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EFFECTS OF pH AND CONCENTRATION ON THE SELF-ASSOCIATION OF MALVIN QUINONOIDAL BASE --- ELECTRONIC AND CIRCULAR DICHROIC STUDIES[1]

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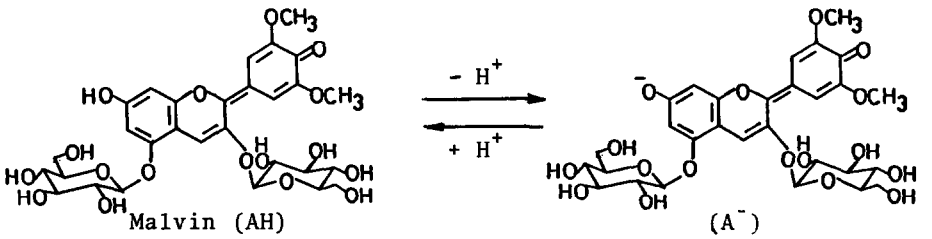
The self-association of malvin in aqueous solutions above pH 6 causes suppression of ionization of the phenolic hydroxyl group in its quinonoidal nucleus. Malvin (AH) in 5 x 10^{-3} M solution shows two isosbestic points between pH 6 and 9 in its electronic spectra, indicating existence of the equilibria, $AHAH \rightleftarrows AHA^- \rightleftarrows A^-A^-$, which is also supported by CD studies; thus, hetero-association as well as homo-association occurs.

Anthocyanin pigments are responsible for a variety of colors in flowers and fruits. The acidity in flower cell vacuoles most frequently encountered in nature are in the range 4 to 6.5.[2] Therefore, flower color would be expressed mainly in terms of quinonoidal form of anthocyanins. In dilute aqueous solutions in the pH range such a quinonoidal form of anthocyanins, however, is very unstable and easily hydrated into the corresponding colorless pseudobase.[3,4]

In the previous papers,[5-8] we have reported a new stabilization mechanism of natural anthocyanins, i.e., self-association of the quinonoidal bases at pH 7.0. When the concentration of anthocyanidin 3,5-diglucosides is increased, a blue shift of visible absorption maximum and the exciton type circular dichroism (CD) around the visible absorption band become apparent.[7,8] These optical phenomena clearly indicate that two or more of the anthocyanidin chromophores are in a chiral stacking, possibly a vartical stacking of aromatic nuclei, as supported by the NMR study.[8] Such a self-association sterically suppresses hydration of the quinonoidal base,[8] thus stabilizes anthocyanin quinonoidal base.

Investigation of electronic spectra indicated that at pH 7 the anthocyanin diglucosides exist in an equilibrium between the neutral (AH) and the negatively charged (A^-) species (Fig. 2).[9] Therefore, our previous papers,[5-8] in which a buffer of pH 7 was used throughout, had dealt with the self-association in a mixture of AH and A^- species. This paper deals with the effect of pH on the chiral stacking of anthocyanidin chromophores, i.e., whether AH associates with AH (homo-association) and/or with A^- (hetero-association) using malvin as a typical natural anthocyanidin 3,5-diglucoside; peonin,

delphin, petunin, and hirsutin, but not pelargonin and cyanin,[7,11] behave similarly.[7,10]



Malvin (AH) (A^-)

Electronic spectra of malvin at various acidities above 6 are shown in Fig. 1A. Plots of ε and λmax against pH at different pigment concentrations are depicted in Fig. 2. These spectrophotometric titration curves determine the ionization constant (pKa) of the phenolic hydroxyl group in the quinonoidal base. Fig. 2 indicates that apparent pKa between A and A^- is increased by increasing concentration, thus indicating that the self-association gives rise to supression of the ionization of AH to A^-. We have previously reported that the self-association is caused by the hydrophobic interaction between anthocyanidin chromophores.[8] It is well known that ionizable functional groups surrounded by hydrophobic area are hard to dissociate in aqueous media.[12]

