Color Stability and Structural Transformations of Cyanidin 3,5-Diglucoside and Four 3-Deoxyanthocyanins in Aqueous Solutions

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The acidity constants together with the equilibrium distribution of the flavylium cation (AH^+), quinoidal base (A), carbinol pseudobase (B), and chalcone (C) of cyanidin 3,5-diglucoside and four related 3-deoxyflavylium salts in water at 25 °C have been determined by the pH-jump method. For cyanidin 3,5-diglucoside, the equilibrium constants for the acid–base, hydration, and tautomeric equilibria were 4.36×10^{-4} M (pK_a' 3.38), 5.85×10^{-3} M (pK_h' 2.23) and 0.16, respectively. The acidity constant for the chalcone formation reaction starting from the flavylium cation of cyanin was 9.54×10^{-4} M (pK_c' 3.02). Thus, in acidic aqueous media and at 25 °C, the most favored species for this anthocyanin is the colorless carbinol pseudobase. 4′-Hydroxy-, 4-methyl-7-hydroxy-, and 4′-methoxy-4-carboxyl-7-hydroxyflavylium salts were essentially stable in the form of flavylium cation in fast equilibrium with the quinoidal base. Apigeninidin existed primarily in the flavylium cationic form at pH ≤4 and as a mixture of the three neutral forms at higher pH. Unlike the common anthocyanin, then, the 3-deoxyflavylium salts could be of advantage to color foods and beverages at neutral pH.

The last decade has seen the development of an increased interest in the chemistry of anthocyanins, a family of glycosidic pigments responsible for most of the orange, red, and blue colors of flowers and fruits. The reasons behind this interest are several: the most important perhaps is the need to develop safe, economical, and efficient food colors to replace banned coal tar or azo dyes (Meggos, 1984; Francis, 1984). For this purpose anthocyanins possess the following advantages: they have been consumed by man for countless generations without apparent adverse effects to health; they are brightly colored especially in the red region; and they are rather soluble, which simplifies their incorporation in aqueous food systems. But they also have disadvantages in that they are not very stable chemically, they are difficult to purify, and their tinctorial power is nearly 100 times lower than the coal tar dyes (Riboh 1977).

In addition to the red anthocyanins there are a few natural yellow anthocyanidins (apigeninidin, luteolinidin, tricetinidin) and several anthocyanin analogues whose structures differ from the natural pigments either in the hydroxylation pattern of the A ring or on the presence of unusual substituents attached to the pyrylium ring (Iacobucci and Sweeny, 1983). The natural yellow anthocyanidins are the chemical ancestors of the red and purple anthocyanidins and are much more stable in slightly acidic solutions than their red and purple counterparts (Sweeny and Iacobucci, 1983). The reason for the difference in stability between these two classes of anthocyanins is obviously structural and appears to be related to two specific structural factors, namely the pattern of aromatic hydroxylation and the chemical substitution at C_4 (Brouillard, 1982; Brouillard et al., 1982). These conclusions, however, were arrived at from the study of a limited number of compounds.

The present paper discusses color stability and structural transformations of cyanidin 3,5-diglucoside, 4′,5,7-trihydroxyflavylium, 4′-hydroxyflavylium, 4-methyl-7-hydroxyflavylium, and 4′-methoxy-4-carboxyl-7-hydroxyflavylium chloride in aqueous solutions.

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THEORY

The transformations anthocyanins, anthocyanidins, 3-deoxyanthocyanins, and 3-deoxyanthocyanidins undergo in aqueous media are illustrated in Figure 1. Upon dissolution, a complex equilibrium is quickly established between two colored species, flavylium cation (AH^+) and quinoidal base (A), and two colorless ones, carbinol pseudobase (B) and chalcone (C), resulting from the hydration of AH^+. Thus, the final color of the solution at equilibrium is the direct consequence of the equilibrium rate constants K_a', K_h', and K_T, controlling the ionization, hydration, and tautomeric reactions, respectively. $K_a' = ([A]/[AH^+])a_{H^+}$, $K_h' = [B]/[AH^+]a_{H^+}$, $K_T = [C]/[B]$, and a_{H^+} is the activity of the hydronium ion ($pH = -\log a_{H^+}$).

