hammett constant (Figure 1). The linear regression of the values of K_h on the Hammett constants is statistically significant (Figure 1). This indicates that the different values of K_h for the benzoylated anthocyanins are due to differences in the electron density in the aromatic ring caused by the substituents. The electron density effects of the substituents can be examined further because $K_h = k_1/k_2$. The linear regression of log k_1 on the Hammett constants is not significant (Figure 2), showing that the electron density in the benzoic acid ring does not affect the rate of formation of the hemiacetal. In contrast, the linear regression of log k_2 on the Hammett constants is highly significant (Figure 3), showing that the rate of

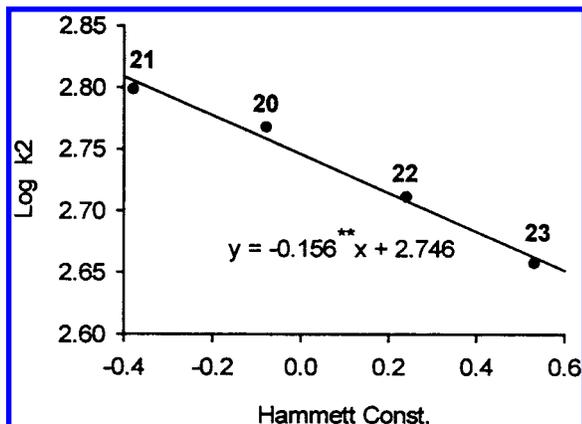


Figure 3. Relationship between log average rates of the dehydration reactions of some benzoylated anthocyanin hemiacetals and the electron-donating capacity of the substituents on the acyl group. The double asterisk indicates that the coefficient was statistically significant ($P < 0.01$). **21** on the graph refers to compound **21**, etc.

conversion of the hemiacetal into the colored flavylium ion is markedly increased as the electron density in the benzoyl ring is increased.

There was no significant linear regression of $\log K_a$ on the Hammett constants, showing that the equilibrium between quinonoid forms and the flavylium ion does not change with changing electron density in the benzoyl ring system.

Inspection of the linear plots of $\log K_h$, $\log k_1$, and $\log k_2$ versus the Hammett constants for the cinnamoylated anthocyanins (not shown) revealed that, when the values of these constants for **18** were plotted using $+0.81$ as the Hammett constant (March, 1992) for the para- NO_2 group, the points did not fall on the line given by the other para-substituted cinnamoylated anthocyanins. The $-\text{NO}_2$ group along with others has altered Hammett constants under some circumstances, due to "through-conjugation" effects (Sykes, 1987). When the modified value of $+1.27$ for the Hammett constant for the $-\text{NO}_2$ group (Sykes, 1987) was used, the values of **18** were at the regression lines for the para-substituted cinnamoylated anthocyanins. All linear regressions reported here have been calculated using $+1.27$ rather than $+0.81$ as the Hammett constant for the $-\text{NO}_2$ group.

The graphs of $\log K_h$, $\log k_1$, and $\log k_2$ against the Hammett constants for the cinnamoylated anthocyanins are shown in Figures 4, 5, and 6, respectively. Although the linear regressions of the values of $\log K_h$ and $\log k_1$ (but not of $\log k_2$) on the Hammett constants for all of the cinnamoylated anthocyanins examined are significant (data not shown), inspection of the graphs suggests that **5–8** and **12** behave differently from the other compounds. These compounds, which are meta-substituted, occupy space on the graphs that is separated from and below that of the other anthocyanins that are either para-substituted or unsubstituted. The linear regressions for the unsubstituted and the para-substituted anthocyanins are also shown in Figures 4–6. These regressions have a smaller value of P and smaller standard deviations of the constants from the regression lines than do the regressions when all cinnamoylated anthocyanins are included. Two conclusions come from these observations. First, most or all of the differences in the constants for the anthocyanins with para-

