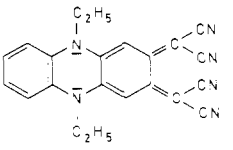
homologue of **3**. This means that two ethyl groups on the B-ring nitrogens in TEPP replace the two methyl groups of **3**. The strong correlations between physical and chemical properties ascertain the structure of TEPP as



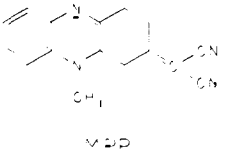
TEPP

The reaction path could be similar as the one proposed for the (2 + 2) cycloaddition of TCNE to cyclic thioenol ether,[19] with the final abstraction of H_2 per mole of adduct.

The structure and the physical properties of this new class of compounds are strongly related to the similar (tricyanovinyl)-amines.[8] (Tricyanovinyl)amines have found widespread interest because of their interesting optical[10] and dyeing[8] properties and because of their capability to yield charge-transfer complexes with donors *and* acceptors.[20] Since **3** and TEPP contain both π-donating and π-accepting groups in the molecule, a similar charge-transfer chemistry can be expected. The high chemical and thermal stability, the symmetry of the molecule, and the presence of two propanedinitrile groups make these dyes expecially interesting.

(20) Sandman, D. J.; Richter, A. F. *J. Am. Chem. Soc.* **1979**, *101*, 7079–7080.

MPP. The reaction product between $MP^+PF_6^-$ and TCNE in acetonitrile is again a deep blue dye which is formulated as



MPP

It can be obtained additionally in an immediate reaction between $MP^+PF_6^-$ and malonodinitrile and from a solution of malonodinitrile with M_2P in DMF or acetonitrile which contains equimolar amounts of bromine as an oxidation agent. The latter reaction leads primarily to M_2P^+ and after partial degradation of M_2P^+ to NMP^+ which is involved in the above mentioned reaction. Similar "oxidative demethylations" of M_2P^+ species have been observed earlier.[12]

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Registry No. 1, 83720-86-9; **2**, 83720-87-0; **3**, 83720-88-1; M_2P, 15546-75-5; TCNE, 670-54-2; TEPP, 83720-89-2; E_2P, 62248-00-4; MDN, 109-77-3; $MP^+\cdot PF_6^-$, 65149-30-6; MPP, 83720-90-5.

Supplementary Material Available: Listing of observed and calculated structure factors and a listing of the anisotropic temperature factors (9 pages). Ordering information is given on any current masthead page.

Chemistry of Anthocyanin Pigments. 9.[1] UV–Visible Spectrophotometric Determination of the Acidity Constants of Apigeninidin and Three Related 3-Deoxyflavylium Salts

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Contribution from the Institut de Topologie et de Dynamique des Systèmes de l'Université Paris VII, associé au CNRS, 1, rue Guy de la Brosse 75005 Paris, France, and the Corporate Research and Development Department, The Coca-Cola Company, Atlanta, Georgia 30301.
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Abstract: The equilibrium constants for the structural transformations of some 3-deoxyanthocyanidins in water at 25 °C have been measured by using the pH-jump method. This method has been described previously.[2] According to their particular substitution patterns, hydroxylated flavylium salts can exist in slightly acidic media in several neutral forms: the quinoidal bases A, the carbinol pseudobase B, and the chalcone pseudobase C. Two of the compounds investigated, namely 4′,5,7-trihydroxyflavylium (apigeninidin) and 4′-methoxy-4-methyl-5,7-dihydroxyflavylium chlorides, exist essentially as a mixture of the three neutral forms A, B, and C, the colored species A being the most abundant. As expected, 4′,7-dihydroxyflavylium chloride is stable in the open chalcone structure C. This result is in good agreement with the catalytic light effect generally observed for the ring-closure reaction of this species leading to the flavylium cation AH^+. Only for the monohydroxylated pigment 4′-methoxy-4-methyl-7-hydroxyflavylium chloride is the quinoidal base A perfectly stable, whatever the pH. In contrast to natural anthocyanins, the hydration of the pyrylium ring is less efficient and occurs, therefore, at much higher pH values (pH 5–6). Proton loss from the phenolic acidic hydroxyl groups of the flavylium cation takes place in the usual acidity range (pH 4–5), indicating that these groups are strongly hydrogen bonded to the surrounding water molecules. The chalcone content is much higher than for the anthocyanins, and for 4′,7-dihydroxyflavylium chloride for instance, the value for the equilibrium ratio of the chalcone to the carbinol is as high as 20.6.

Introduction

In a previous paper, a general method for measuring the equilibrium constants associated to the structural transformations of anthocyanins in aqueous solutions was described.[2] Anthocyanins are glycosylated polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium (flavylium) salts. This method is based on pH-jump experiments, where the position of the system, initially

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(1) Part 8: Brouillard, R. In "Anthocyanins as Food Colors"; Markakis, P., Ed.; Academic Press: New York, 1982; p 1.
(2) Brouillard, R.; Delaporte, B.; Dubois, J. E. *J. Am. Chem. Soc.* **1978**, *100*, 6202.