The concentration of AH^+, A, B, and C can be expressed as a function of the equilibrium constants K_a', K_h', and K_T, the acidity of the medium a_{H^+}, and the overall pigment concentration C_0 as shown by

$$[AH^+] = (a_{H^+}/\delta)C_0 \quad (1)$$

$$[A] = (K_a'/\delta)C_0 \quad (2)$$

$$[B] = (K_h'/\delta)C_0 \quad (3)$$

$$[C] = (K_h'K_T/\delta)C_0 \quad (4)$$

where

$$C_0 = [AH^+] + [A] + [B] + [C] = [AH^+]\frac{K_a' + K_h' + K_h'K_T + a_{H^+}}{a_{H^+}}$$

$$\delta = K_a' + K_h' + K_h'K_T + a_{H^+}$$

Knowing K_a', K_h', K_T, and the acidity, one can calculate the relative amounts of $[X]/C_0$ (where [X] is the concentration of AH^+, A, B, or C) for a particular anthocyanin at a given temperature.

The equilibrium constants K_a', K_h', and K_T can be obtained by a variety of system perturbation techniques, namely temperature jump, pressure jump, pH jump, and concentration jump (Bernasconi, 1976). For this work we used the pH-jump method (Brouillard et al., 1978).

EXPERIMENTAL SECTION

Cyanidin 3,5-diglucoside was purchased from Carl Roth GmbH Co., Karlsruhe, and repurified by polyvinylpolypyrrolidone (PVP) column chromatography (Wrolstad and Struthers, 1970). The purity (≥98%) of the anthocyanin was checked by proton magnetic resonance (^1H NMR)

0021-8561/87/1435-0422$01.50/0 Published 1987 by the American Chemical Society

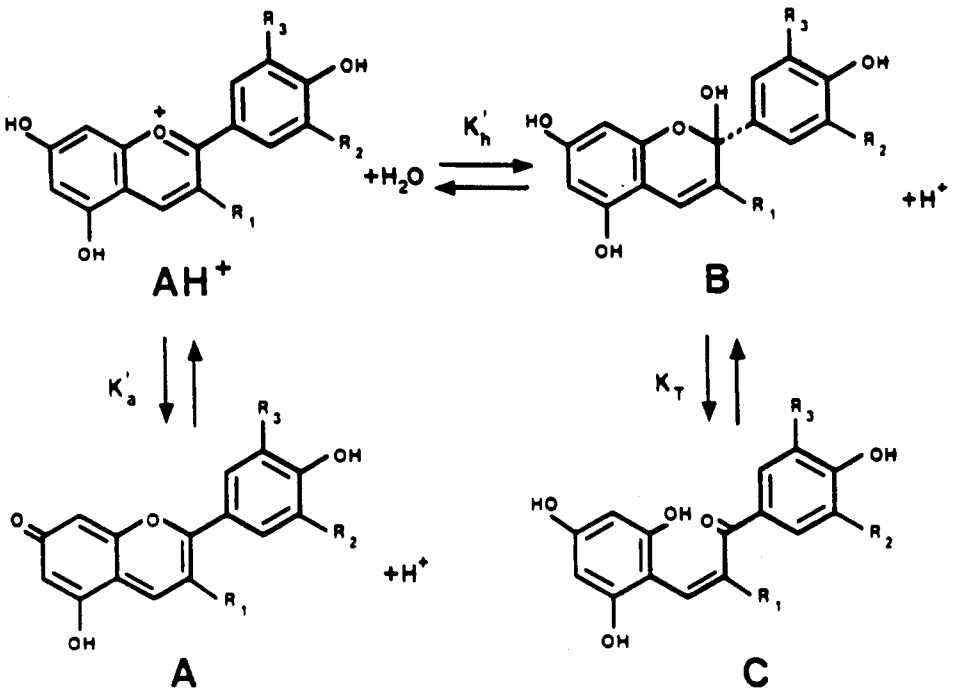

Figure 1. Structural transformations of anthocyanins. R_1 is usually a glycosyl, and R_2 and R_3 are H, OH, or OCH_3.

following the procedure of Goto et al. (1978). The 3-deoxyanthocyanins were supplied by G. A. Iacobucci (The Coca Cola Co., Atlanta, GA).