At a given pH, hypsochromism (Fig. 2B) as well as hypochromism (Fig. 2A) are observed by increasing pigment concentration. Thus, negatively charged A^- species as well as neutral AH is capable of the self-association. The pH range at which AH and A^- species coexist was investigated in order to elucidate whether AH associates with A^- (hetero-association) or not. At the pigment concetration as low as 2.5 x 10^{-5}M, where malvin occurs mostly as the monomer, only one isosbestic point (554 nm) is observed in the electronic spectra as a function of pH when measured at 5 °C, which is indicative of the simple acid-base equilibrium between monomeric AH and A^- species. On the other hand, two isosbestic points are observed in the electronic spectra of the pigment in a higher concentration (Fig. 1A). The isosbestic point at the shorter wavelength (532 nm) would indicate the equilibrium between AHAH and AHA^- species[13] (approximate pKa 7.0), and the longer wavelength point (544 nm) the equilibrium between AHA^- and A^-A^- species (approximate pKa 8.2).[14] At pH 7.55 both isosbestic points are involved in the spectrum, where hetero-association of AH with A^- species would be maximal.

Study of CD spectra gave a similar conclusion. As shown in Fig. 1B, in 5 x 10^{-3}M solution two isosbestic points are observed at 504 nm and at 526 nm, which are consistent with the electronic spectra (Fig. 1A). Thus, the following equilibria as a function of pH are established by these results: AHAH $\rightleftharpoons$ AHA^- $\rightleftharpoons$ A^-A^-.

Fig. 3 shows as a function of pH "A value", which expresses the difference in $\Delta\varepsilon$ of split extremes. The 5 x 10^{-5}M solution shows an apparent single pKa around 7, whereas

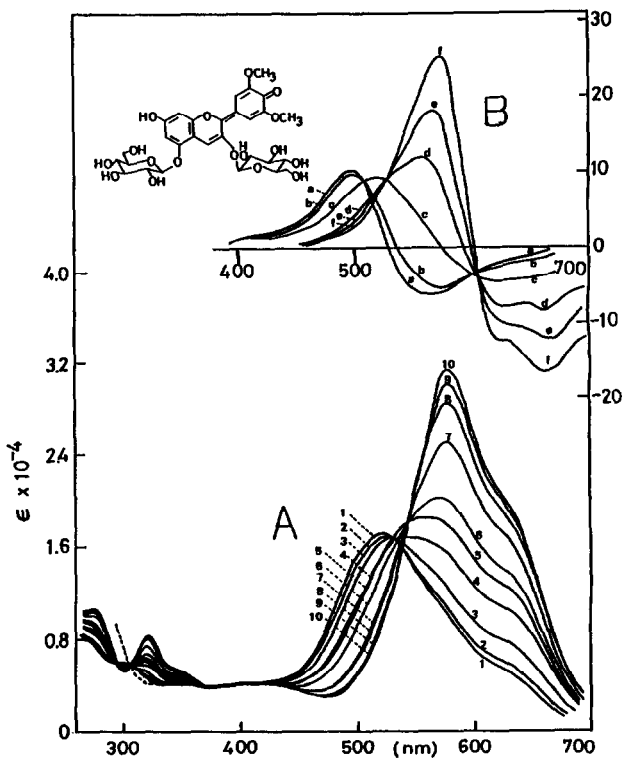


Fig. 1. Electronic spectra (A) and circular dichroism (B) of malvin quinonoidal base at 5 x 10^{-3}M aq. soln. as a function of pH, measured within 30 sec after dissolving at 25 °C.

pH = (1) 6.03; (2) 6.37; (3) 6.90; (4) 7.37; (5) 7.55; (6) 7.73; (7) 8.24; (8) 8.40; (9) 8.82 (10) 9.12. pH = (a) 6.12; (b) 6.70; (c) 7.52; (d) 7.80; (e) 8.30; (f) 8.90.