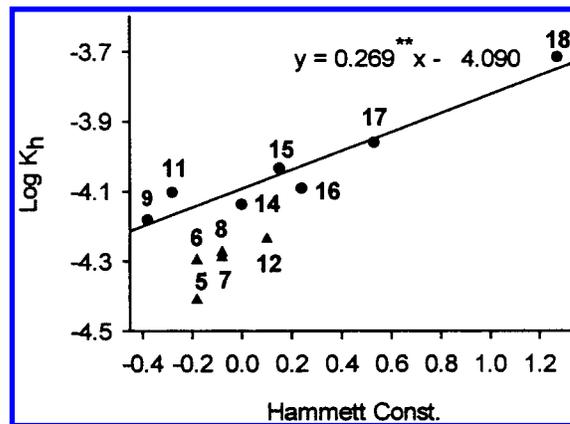


Figure 4. Relationship between log average equilibrium constants of the hydration reactions of some cinnamoylated anthocyanins and the electron-donating capacity of the substituents on the acyl group. The circles identify anthocyanins acylated with para-substituted cinnamic acids. The triangles identify those acylated with meta- or meta + para-substituted cinnamic acids. The double asterisk indicates that the coefficient was statistically significant ($P < 0.01$). **9** on the graph refers to compound **9**, etc.

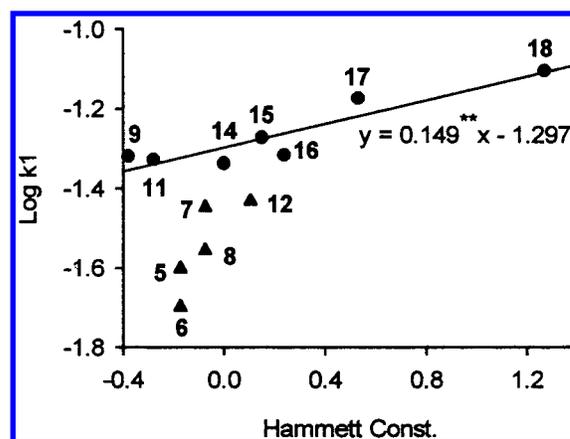


Figure 5. Relationship between log average rates of the hydration reactions of some cinnamoylated anthocyanins and the electron-donating capacity of the substituents on the acyl group. The circles identify anthocyanins acylated with para-substituted cinnamic acids. The triangles identify those acylated with meta- or meta + para-substituted cinnamic acids. The double asterisk indicates that the coefficient was statistically significant ($P < 0.01$). **9** on the graph refers to compound **9**, etc.

substituted cinnamoyl groups are due to differences in electron density in the acyl ring system. Second, for the anthocyanins with meta-substituted acyl groups some of the differences in their constants are due to differences in electron density in the acyl ring system and some are due to other factors. The possibility that steric effects alter the constants of the anthocyanins with meta-substituted acyl groups is indicated by the systematic changes in the constants for **11**, **12**, and **13**, which have the *p*-, *m*-, and *o*-methoxycinnamoyl acyl groups. Of these three compounds, the ortho-substituted **13** has the smallest K_h and k_1 and largest k_2 values, indicating that it maintains a higher concentration of the colored flavylium ion under the influence of pH compared to **11** and **12**. Steric effects of ortho-substituents have been observed in many reactions (March, 1992; Sykes, 1987).

There was no significant linear regression of $\log K_a$ on the Hammett constants, showing that the equilibri-

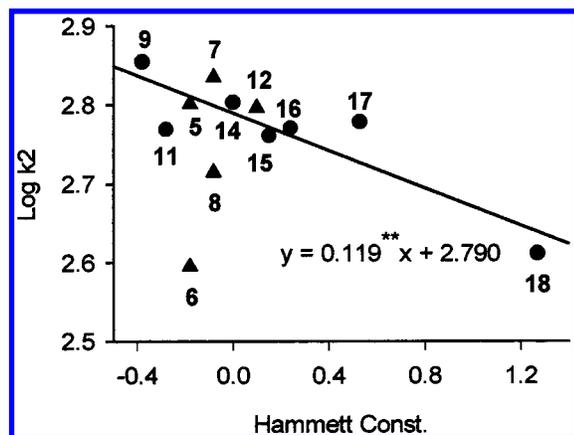


Figure 6. Relationship between log average rates of the dehydration reactions of some cinnamoylated anthocyanin hemiacetals and the electron-donating capacity of the substituents on the acyl group. The circles identify anthocyanins acylated with para-substituted cinnamic acids. The triangles identify those acylated with meta- or meta- + para-substituted cinnamic acids. The double asterisk indicates that the coefficient was statistically significant ($P < 0.01$). 9 on the graph refers to compound 9, etc.