For all the experiments the required amount of pigment (analytical concentration, 2×10^{-6}–5×10^{-5} M) was dissolved in distilled water and the resultant solution allowed to equilibrate in the dark at 25 °C. When necessary, the solution was brought to pH 1–4 by adding a few microliters of concentrated hydrochloric acid solution. pH-jump absorbance changes were induced as described by Brouillard et al. (1978) and recorded on a Perkin-Elmer Lambda 5 UV/vis spectrophotometer (Perkin-Elmer, Analytical Instruments, Norwalk, CT) fitted with a thermostated 1.0-cm path length cuvette and a magnetic stirring device. Throughout the whole experiment the temperature was kept at 25 °C.

RESULTS AND DISCUSSION

Typical UV/vis spectra of cyanidin 3,5-diglucoside (2.8×10^{-5} M in water) are presented in Figure 2. In accordance with the outlined theory, the intensity of the absorption at 509 nm (the λ_{max} of cyanin in water) markedly decreased as the pH was raised. Simultaneously, the absorption at 275 nm slightly increased as a result of the formation of a new compound with a peak at this wavelength. This change in absorbance with increasing pH reflects the conversion of the flavylium cation (AH^+) to the colorless carbinol pseudobase (B). The lack of well-defined isobestic points between the spectral curves at pH 2.2–3.8, however, indicates that in the solutions at these pHs there were more than two chemical species (Morton, 1962). As the pH was raised further, the presence of two quinoidal bases (A, A^-) and two chalcone forms (C, C^-) was evident. Thus, from the spectral characteristics at varying pH, six species of cyanidin 3,5-diglucoside were detected.

From absorbance values of equilibrated solutions at known pHs the overall acidity constant, K', of each pigment studied was calculated from

$$pK' = \log\left[\frac{D_{acid} - D}{D}\right] - pH \qquad (5)$$

where D_{acid} is the absorbance at the visible λ_{max} of a pigment solution that is totally converted to the flavylium structure (AH^+) and D is the absorbance at the visible λ_{max} and at the equilibrium pH. This method for determining K' is well adapted to all anthocyanins and structurally related compounds and is independent of the relative quantities of the neutral species A–C (Brouillard, 1982). Knowledge of K' is useful not only because it is a measure of the stability of the flavylium cation as a function of pH but also because it simplifies the calculation of K_a', K_h', and K_T:

$$K' = K_a' + K_h' + K_c' \qquad (6)$$

K_c' is the acidity constant for the chalcone pseudobase (C) formation reaction starting from the flavylium cation AH^+: $K_c' = ([C]/[AH^+])a_{H^+} = K_h'K_T$. Once K' is known, one can evaluate the ratios of the values of the different acidity constants K_a', K_h', and K_c' in order to obtain their absolute values. Thus

$$K_a'/K_h' = (D_0 - D_1)/(D_1 - D_2) \times 1/(1 - \epsilon_A/\epsilon_{AH^+}) \qquad (7)$$

$$K_h'/K_c' = 1/K_T = (D_1 - D_2)/(D_2 - D_f) \qquad (8)$$

D_0, D_1, D_2, and D_f are the absorbances at the visible λ_{max} at the initial pH (pH_0), immediately after the proton-transfer equilibrium is reached, immediately after the hydration equilibrium is attained, and at the final pH (pH_f), respectively. Their values are obtained directly from experimental pH-jump relaxation spectra such as that presented in Figure 3. The term $\epsilon_A/\epsilon_{AH^+}$ is the ratio of the molar extinction coefficients of A and AH^+. Its value is practically identical with the reciprocal of the ratio of the initial absorbance D_0 of a sufficiently acidified solution to the absorbance D_1 of the same solution rapidly brought to a slightly acidic pH_f, so as to fully convert the initially present flavylium cation AH^+ to the quinoidal base.

The values of the equilibrium constants for the presently investigated pigments are presented in Table I. With the exception of the pK' value for cyanidin 3,5-diglucoside (Timberlake and Bridle, 1967), the equilibrium constants for apigeninidin (Brouillard et al., 1982), and the equilibrium constants for 4′-hydroxyflavylium (McClelland and McGall 1982), none of these values have been reported prior to this work. The pK' value for cyanin reported by