0002-7863/82/1504-7585$01.25/0

Table I. Acidity Constants at 25 °C in the Dark and $I < 10^{-2}$ M[a]

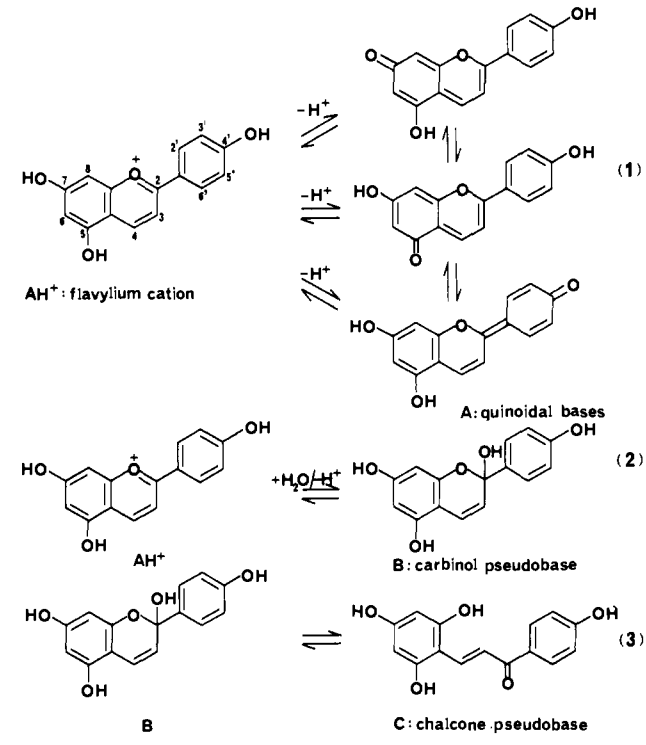
pigment	K', M pK'	K'_a, M pK'_a	K'_h, M pK'_h	K_T	K'_c, M pK'_c
4',7-dihydroxyflavylium chloride (1)	$8.9\ (\pm0.4) \times 10^{-4}$ 3.05 (±0.02)	$5.0\ (\pm0.5) \times 10^{-5}$ 4.30 (±0.05)	$3.9\ (\pm0.4) \times 10^{-5}$ 4.40 (±0.05)	20.6 (±1)	$8.0\ (\pm0.4) \times 10^{-4}$ 3.10 (±0.02)
4',5,7-trihydroxyflavylium chloride (apigeninidin) (2)	$1.0\ (\pm0.1) \times 10^{-4}$ 4.0 (±0.05)	$6.2\ (\pm0.5) \times 10^{-5}$ 4.20 (±0.05)	$7.0\ (\pm1.5) \times 10^{-6}$ 5.15 (±0.1)	4.4 (±0.5)	$3.1\ (\pm0.4) \times 10^{-5}$ 4.50 (±0.05)
4'-methoxy-4-methyl-5,7-dihydroxyflavylium chloride (3)	$7.9\ (\pm0.5) \times 10^{-5}$ 4.10 (±0.03)	$4.9\ (\pm0.5) \times 10^{-5}$ 4.30 (±0.05)	$9\ (\pm2) \times 10^{-6}$ 5.05 (±0.1)	2.2 (±0.2)	$2.0\ (\pm0.3) \times 10^{-5}$ 4.70 (±0.07)
4'-methoxy-4-methyl-7-hydroxyflavylium chloride (4)	$1.6\ (\pm0.1) \times 10^{-5}$ 4.80 (±0.03)	$1.4\ (\pm0.1) \times 10^{-5}$ 4.85 (±0.04)	$8.4\ (\pm2) \times 10^{-7}$ 6.07 (±0.1)	0.8 (±0.3)	$8.2\ (\pm2) \times 10^{-7}$ 6.08 (±0.1)
malvidin 3-glucoside chloride[b] (Mv)	$2.8\ (\pm0.2) \times 10^{-3}$ 2.55 (±0.03)	$5.7\ (\pm1) \times 10^{-5}$ 4.25 (±0.1)	$2.5\ (\pm0.1) \times 10^{-3}$ 2.60 (±0.02)	0.12 (±0.01)	$3.0\ (\pm0.3) \times 10^{-4}$ 3.52 (±0.04)

[a] Experimental uncertainties are the mean standard deviations. [b] At 25 °C and I = 0.2 M, from Brouillard and Delaporte (ref 7).

at equilibrium at T and pH_o, is shifted to a new equilibrium state at T and pH_f. The corresponding relaxation amplitudes are recorded by means of UV–vis absorption spectrophotometry. For two natural anthocyanins (malvidin 3-glucoside and malvidin 3,5-diglucoside), Brouillard et al. demonstrated that there was an excellent agreement between the calculated spectrophotometric relaxation amplitudes and the observed ones (Table I, ref 2). We have now applied the pH-jump method to four 3-deoxyflavylium salts, one natural product (apigeninidin) and three synthetic analogues, and directly measured their acidity constants.

At present much work is being carried out on the use of anthocyanins as food coloring materials.[3] However, most natural anthocyanins, like the previously investigated malvidin 3-glucoside and malvidin 3,5-diglucoside, tend to exist in acidic solns. (pH range 2.5–6.0 for most food systems) predominantly as the colorless forms B and C (Scheme I). Therefore, it is of interest for food application purposes to find anthocyanins or related flavylium salts that would exist, under those pH conditions, largely in the colored forms AH^+ and A. This paper documents the important effects that variations of the molecular structure have on achieving this goal. The pigments studied are 4',7-dihydroxy-, 4',5,7-trihydroxy- (apigeninidin), 4'-methoxy-4-methyl-5,7-dihydroxy- and 4'-methoxy-4-methyl-7-hydroxyflavylium chlorides, **1–4**, respectively. Due to their hydroxylation patterns, the two former pigments have frequently been selected as model compounds for investigating the stability and the reactivity of natural anthocyanins. We now show that these classes of pigments essentially differ as to their most stable neutral species. Equilibrium distribution for the colored and for the colorless structures at 25 °C and an ionic strength lower that 10^{-1} M are given for each pigment even in the case where very small amounts of a structure are present.

Scheme I



Experimental Section

Flavylium Salts. 4',7-Dihydroxyflavylium chloride,[4a] apigeninidin chloride,[4b] 4'-methoxy-4-methyl-5,7-dihydroxyflavylium chloride,[4c] and 4'-methoxy-4-methyl-7-hydroxyflavylium chloride[4c] are prepared as described in the literature.