CD spectra were measured with a Jasco J-500C spectropolarimeter equipped with a DP-500 data processor.

0.1M phosphate and NH_4OH-NH_4Cl buffer were used at pH below 8 and above 8, respectively, and the ionic strength was adjusted to 0.3 with KNO_3.

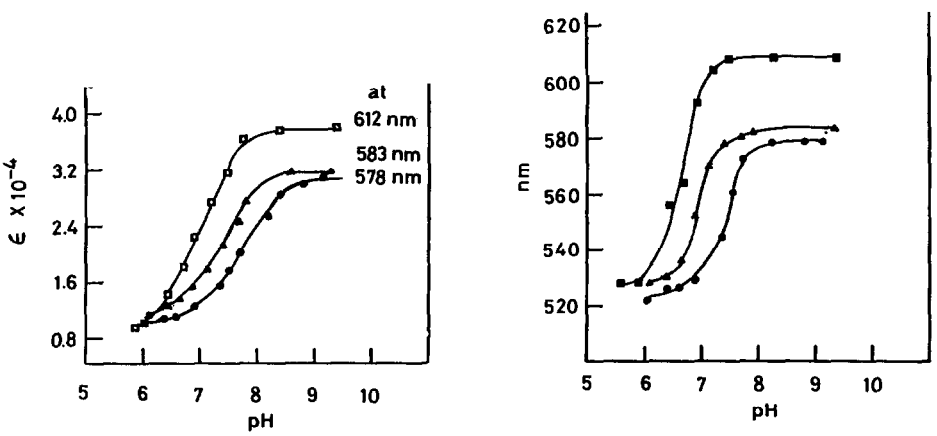


Fig. 2. Change of absorbance and absorption maximum of malvin with pH.

(A) Relationship between pH and the absorbance at the visible λmax of the ionized quinonoidal base; (B) relationship between pH and visible λmax. □ 1 x 10^{-5}M; ■ 2.5 x 10^{-5}M; ▲ 5 x 10^{-4}M; ● 5 x 10^{-3}M.

more concentrated solutions gave a somewhat complex titration curve with a shift of apparent pKa to higher values. Such an irregularity of the curve may be explained by assuming that two equilibria, $AHAH \rightleftarrows AHA^-$ and $AHA^- \rightleftarrows A^-A^-$, are present and that strong association suppresses the ionization. Thus, 5×10^{-3}M solution exibits (Fig. 1B and 3) that there are two pKa's around 7 ($AHAH \rightleftarrows AHA^-$) and 8.2 ($AHA^- \rightleftarrows A^-A^-$) and that A values of AHAH and $(AHA^-)n$ are nearly equal, whereas A^-A^- has a large A value compared to that of AHAH and AHA^-.

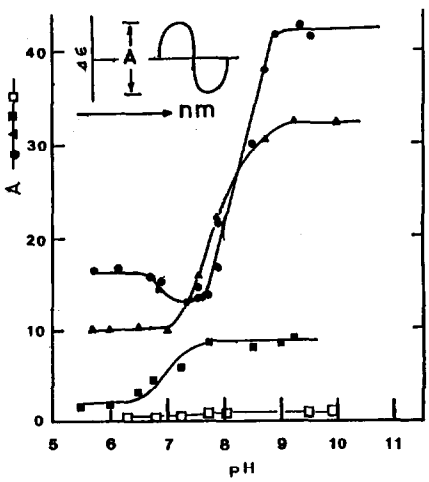


Fig. 3. Change of "A values" with variation of pH and pigment concentrations. Closed circle, triangle and cube refer to 5×10^{-3}M, 5×10^{-4}M, and 5×10^{-5}M, respectively, of malvin. Open cube refers to the A value of malvin at 5×10^{-4}M in the presence of 7M urea.

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