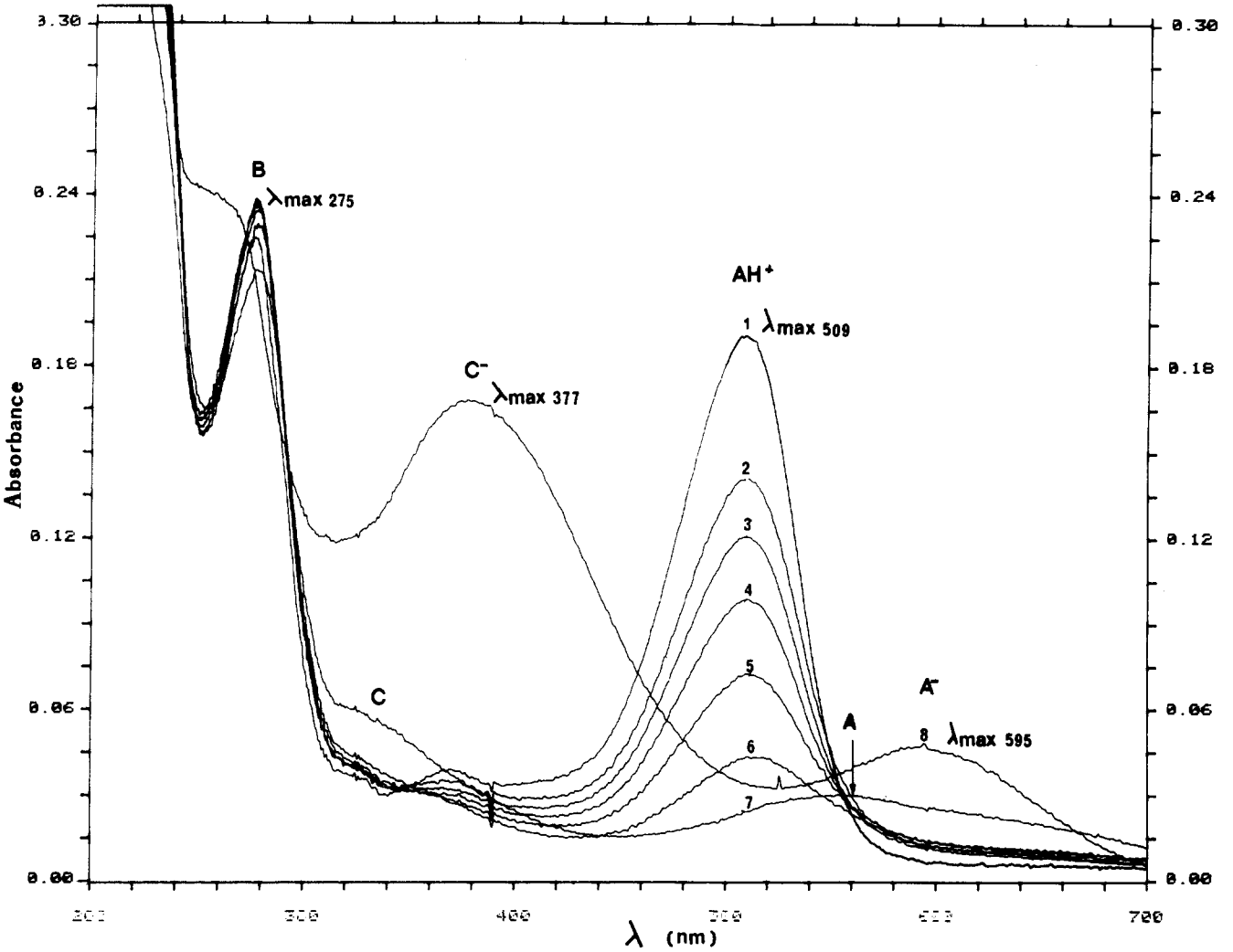

Figure 2. Spectra of cyanidin 3,5-diglucoside at varying pH values: (1) 2.21, (2) 2.49, (3) 2.58, (4) 2.72, (5) 2.91, (6) 3.77, (7) 4.81, (8) 10.66. Solutions were left 30 min in the dark to equilibrate.

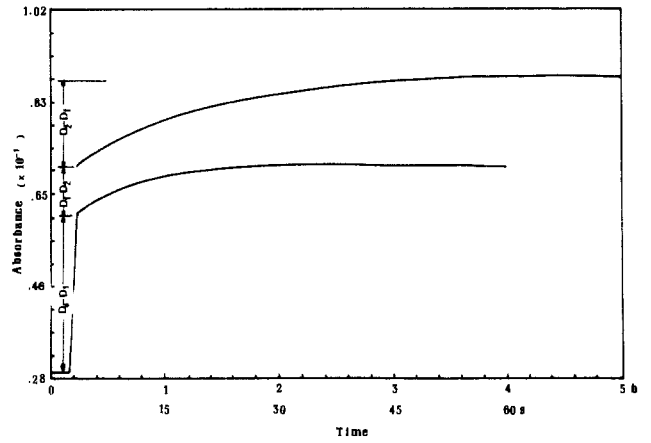

Figure 3. Variation of absorbance at 467 nm with time on acidification to pH 1.3 of a pH 5.5 solution of apigeninidin. Lower curve refers to lower time scale; upper curve refers to upper time scale.