Stock Solutions. For most of the experiments the required amount of the pigment (analytical concentrations ranged from 2×10^{-6} to 2×10^{-5} M) is dissolved in distilled water. The solution is kept in the dark at 25 °C and sufficient time is allowed for complete equilibrium to be attained. When necessary the pH of the solution is brought to values close to 1–3 by injecting a few microliters of a concentrated hydrochloric acid solution (Merck Suprapur). Thus, the initial pH values of the stock solutions range from about 1 to 6.

pH-Jump Experiments and Absorbance Measurements. For recording pH-jump-induced absorbance changes a UV–visible spectrophotometer (Cary 118) fitted with a thermostated sample cell with a magnetic stirring device is used. From a completely equilibrated stock solution an aliquot is taken and put into the thermostated sample cell. The pH jump is produced by injecting into the sample cell a few microliters of aqueous hydrochloric acid or sodium hydroxide solutions. Throughout the whole experiment the temperature is kept at 25 (±0.2) °C. The absorbance changes are recorded in the visible region where strong absorption by the flavylium cation and the quinoidal bases takes place. Before and after each experiment the pH is measured directly in the sample cell with a pH meter (Knick) equipped with a small combined glass electrode (Metrohm EA 125). The buffers used for calibration are pH 7.00 and 4.00 NBS standards (Beckman).

Results and Discussion

Structural Transformations. The elementary reactions for anthocyanins, anthocyanidins, 3-deoxyanthocyanins, and 3-deoxyanthocyanidins structural transformations in aqueous media are illustrated in Scheme I in the case of apigeninidin. In the pH range (1–6) investigated, the neutral species A, B, and C do not deprotonate to an appreciable extent. Equilibria 1 are fast proton-transfer diffusion-controlled reactions; the protons transferred are from an oxygen atom at C-5, C-7, or C-4' hydroxyl groups of the flavylium cation AH^+ to a water molecule.[1] This results in the formation of the quinoidal bases denoted by A. Both AH^+ and A strongly absorb visible light and are responsible for the brilliant orange, red, and blue colors of flowers and fruits.[5] If

(3) Timberlake, C. F.; Bridle, P. In "Developments in Food Colours - I"; Walford, J., Ed.; Applied Science Publishers: Barking, England, 1980; p 115. Markakis, P. "Anthocyanins as Food Colors"; Academic Press: New York, 1982.

(4) (a) Jurd, L. U.S. Patent 3 266 903, 1966. (b) Sweeny, J. G.; Iacobucci, G. A. *Tetrahedron* **1981**, *37*, 1481. (c) Timberlake, C. F., German Patent 1 904 810, 1969.

one or two of the free hydroxyl groups of AH^+ are methylated or glycosylated, as is frequently the case for natural anthocyanins, the number of the quinoidal bases reduces to two or one, respectively. Equilibration of reaction 2 occurs much slower than equilibration of reactions 1. The relaxation time for reaction 2 is on the order of a few minutes at room temperature, corresponding to the rate of nucleophilic addition of water to the positively charged pyrylium ring. Preferential attack takes place at C-2,[6] affording colorless carbinol pseudobase B. It has been shown very recently that, owing to unfavorable kinetic and thermodynamic factors, the C-4 water adduct does not form.[1] The positions of equilibria 1 and 2 are dependent on the acidity of the medium and are characterized by two acidity constants K'_a and K'_h, respectively. $K'_a = ([A]/[AH^+])a_{H^+}$ and $K'_h = ([B]/[AH^+])a_{H^+}$, where a_{H^+} is the activity of the hydronium ion. K'_a is a true acidity constant whereas K'_h is an apparent acidity constant. Equilibrium 3 is a base and light-catalyzed, ring–chain tautomerization whose equilibration rate is slow in moderately acidic aqueous solutions compared to the rates of equilibration of reactions 1 and 2.[7] Ring-opened chalcone pseudobase C does not absorb light in the visible range.[8] The position of pH-independent equilibrium 3 is governed by the value of the tautomeric equilibrium constant K_T. Formally, one can also define an apparent acidity constant K'_c for the chalcone pseudobase C formation reaction starting from the flavylium cation AH^+: $K'_c = ([C]/[AH^+])a_{H^+} = K'_hK_T$. Thus the stability of the acidic flavylium cation AH^+ relative to the neutral bases or pseudobases A, B, and C depends on the values of the three constants K'_a, K'_h, and K'_c, respectively. The relative stabilities of A, B, and C depend on the relative values of K'_a, K'_h and K'_c; the larger a given acidity constant, the larger the equilibrium amount of the corresponding base or pseudobase and vice versa.

Absorbance Changes Associated with a pH Jump. When a suitable pH jump occurs, the solution initially equilibrated at T and pH_o evolves toward a new equilibrium state at T and pH_f according to three kinetically distinct steps. The UV–vis theoretical spectrophotometric amplitudes have been established previously and are given by eq 4–6.[2] D_o, D_1, D_2, and D_f are given

$$D_o - D_1 = -(\epsilon_{AH^+} - \epsilon_A)\frac{K'_a(a_{H^+}{}^f - a_{H^+}{}^o)lC_o}{(K' + a_{H^+}{}^o)(K'_a + a_{H^+}{}^f)} \quad (4)$$

$$D_1 - D_2 = -\frac{(\epsilon_{AH^+}a_{H^+}{}^f + \epsilon_A K'_a)K'_h(a_{H^+}{}^f - a_{H^+}{}^o)lC_o}{(K'_a + a^f{}_{H^+})(K' + a_{H^+}{}^o)(K'_a + K'_h + a_{H^+}{}^f)} \quad (5)$$

$$D_2 - D_f = -\frac{(\epsilon_{AH^+}a_{H^+}{}^f + \epsilon_A K'_a)K'_c(a_{H^+}{}^f - a_{H^+}{}^o)lC_o}{(K'_a + K'_h + a_{H^+}{}^f)(K' + a_{H^+}{}^o)(K' + a_{H^+}{}^f)} \quad (6)$$

by eq 7–10, where $a_{H^+}{}^o = 10^{-pH_o}$ and $a_{H^+}{}^f = 10^{-pH_f}$. The overall