um between quinonoid forms and the flavylium ion does not change with changing electron density in the cinnamoyl ring system.

The intercepts of the regression lines for $\log K_h$, k_1 , and the average of the K_a values for the benzoylated compounds are numerically larger than those for the cinnamoylated compounds, whereas the intercepts for the regressions of the $\log k_2$ values are very similar. This shows that the benzoylated compounds have less flavylium ion in equilibrium with the other species than do the cinnamoylated compounds; that is, the benzoylated compounds retain less color under the influence of pH than do the cinnamoylated compounds. This confirms the importance of the exocyclic double bond in the color retention of the acylated anthocyanins.

DISCUSSION

The anthocyanins acylated with benzoic acids display a greater tendency to form hemiacetals than do the anthocyanins acylated with the corresponding cinnamic acids, which identifies the importance of the exocyclic double bond in color retention. In their ^1H NMR spectra, anthocyanins with both types of acyl groups display an upfield shift of the H-4 of the flavylium nucleus relative to the nonacylated anthocyanins, but only the anthocyanins acylated with cinnamic acids display an upfield shift of the H-8 resonance of the flavylium nucleus (Dougall et al., 1998). These observations suggest that the two types of acyl groups interact differently with the flavylium nucleus.

The rates and the equilibrium constant for the hydration reaction and thus color retention by these anthocyanins are altered by the electron-donating capacity of substituents on the acyl group and by at least one additional factor associated with meta- and ortho-substitution on the acyl group. The latter may be steric effects.

For both the benzoylated and the cinnamoylated anthocyanins, the higher the electron density on the aromatic ring, the smaller the K_h for that compound and the smaller the tendency of that compound to lose its

color by hydration. This conclusion parallels that of Dangles and Elhajji (1994), who examined the effects of substituents attached to the flavylium nucleus.

In the anthocyanins studied here the substituents are not directly attached to the flavylium nucleus but are at the opposite end of a disaccharide chain from the flavylium nucleus. The explanation of the electronic effects of the acyl group substituents on the reactions of the flavylium nucleus is not immediately obvious. There is no direct pathway through the carbon framework by which electronic effects from the acyl group can exert an influence on the flavylium nucleus, and, as a result, other explanations are needed. However, the acyl groups in these molecules are physically near the flavylium nucleus (at least part of the time) as shown by the chemical shifts in the ^1H NMR spectra of the acylated versus the nonacylated anthocyanins (Dougall et al., 1998; Gakh et al., 1998). This has been interpreted in terms of "intramolecular stacking" (Dangles et al., 1993; Goto and Kondo, 1991) with the acyl group near the flavylium nucleus in the stacked form, the acyl group away from the flavylium nucleus in the unstacked form, and these two forms in equilibrium with each other (Dangles et al., 1993). This model of the monoacylated anthocyanin molecule is inadequate because it always leaves one face of the flavylium ion available to attack by water. We are developing models of monoacylated anthocyanins using NMR and quenched molecular dynamics to attempt to understand the effects of the acyl groups on color retention and other properties of the anthocyanins.

ACKNOWLEDGMENT

We thank Dr. Elizabeth Howell, Biochemistry Department, for access to the UV-vis spectrometer and for help and guidance with the measurements.

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Received for review December 14, 1998. Revised manuscript received June 11, 1999. Accepted June 14, 1999. USDA-NRICGP Grant 94-37500-0512 supported this work.

JF9813485