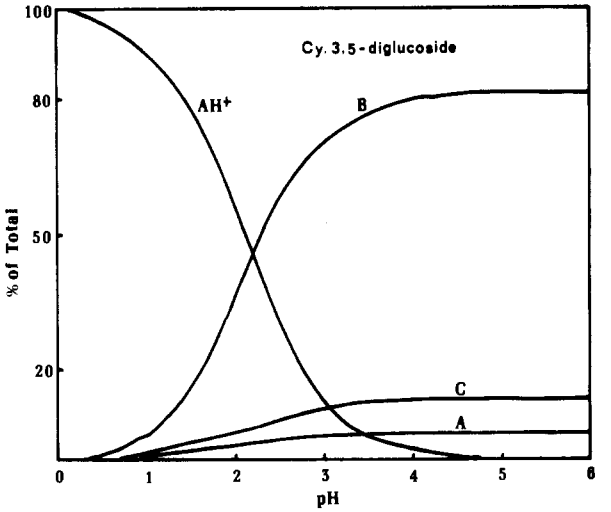

Figure 4. Equilibrium distribution of AH^+, A, B, and C forms for cyanidin 3,5-diglucoside as a function of pH.

Timberlake and Bridle (1967) is 2.15 ± 0.06, which is essentially the same as the value of 2.08 ± 0.04 measured in this study.

The equilibrium distributions of AH^+, A, B, and C for cyanidin 3,5-diglucoside are presented in Figure 4. In very acidic solutions (pH <0.5) the red cation AH^+ was the sole structure. With increasing pH its concentration decreased as hydration of the colorless carbinol pseudobase occurred, the equilibrium being characterized by a pK_h' value of 2.23 ± 0.10, when equal amounts of both forms existed. Already at this pH small amounts of the colorless chalcone C and the blue quinoidal base A were also present, and the proportions of these and the carbinol base increased with increasing pH at the expense of the red cationic form AH^+ to about pH 4.5. Between pH 4 and 6 very little color remained in the solution since the amounts of both colored forms, AH^+ and A, were very small. The equilibrium

Table I. Acidity Constants of Cyanidin 3,5-Diglucoside and Four 3-Deoxyanthocyanins at 25 °C in the Dark

pigment		K', M (pK')	K_a', M (pK_a')	K_h', M (pK_h')	K_T	K_c', M (pK_c')
cyanidin 3,5-diglucoside		$(8.5 \pm 0.8) \times 10^{-3}$ (2.08 ± 0.04)	$(4.36 \pm 1.0) \times 10^{-4}$ (3.38 ± 0.15)	$(5.85 \pm 0.5) \times 10^{-3}$ (2.23 ± 0.10)	0.16 ± 0.02	$(9.54 \pm 0.20) \times 10^{-4}$ (3.02 ± 0.09)
4',5,7-trihydroxyflavylium chloride (apigeninidin)	*a*	$(1.11 \pm 0.19) \times 10^{-4}$ (3.96 ± 0.08)	$(6.77 \pm 0.32) \times 10^{-5}$ (4.17 ± 0.01)	$(8.07 \pm 1.27) \times 10^{-6}$ (5.10 ± 0.13)	4.89 ± 1.33	$(3.53 \pm 0.5) \times 10^{-5}$ (4.46 ± 0.06)
	b	$(1.0 \pm 0.1) \times 10^{-4}$ (4.0 ± 0.05)	$(6.20 \pm 0.5) \times 10^{-5}$ (4.20 ± 0.05)	$(7.0 \pm 1.5) \times 10^{-6}$ (5.15 ± 0.10)	4.4 ± 0.5	$(3.1 \pm 0.4) \times 10^{-5}$ (4.50 ± 0.05)
4'-hydroxyflavylium chloride		$(6.39 \pm 1.03) \times 10^{-5}$ (4.20 ± 0.07)	$(3.18 \pm 2.0) \times 10^{-5}$ (4.61 ± 0.45)	$(3.61 \pm 1.92) \times 10^{-6}$ (5.48 ± 0.21)	0.61 ± 0.17	$(2.22 \pm 1.17) \times 10^{-6}$ (5.70 ± 0.21)
4-methyl-7-hydroxyflavylium chloride		$(2.37 \pm 0.67) \times 10^{-5}$ (4.64 ± 0.12)	$(2.20 \pm 1.0) \times 10^{-5}$ (4.66 ± 0.10)	$(1.08 \pm 1.00) \times 10^{-6}$ (5.97 ± 0.08)	2.00 ± 0.90	$(2.37 \pm 1.53) \times 10^{-6}$ (5.62 ± 0.20)
4'-methoxy-4-carboxyl-7-hydroxyflavylium chloride		$(1.71 \pm 0.38) \times 10^{-5}$ (4.78 ± 0.10)	$(1.24 \pm 0.32) \times 10^{-5}$ (4.92 ± 0.12)	$(6.39 \pm 3.0) \times 10^{-7}$ (6.37 ± 0.15)	7.08 ± 1.30	$(4.50 \pm 2.02) \times 10^{-6}$ (5.40 ± 0.12)