$$D_o = \frac{(\epsilon_{AH^+}a_{H^+}{}^o + \epsilon_A K'_a)lC_o}{(K' + a_{H^+}{}^o)} \quad (7)$$

$$D_1 = \frac{(\epsilon_{AH^+}a_{H^+}{}^f + \epsilon_A K'_a)(K'_a + a_{H^+}{}^o)lC_o}{(K' + a_{H^+}{}^o)(K'_a + a_{H^+}{}^f)} \quad (8)$$

$$D_2 = \frac{(\epsilon_{AH^+}a_{H^+}{}^f + \epsilon_A K'_a)(K'_a + K'_h + a_{H^+}{}^o)lC_o}{(K' + a_{H^+}{}^o)(K'_a + K'_h + a_{H^+}{}^f)} \quad (9)$$

$$D_f = \frac{(\epsilon_{AH^+}a_{H^+}{}^f + \epsilon_A K'_a)lC_o}{(K' + a_{H^+}{}^f)} \quad (10)$$

acidity constant $K' = K'_a + K'_h + K'_c$. C_o is the analytical concentration of the pigment. D_o and D_f are the absorbances, in the visible range, when the solution is completely equilibrated at pH_o and pH_f, respectively. D_1 and D_2 are the absorbances, in the visible range and at pH_f, immediately after (a) the proton-transfer equilibria 1 are reached and, (b) the hydration equlibrium 2 is attained, respectively. ϵ_{AH^+} and ϵ_A are the molecular extinction coefficients of AH^+ and A, respectively. l is the optical path length. Equations 4–10 are rather complex; however, they can easily be checked by setting $a_{H^+}{}^o = a_{H^+}{}^f$, which leads to $D_o = D_1 = D_2 = D_f$.

Methods for Measuring the Overall Acidity Constant K'. (a) Method A. When a pigment solution is completely equilibrated at a given pH, the absorbance D is expressed by eq 11, which is

$$D = \frac{(\epsilon_{AH^+}a_{H^+} + \epsilon_A K'_a)lC_o}{K' + a_{H^+}} \quad (11)$$

identical with eq 7 and 10, $a_{H^+}{}^o$ and $a_{H^+}{}^f$ being replaced by a_{H^+}. K'_a is usually not larger than 10^{-4} M.[9] Thus, for sufficiently acidified solutions ($a_{H^+} > 10^{-2}$ M) and for ϵ_{AH^+} values larger than ϵ_A, eq 11 reduces to $D = (\epsilon_{AH^+}a_{H^+}lC_o)/(K' + a_{H^+})$. The term $\epsilon_{AH^+}lC_o = D_{acid}$ is the absorbance in the visible range of a pigment solution that is totally converted to the flavylium structure AH^+ ($a_{H^+} \gg K'$). Thereafter, the result can be conveniently expressed in the form of eq 12, which is an extension to an equilibrated

$$\log\left[\frac{D_{acid} - D}{D}\right] + pK' = pH \quad (12)$$

multistep system of the classical Henderson–Hasselbach equation for a single acid–base equilibrium. By plotting $\log [(D_{acid} - D)/D]$ as a function of pH, one gets the pK' value by the intersection of the straight line with the x axis.

(b) Method B. Usually, the quinoidal bases A absorb light at longer wavelengths than the flavylium form AH^+.[10] In the spectral range where $\epsilon_{AH^+} = 0$ and $\epsilon_A \neq 0$, eq 7 and 10 become eq 13 and 14, respectively. Combining these two equations gives

$$D_o = \epsilon_A K'_a lC_o/(K' + a_{H^+}{}^o) \quad (13)$$

$$D_f = \epsilon_A K'_a lC_o/(K' + a_{H^+}{}^f) \quad (14)$$

$D_o/D_f = (K' + a_{H^+}{}^f)/(K' + a_{H^+}{}^o)$, which can finally be rearranged in the form of eq 15. By measuring D_o and D_f in the suitable

$$K' = \frac{D_f a_{H^+}{}^f - D_o a_{H^+}{}^o}{D_o - D_f} \quad (15)$$

region of the visible spectrum and $a_{H^+}{}^o$ and $a_{H^+}{}^f$, one obtains directly the value of the overall acidity constant K'.

Method A is well adapted to all anthocyanins and structurally related compounds, no matter the relative quantities of the neutral species A, B, and C. Method B is most suitable for pigments possessing sufficient amounts of quinoidal bases at equilibrium, i.e., when K'_a is of the same magnitude or larger than the sum of K'_h and K'_c. Presently, method B only fails in the case of 4′,7-dihydroxyflavylium chloride. For this compound K'_h plus K'_c is more than 16 times larger than K'_a, and at equilibrium there are only a few percent of the analytical concentration present as the quinoidal bases. What has generally been measured in the past is the overall acidity constant K' corresponding to the overall transformation $AH^+ \rightleftarrows (A + B + C) + H^+$.[11] However, McClelland and Gedge have reported very recently[12] equilibrium constants for the hydration and ring–chain tautomeric processes of nonhydroxylated flavylium perchlorates, in acidic and alkaline aqueous solutions. The overall acidity constant K' does not reveal information about the mechanisms of the interconversions represented in Scheme I. Rather, it is only a measure of the stability

(5) Harborne, J. B. "Comparative Biochemistry of the Flavonoids"; Academic Press: New York, 1967. Timberlake, C. F.; Bridle, P. In "The Flavonoids"; Harborne, J. B., Mabry, T. J., Marbry, H., Eds.; Chapman and Hall: London, 1975; p 214. Asen, S. *J. Am. Soc. Hort. Sci.* **1979**, *104*, 223. Timberlake, C. F. In "Recent Advances in the Biochemistry of Fruits and Vegetables"; Friend, J., Rhodes, M. J. C., Eds.; Academic Press: London, 1981; p 221.

(6) Brouillard, R.; Dubois, J. E. *J. Am. Chem. Soc.* **1977**, *99*, 1359.

(7) Brouillard, R.; Delaporte, B. *J. Am. Chem. Soc.* **1977**, *99*, 8461. Preston, N. W.; Timberlake, C. F. *J. Chromatogr.* **1981**, *214*, 222.

(8) Brouillard, R.; Delaporte, B.; El Hage Chahine, J. M.; Dubois, J. E. *J. Chim. Phys. Phys.-Chim. Biol.* **1979**, *76*, 273.

(9) Brouillard, R. *Phytochemistry* **1981**, *20*, 143.

(10) Asen, S.; Stewart, R. N.; Norris, K. H. *Phytochemistry* **1977**, *16*, 1118. Yoshitama, K. *Bot. Mag.* **1978**, *91*, 207.

(11) Sondheimer, E. *J. Am. Chem. Soc.* **1953**, *75*, 1507. Jurd, L. *J. Org. Chem.* **1963**, *28*, 987. Timberlake, C. F.; Bridle, P.; *J. Sci. Food Agric.* **1967**, *18*, 473.

(12) McClelland, R. A.; Gedge, S. *J. Am. Chem. Soc.* **1980**, *102*, 5838.

of the flavylium cation as a function of pH.

Methods for Measuring the Ratios K'_a/K'_h, K'_h/K'_c and K'_a/K'_c. 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. For K'_a/K'_h this is readily achieved by considering the ratio of the fast $D_0 - D_1$ (eq 4) to the intermediate $D_1 - D_2$ (eq 5) relaxation amplitudes. For any pH jump eq 16 is valid.

$$\frac{D_0 - D_1}{D_1 - D_2} = \frac{(\epsilon_{AH^+} - \epsilon_A)K'_a(K'_a + K'_h + a_{H^+}{}^f)}{(\epsilon_{AH^+}a_{H^+}{}^f + \epsilon_A K'_a)K'_h} \quad (16)$$

Whenever $a_{H^+}{}^f \gg (K'_a + K'_h)$ and $\epsilon_{AH^+} > \epsilon_A$, eq 16 becomes eq 17. Following the same procedure as above, the ratio of the

$$\frac{D_0 - D_1}{D_1 - D_2} \simeq (1 - \epsilon_A/\epsilon_{AH^+})K'_a/K'_h \quad (17)$$

intermediate relaxation amplitude, $D_1 - D_2$ (eq 5), to the slow relaxation amplitude, $D_2 - D_f$ (eq 6), leads to eq 18, which reduces

$$\frac{D_1 - D_2}{D_2 - D_f} = \frac{K' + a_{H^+}{}^f}{K_T(K'_a + a_{H^+}{}^f)} \quad (18)$$

to eq 19, assuming that $a_{H^+}{}^f \gg K'$. Finally, the ratio of the

$$\frac{D_1 - D_2}{D_2 - D_f} \simeq \frac{1}{K_T} = \frac{K'_h}{K'_c} \quad (19)$$

amplitude of the fast relaxation, $D_0 - D_1$ (eq 4), to the amplitude of the slow relaxation, $D_2 - D_f$ (eq 6), is given by eq 20, providing

$$\frac{D_0 - D_1}{D_2 - D_f} \simeq (1 - \epsilon_a/\epsilon_{AH^+})K'_a/K'_c \quad (20)$$

that $a_{H^+}{}^f \gg K'$ and $\epsilon_{AH^+} > \epsilon_A$. The term $\epsilon_A/\epsilon_{AH^+}$ is practically identical with the reciprocal of the ratio of the initial absorbance D_0 (eq 7) of a sufficiently acidified solution ($a_{H^+}{}^o \gg K'_a$) to the absorbance D_1 (eq 8) of the same solution rapidly brought to a slightly acidic pH_f ($a_{H^+}{}^f \ll K'_a$), so as to fully convert the initially present flavylium cation AH^+ to the quinoidal bases A.

The amplitudes D_0-D_1, $D_1 - D_2$, and $D_2 - D_f$ are proportional to the initial equilibrium amounts at pH_0, $[A]_o$, $[B]_o$, and $[C]_o$ of A, B, and C, respectively. One can readily demonstrate that $[A]_o = K'_aC_o/(K' + a_{H^+}{}^o)$, $[B]_o = K'_hC_o/(K' + a_{H^+}{}^o)$, and $[C]_o = K'_cC_o/(K' + a_{H^+}{}^o)$. In order to get the best signal-to-noise ratio and therefore the best accuracy, it is necessary to induce relaxation processes with the largest possible amplitudes. This can be achieved by preparing solutions where the pigment is only present as the neutral bases A, B, and C ($a_{H^+}{}^o \ll K'$), which are completely converted into the flavylium cation after a suitable pH jump has occurred ($a_{H^+}{}^f \gg K'$). The amplitudes are the largest possible when the pH jump is the largest possible, i.e., when the jump goes from a very slightly acidic medium ($a_{H^+}{}^o \simeq 10^{-6}$ M) to a medium where the pigment is only stable in the flavylium form ($a_{H^+}{}^f \simeq 10^{-1}$ M). For the presently investigated 3-deoxyanthocyanidins with this type of experiment, all the neutral species could be detected, even when they were very minor components. The method is so sensitive that we estimated the limit for the dectection of the concentration of a given neutral species to be ca. 1% of the analytical pigment concentration. For the more favorable cases this limit could be even lower. Except for method B, the absorbance changes were recorded in the visible range at the maximum of absorption of the flavylium cation where $\epsilon_{AH^+} > \epsilon_A$.