[a] This study. [b] Brouillard et al. (1982).

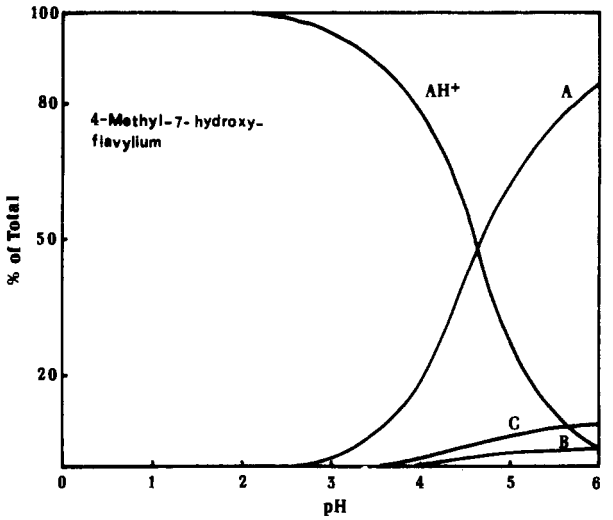

Figure 5. Equilibrium distribution of 4-methyl-7-hydroxyflavylium chloride as a function of pH.

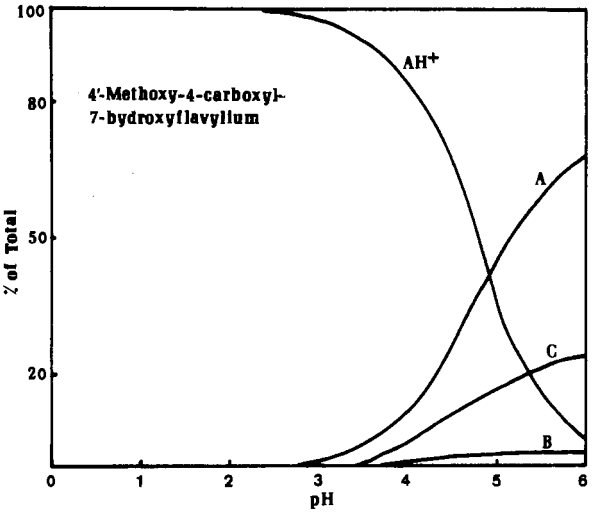

Figure 6. Equilibrium distribution of 4'-methoxy-4-carboxyl-7-hydroxyflavylium chloride as a function of pH.

between these two species was characterized by a pK_a' value of 3.38 ± 0.15.

A comparison of the acidity constants of cyanidin 3,5-diglucoside, presented in Table I, with the acidity constants of malvidin 3-glucoside (Brouillard et al., 1977) and malvidin 3,5-diglucoside (Timberlake and Bridle, 1967; Brouillard and Dubois, 1977) reveals that the hydration constant K_h' of cyanidin 3,5-diglucoside is lower than that of malvidin 3-glucoside, but higher than that of malvidin 3,5-diglucoside. Thus, the color stability of cyanin is higher than that of malvin but lower than that of malvidin 3-glucoside. This is probably due to the effect of the OH group at carbon 3' of cyanin. Also, the presence of the 5-glycosyl may decrease color stability by improving the efficiency of the hydration process. At 25 °C, K_h' is 2.5 × 10^{-3} and 5.85 × 10^{-3} M for malvidin 3-glucoside and cyanidin 3,5-diglucoside, respectively. The corresponding values of K_c' are 3.0 × 10^{-4} and 9.54 × 10^{-4} M.