Factors Influencing the Stability of the Neutral Bases A and the Neutral Pseudobases B and C. The values of the equilibrium constants for the presently investigated pigments, together with results previously obtained for malvidin 3-glucoside (Mv), a natural anthocyanin, are reported in Table I. With the exception of the equilibrium constants for Mv and of the pK' and pK'_a values for 4′,7-dihydroxyflavylium chloride (**1**), none of these values have been reported prior to this work. For the later compound, in their 1967 paper Timberlake and Bridle measured $pK' = 3.3$ at 20 °C and I = 0.1 M.[11] The higher pK' value found in this case by these authors may reflect the temperature and ionic strength dependencies upon the position of the overall transformation $AH^+ \rightleftharpoons (A + B + C) + H^+$. For the same pigment, Sperling et al.[13] reported the value of pK'_a to be 4.3 at 20 °C in a 10% methanolic aqueous solution.

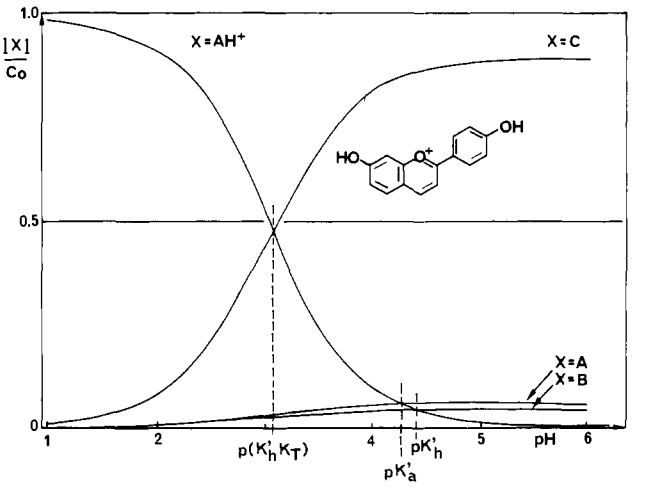


Figure 1. Equilibrium distribution in the dark at 25 °C of AH^+, A, B, and C for 4′,7-dihydroxyflavylium chloride (**1**) as a function of pH.

(A) 4′,7-Dihydroxyflavylium Chloride (1). Due to a hydroxylation pattern similar to that encountered in natural anthocyanins and the ease of its synthesis, the 4′,7-dihydroxyflavylium cation has been most investigated. The equilibrium distributions of AH^+, A, B, and C as a function of pH are represented for this pigment in Figure 1. At equilibrium, and in slightly acidic solutions (pH 4–6), the chalcone pseudobase C accounts for about 90% of the analytical concentration. Such a strong predominance of C over the two other neutral bases A and B results from the extraordinarily large value (20.6) of the tautomeric ring–chain equilibrium constant K_T. Consequently, K'_c is 20.6 times greater than K'_h, which, in turn, is of the same magnitude as K'_a. pK' is close to pK'_c; therefore, at equilibrium, the apparent reaction is $AH^+ \rightleftharpoons C + H^+$ (Figure 1).

Previously, the carbinol pseudobase of **1** could not be detected by conventional spectrophotometric and polarographic techniques, and its existence was, therefore, questioned.[14] We now demonstrate that only very small amounts of the pseudobase B can be formed and we explain it in the following manner. Since K'_a is a little larger than K'_h and since the quinoidal bases A are always formed first during a pH jump from pH_0 1–2 to pH_f 5–6, the initially present flavylium cation AH^+ transforms, very rapidly and almost completely, into the quinoidal bases A. Since K'_c is much larger than K'_a, however, the quinoidal bases A slowly convert into the chalcone pseudobase C, the most stable neutral species for **1** in slightly acidic solutions. The carbinol pseudobase B, which is neither the kinetic nor the thermodynamic product in a pH-jump experiment, cannot accumulate. In this connection, it is clear that **1** differs considerably from the usual natural anthocyanins whose K_T values are much lower than unity and which, at the same time, possess great amounts of the carbinol pseudobase owing to large K'_h values.

It has been demonstrated that the reaction forming the pyrylium ring is faster in the daylight than in the dark and that consequently pK'_c and pK' are lower in the dark than in the light.[15] It also has been shown that in slightly acidic solutions the ring-closure step is rate determining. Those observations are consistent with Jurd's proposal[16] that form C is more stable as the *trans*-chalcone and that light enhances its rate of conversion to the *cis*-chalcone. The isomerization step necessarily takes place prior to the pyranol ring formation. Flavylium salts where pK' is governed by pK'_h and/or pK'_a rather than by pK'_c are not expected to show light

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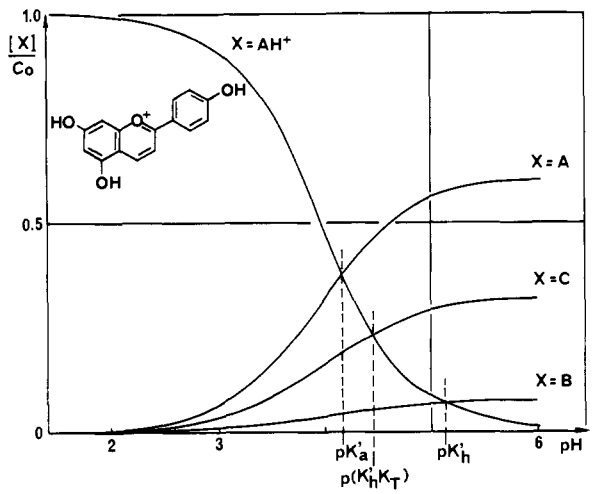

Figure 2. Equilibrium distribution in the dark at 25 °C of AH^+, A, B, and C for apigeninidin chloride (**2**) as a function of pH.

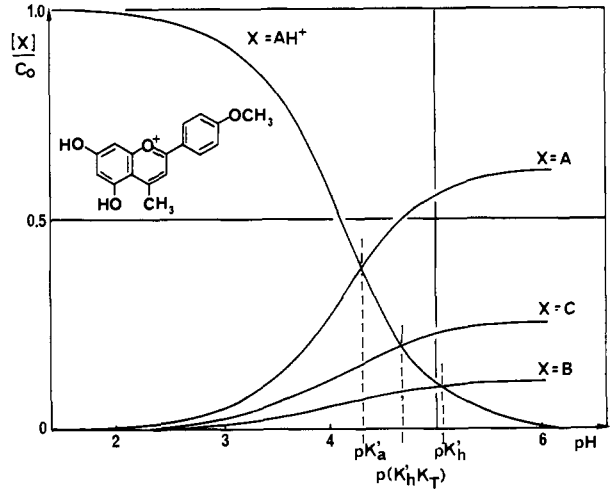

Figure 3. Equilibrium distribution in the dark at 25 °C of AH^+, A, B, and C for 4′-methoxy-4-methyl-5,7-dihydroxyflavylium chloride (**3**) as a function of pH.

sensitivity, since the equilibria forming A and B are not photocatalyzed. Consequently, pigments with large amounts of the neutral chalcone C should be easily detected. Suitably acidified solutions should be less intensely colored in the dark than in the light.

It is interesting to compare the stabilities of the neutral bases of **1** to the stabilities of the neutral bases of malvidin 3-glucoside (Mv), a typical natural anthocyanin. K_T is about 170 times lower for Mv than for **1**. This is in good agreement with the general observation that for ring–chain tautomerism introduction of a substituent in the chain always favors the cyclic structure and that compounds without a substituent in the chain are frequently present in the open form.[17] At the same time, the value of the acidity constant K'_h is multiplied by 64 from **1** to Mv. Consequently, K'_c is only 2.6 times lower for Mv than for **1**. Due to the large K'_h value for Mv, however, the more stable neutral species for this natural pigment is the carbinol pseudobase B. One can therefore conclude that the introduction of the glucosyl group at C-3 considerably reduces the chalcone amount and greatly enhances the stability of the carbinol, the pK'_a value being not changed to an appreciable extent (pK'_a is 4.30 and 4.25 for **1** and Mv, respectively). This raises the intriguing question: how can the 3-glucosyl group act in order to favor so much the hydration process without modifying the position of the flavylium cation–quinoidal bases equilibria? The extent of the nucleophilic addition of water to the pyrylium nucleus has often been related to the high positive net charges on C-2 and C-4.[18] We do not believe that this presently applies since the K'_a values for **1** and Mv are identical, probably indicating that no charge redistribution over the flavylium system has taken place from one compound to the other. Furthermore, it is well-known that anthocyanidins, which always bear a free hydroxyl at C-3, hydrate almost completely at low pH values (pH 2–4). Therefore, one can assume that in the presence of a 3 oxygen atom, an intramolecular hydrogen bond is formed between the 2-OH and this oxygen. The main effect is that the carbinol pesudobase is more stable. On the other hand, the hydroxyl groups of the flavylium heterocycle, which are strongly solvated by hydrogen bonding to the water molecules in both pigments, remain completely unaffected. With the exceptions of the newly discovered polyacylated anthocyanins,[9,19] similar high K'_h values have been found for natural anthocyanins which always bear a sugar at C-3.

(B) 4′,5,7-Trihydroxyflavylium Chloride (Apigeninidin) (2). Apigeninidin is characterized by an hydroxylation pattern common to all anthocyanin 3-glycosides which are the most abundant natural anthocyanins. The equilibrium distributions of AH^+, A, B, and C for apigeninidin chloride, are represented in Figure 2. It is easily seen that the main effect resulting from the existence of the 5-OH is to considerably reduce the equilibrium amount of the chalcone pseudobase C (K_T is 20.6 and 4.4 for **1** and **2**, respectively). On the other hand, the value of K'_h is significantly diminished compared to the value for **1**. The net result is that the carbinol pseudobase is a little more stable for **2** as for **1**. It is remarkable that the 5-OH, only very slightly modifies the position of the flavylium cation–quinoidal bases system (pK'_a is 4.30 and 4.20 for **1** and **2**, respectively). In this connection, the quinoidal bases become the most stable neutral species owing to the following ordering of the acidity constants: $K'_a > K'_c > K'_h$. Due to the lowering of K'_c, the flavylium cation becomes more stable at higher pH values. Some 20 years ago, it was reported that the color of apigeninidin in slightly acidic aqueous solutions does not fade on long standing.[20] Indeed, the coloring power of apigeninidin is far better than the coloring power of **1**. Inspection of Figure 2 shows, however, that about 40% of the overall pigment is still present in the colorless forms B and C. One can now understand that the methylated analogue of apigeninidin (4′,5,7-trimethoxyflavylium cation) exists mainly in the chalcone structure, since no quinoidal bases can be formed and since its K'_h value is probably lower than its K'_c value. Our reasoning agrees well with the observation that the 4′,5,7-trimethoxyflavylium cation gives rise to an acid–base equilibrium whose position is light dependent.[16]

(C) 4′-Methoxy-4-methyl-5,7-dihydroxyflavylium Chloride (3). Simultaneous methylation at the 4′-OH and at the C-4 does not appreciably change the K'_a and K'_h values, which are almost identical with the values found for apigeninidin. Only K_T is reduced by a factor of 2 (4.4 and 2.2 for **2** and **3**, respectively). The K_T value is still much higher for **3** than for 3-glycosides, however. A 4-methyl is therefore not so effective in reducing the chalcone content of a flavylium salt as is a 3-glycosyl. In fact the greater stability of the quinoidal bases of **2** and **3** compared to the stability of the quinoidal bases of Mv results essentially from the poor efficiency of the hydration process of the hydrophobic pyrylium ring in the case of the 3-deoxyanthocyanidins. The equilibrium distributions of AH^+, A, B, and C (Figure 3) for **3** are, therefore, very similar to the equilibrium distributions of AH^+, A, B, and C for **2** (Figure 2). Again, this pigment is suitable for coloring acidic and slightly acidic media though about 40% of it is in a colorless state above pH 5.