By varying the substitution pattern of the flavylium ring anthocyanidins that can exist primarily in the colored quinoidal form, A can be prepared. This is exemplified by 4-methyl-7-hydroxyflavylium (Figure 5) and 4'-methoxy-4-carboxyl-7-hydroxyflavylium chloride (Figure 6). As can be noted, these compounds are essentially stable in the form of flavylium cation in fast equilibrium with the quinoidal base. At equilibrium and in nearly neutral solutions (pH 6), the colored quinoidal forms account for about 83% and 67% of the analytical concentration of the 4-methyl-7-hydroxyflavylium and 4'-methoxy-4-carboxyl-7-hydroxyflavylium chloride, respectively. For 4-methyl-7-hydroxyflavylium chloride the strong predominance of A over the two other neutral bases B and C results from the relatively larger value of K_a' that is 20 and 10 times greater than K_h' and K_c', respectively.

Methoxylation at carbon 4' and carboxylation at carbon 4 further improved the stability of the flavylium cation (Figure 6). Although, at pH 6, the concentrations of the colorless forms B and C were higher for 4'-methoxy-4-carboxyl-7-hydroxy- than for 4-methyl-7-hydroxyflavylium chloride. The difference in equilibrium distribution of the AH^+, A, B, and C forms of these two compounds results primarily from the approximately 4 times lower value of the tautomeric ring-chain equilibrium constant, K_T, of 4-methyl-7-hydroxyflavylium chloride.

A comparison of the equilibrium distribution of the four species AH^+, A, B, and C, of 4'-methoxy-4-carboxyl-7-hydroxyflavylium (Figure 6) to that of the analogue 4'-methoxy-4-methyl-7-hydroxyflavylium reported by Brouillard et al. (1982) reveals that the 4-carboxyl has no influence on the stability of the flavylium cation, but considerably increases the chalcone content. Therefore, one can assume that in the presence of CO_2H or CO_2^- at C-4 and pH ≥4 the equilibrium between A and C is shifted toward the C form. The increase in chalcone content is also caused by the difference in K_T, which is about 9 times larger for the carboxylated compound than for the methylated analogue.

Another flavylium salt whose cationic form AH^+ is present in significant concentration at pH 6, and whose quinoidal form A dominates the mixture at pH 4.5–5.0, is 4'-hydroxyflavylium (Figure 7). This pigment is interesting because it has only one free hydroxyl and deprotonation can occur only at carbon 4'. The consequences of this unique structural characteristic are (i) that its

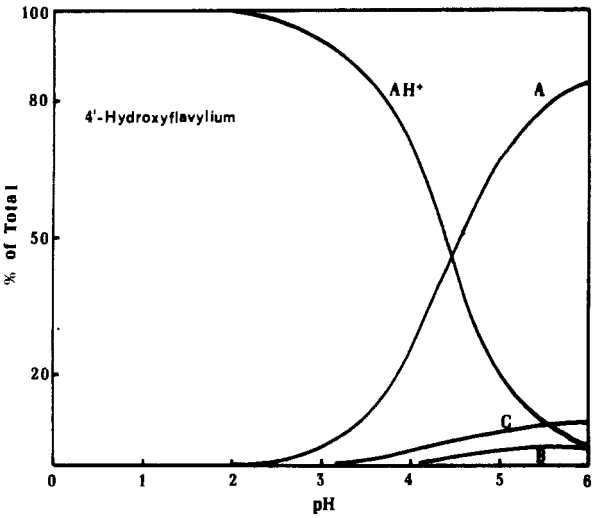

Figure 7. Equilibrium distribution of 4′-hydroxyflavylium chloride as a function of pH.

acid–base equilibrium constant K_a' is related only to the proton loss at carbon 4′ and (ii) that the methoxyl, glycosyl, or carboxyl groups play no role in determining the magnitude of K_a'. Thus, the relatively lower value of K_a' for the 4-methyl-7-hydroxyl and 4′-methoxy-4-carboxyl-7-hydroxyflavylium chloride as compared to 4′-hydroxyflavylium chloride (Table I) suggests that proton loss at carbon 7 occurs more easily than at carbon 4′. Structural modifications, however, by virtue of electronic and steric effects, as well as solvent and salt effects, may also play a role in determining the values of the equilibrium constants of different compounds.