(D) 4′-Methoxy-4-methyl-7-hydroxyflavylium Chloride (4). This is the most efficient pigment of the series studies, and as shown

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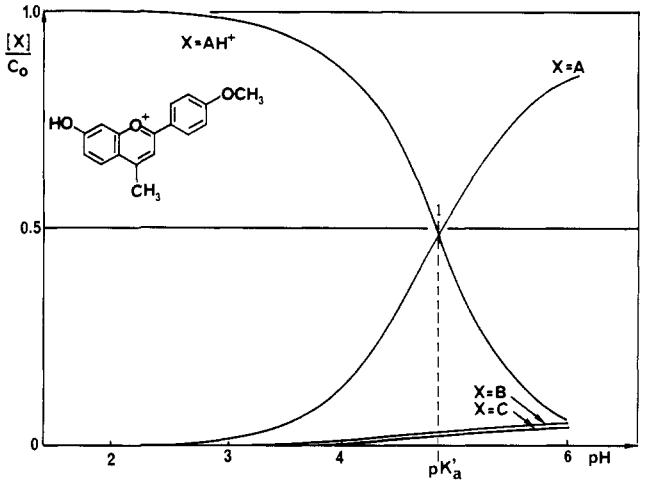

Figure 4. Equilibrium distribution in the dark at 25 °C of AH^+, A, B, and C for 4′-methoxy-4-methyl-7-hydroxyflavylium chloride (**4**) as a function of pH.

by Figure 4, it is essentially stable in the form of the flavylium cation in fast equilibrium with the quinoidal base. Due to very low K'_h and K'_c values (8.4×10^{-7} and 8.2×10^{-7} M, respectively), B and C are very hard to detect. Therefore the 4-methyl group, in the absence of a 5-hydroxyl group, considerably reduces the carbinol and chalcone contents. The K'_a value is significantly diminished compared to the values for the other pigments. This low K'_a value could be interpreted as resulting from the poor solvation of the quinoidal base of **4**, due to the lack of a free hydroxyl in this neutral species.

From mutagenicity studies using the Ames test, it has been shown that **4** induces a mutagenic effect.[21] No such effect has been observed for pigments **1–3**, and it has been pointed out that the basic structural requirement for mutagenicity is never found in natural anthocyanins.[21]

At one time it was thought that deprotonation of the flavylium cation occurs exclusively at the 4′-OH.[22] Later, on the basis of the similarity between the UV–vis absorption spectra of the quinoidal bases of 4′,7-dihydroxyflavylium chloride and its C-4′ methylated analogue, it was proposed that deprotonation takes place exclusively at the 7-OH. Inspection of the K'_a values reported in Table I clearly indicates that deprotonation occurs to some extent at any of the 3 hydroxyl groups at C-4′, C-5, and C-7, leading to several quinoidal bases in fast acid- and base-catalyzed prototropic tautomeric equilibrium. Currently nothing is known regarding the respective amount of each tautomer in any of the anthocyanin pigments.

Conclusion

Dissolving a flavylium salt in a slightly acidic aqueous solution at room temperature, i.e., under the physicochemical conditions found in living cells, gives rise to three equilibrated chemical transformations; namely a prototropic tautomeric equilibrium, an hydration equilibrium, and a prototropic ring–chain equilibrium. Among these species, only the flavylium cation and the quinoidal bases strongly absorb visible light. The two neutral carbinol and chalcone pseudobases are colorless. Some of the structural and solvent factors stabilizing the color have been discussed. One of the most striking features is the poor efficiency of the covalent hydration process of the pyrylium ring for compounds **1–4** (low K'_h values) compared to the high efficiency of the same process for natural anthocyanins (large K'_h values). It is also noteworthy that the position of the flavylium cation–quinoidal bases tautomeric system (K'_a) is quite insensitive to structural effects as long as a free hydroxyl is present in the neutral quinoidal bases. Further equilibrium studies will have to be performed on the natural and synthetic flavylium salts, however, before we can completely understand the chemical and biochemical mechanisms which enhance the color both in vitro and in vivo. It seems plausible that some of the in vivo color variations may be related to the variations in the acidity of the vacuolar sap.[23] This produces modifications in the ratios of the concentrations of the flavylium cation, the quinoidal bases, and the ionized quinoidal bases, changing the color to longer wavelengths when the pH increases.

Pigments yielding large amounts of one of the three neutral species A, B, and C are now known. Surprisingly, most of the natural pigments which give rise to the numerous and brilliant orange, red, and blue colors of flowers and fruits are stable in vitro in the form of the colorless carbinol pseudobase B. This result is not a paradoxical one, since in the cell vacuole there exist mechanisms, not present in our model solutions, strongly favoring the colored forms at the expense of the carbinol. The more important of these effects is probably related to the protection of the pyrylium nucleus against the approach of the water molecule.[1] This could be achieved either by self-association, when the pigment concentration is great ehough,[23a,b] or by association with others flavonoids, generally devoid of color but always present in large amounts in the vacuoles.[24] The most efficient protection against hydration of the pyrylium nucleus, however, seems to be due to the presence of two or more derivatives of the cinnamic acid series (coumaric, caffeic, and ferulic acids), covalently bound to the sugars.[25] In this last case, the pigment can adopt a configuration where the aromatic rings of the cinnamic esters stack with the pyrylium ring.[9] The net result of these associations is, sometimes, to considerably reduce the value of the constant K'_h.

Registry No. **1**, 4082-08-0; **1** (form A), 83615-99-0; **1** (form B), 83616-03-9; **1** (form C), 83616-07-3; **2**, 1151-98-0; **2** (form A), 83616-00-6; **2** (form B), 83616-04-0; **2** (form C), 2435-75-8; **3**, 20931-21-9; **3** (form A), 83616-01-7; **3** (form B), 83616-05-1; **3** (form C), 83616-08-4; **4**, 83615-98-9; **4** (form A), 83616-02-8; **4** (form B), 83616-06-2; **4** (form C), 83616-09-5.

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