McClelland and McGall (1982) reported that at pH 7.2 the 4′-hydroxyflavylium ion exists as a mixture of 37% pseudobase, 33% *cis*-chalcone, and 30% quinoidal base. These results are different from ours, and the reason for the difference is most likely related to the high pH (7.2) of their starting solution that we believe contained ionized chalcone that was not converted to pseudobase and quinoidal forms when the solution was acidified to pH 2.7. Therefore, the end result was observation of lower concentration of quinoidal base and higher concentrations of pseudobase and chalcone. Our starting solutions were at pH 5–6, and they were acidified to pH 1–2.

Apigeninidin chloride, the last pigment of this series, was included in this study to ascertain that the pH-jump method, used to determine the acidity constants, was reproducible over time and that the results of this study could be compared to those reported by Brouillard et al. (1977, 1982). As can be noted from the two sets of acidity constants of apigeninidin presented in Table I, there was essentially no difference between our results and those of Brouillard et al. (1982).

CONCLUSIONS

Depending on their particular substitution pattern and pH, anthocyanins and related compounds can exist in the form of the red or yellow flavylium cation (AH^+), red or blue quinoidal base (A), colorless carbinol pseudobase (B), and chalcone (C). The relative amounts of AH^+, A, B, and C at equilibrium are determined by the rate constants K_a', K_h', and K_T. These constants, together with the equilibrium distribution of AH^+, A, B, and C of cyanidin 3,5-diglucoside and four related 3-deoxyflavylium salts were determined. For cyanidin 3,5-diglucoside the equilibrium between the flavylium cation and the three neutral forms A–C occurred at pK 3.38, 2.23, and 3.02, respectively. Thus, in acidic aqueous media (pH 1–6) and 25 °C, the most favored neutral species for this pigment is the colorless carbinol pseudobase. 4-Hydroxy-, 4-methyl-7-hydroxyl-, and 4′-methoxy-4-carboxyl-7-hydroxyflavylium salts were essentially stable in the form of flavylium cation in fast equilibrium with the quinoidal base. Thus, unlike natural anthocyanins these 3-deoxyflavylium salts could be of advantage to color foods and beverages at neutral pH.

LITERATURE CITED

Bernasconi, C. F. *Relaxation Kinetics*; Academic: New York, 1976.
Brouillard, R. *Anthocyanins as Food Colors*; Markakis P., Ed.; Academic: New York, 1982; p 1.
Brouillard, R.; Dubois, J. E. *J. Am. Chem. Soc.* **1977**, *99*, 1359.
Brouillard, R.; Delaporte, B.; Dubois, J. E. *J. Am. Chem. Soc.* **1977**, *99*, 8461.
Brouillard, R.; Delaporte, B.; Dubois, J. E. *J. Am. Chem. Soc.* **1978**, *100*, 6202.
Brouillard, R.; Iacobucci, G. A.; Sweeny, J. G. *J. Am. Chem. Soc.* **1982**, *104*, 7585.
Francis, F. J. *Developments in Food Colors*; Walford, G., Ed.; Elsevier Applied Science: London, 1984; Vol. 2, p 233.
Goto, T.; Takase, S.; Kondo, T. *Tetrahedron Lett.* **1978**, *27*, 2413.
Iacobucci, G. A.; Sweeny, J. G. *Tetrahedron* **1983**, *39*(19), 3005.
McClelland, R. A.; McGall, G. H. *J. Org. Chem.* **1982**, *47*, 3730.
Meggos, H. N. *Food Technol.* **1984**, *34*(1), 20.
Morton, R. A. *Nature (London)* **1962**, *193*, 314.
Riboh, M. *Chilton's Food Eng.* **1977**, *49*(5), 66.
Sweeny, J. G.; Iacobucci, G. A. *J. Agric. Food. Chem.* **1983**, *31*, 531.
Timberlake, C. F.; Bridle, P. *J. Sci. Food Agric.* **1967**, *18*, 473.
Wrolstad, R. E.; Struthers, B. J. *J. Chromatogr.* **1970**, *55*, 405.

Received for review June 30, 1986. Accepted November 10, 1986.