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EVIDENCE FOR THE SELF-ASSOCIATION OF ANTHOCYANINS IV. PMR SPECTROSCOPIC EVIDENCE FOR THE VERTICAL STACKING OF ANTHOCYANIN MOLECULES

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Summary; Vertical stacking structure is proposed for the self-association of anthocyanins on the basis of PMR shift data due to the ring current effect of aromatic nuclei of anthocyanins.

In the previous papers,[1,2,3] we have presented definite evidence for the occurrence of anthocyanin self-association, i. e. color variation (hypsochromic or bathochromic shift) and appearance of typical exciton coupling type circular dichroism (CD) with increase of anthocyanin concentration in aq. solution. The present concern has been centered, insofar as possible, upon the elucidation of the mechanism and structure of the self-association of anthocyanin molecules. For the complex formation of anthocyanins with flavonoid copigments (co-pigmentation), two mechanism have already been proposed: vertical stacking of anthocyanidin rings with flavone nuclei due to hydrophobic and/or π-electron interactions proposed by us[4] and horizontal interactions due to hydrogen bonding.[5] The mechanism for the self-association, however, still remains to be established. The changes of visible absorption and the splitting CD at visible region by formation of the self-aggregates suggest presence of the interaction between $\pi \rightarrow \pi^*$ electronic transition moments of each chromophore. Disaggregation with urea or dimethylsulfoxide, which presumably acts to disrupt 'structured water' surrounding the self-aggregates, indicates that attractive force comes mainly from hydrophobic interaction between aromatic nuclei of anthocyanins. On the basis of these points of view, we have postulated a vertical stacking structure for the self-association.[1,2,3]

To obtain more conclusive experimental evidence for the stacking interactions of anthocyanin self-association, we have embarked on NMR studies of anthocyanin quinonoidal bases, since concentration dependence of the NMR shielding is a very sensitive probe to distinguish vertical interaction between aromatic nuclei from horizontal interaction. Here, we report experimental evidence that self-aggregates occur by the vertical stacking.

Some PMR spectra of flavylium ions have been reported,[6] but no one has reported analyzable PMR spectra of anthocyanin quinonoidal bases because of extreme broadening of their signals. After extensive trials, we have succeeded in obtaining the interpretable PMR spectra of malvin quinonoidal base. Malvin chloride was dissolved in dilute DCl-D_2O and the solution dried up in vacuo. After repetition of this procedure, deuteriated malvin chloride thus obtained was dissolved at 21°C in 0.1 M NaD_2PO_4-Na_2DPO_4 of pH 7.0 [7] (uncorrected for deuterium isotope effect) and the solution was used for PMR measurements (JEOL JNM-FX 100 FT-NMR spectrometer operated at 100 MHz) within 15 min after dissolving; under this condition, the transformation of the colored quinonoidal base to the colorless pseudobase was negligibly small (< 5%) at more than 5×10^{-3}M of anthocyanin concentration.

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Fig. 1 shows the PMR spectra of malvin quinonoidal base at different concentrations. Line broadening suggests molecular aggregation. The proton resonances[8] of aromatic nucleus were all shifted [9] to higher fields as the solute concentration increased (Fig. 2). Shifts to high fields with increase of anthocyanin concentration are attributable to the diamagnetic anisotropy caused by ring currents in neighboring aromatic molecules; it is thus strongly suggested that anthocyanin molecules stack vertically and do not associate horizontally. The magnitudes of chemical shift changes are comparable to those of base protons of nucleosides or nucleotides,[10] which form vertical stacking in nearly maximal overlap of aromatic portions, indicating the vertical stacking in a closest approach of each aromatic anthocyanidin ring. The vertical stacking structure is further supported by the evidence that hirsutin (7-O-methylmalvin) quinonoidal base does self-associate in spite of the lack of phenolic OH group which might participate in hydrogen bonding.[2] We previously reported that urea weaken the self-association.[2] Chemical shift behaviors were investigated in D_2O-d_4-urea mixture (pH 7.0) ranging from 0 to 7M urea at the fixed concentration of 6.2×10^{-2}M. Increase of urea concentration shifted all aromatic signals to down fields (Table 1), suggesting that urea destacks anthocyanin self-aggregates.

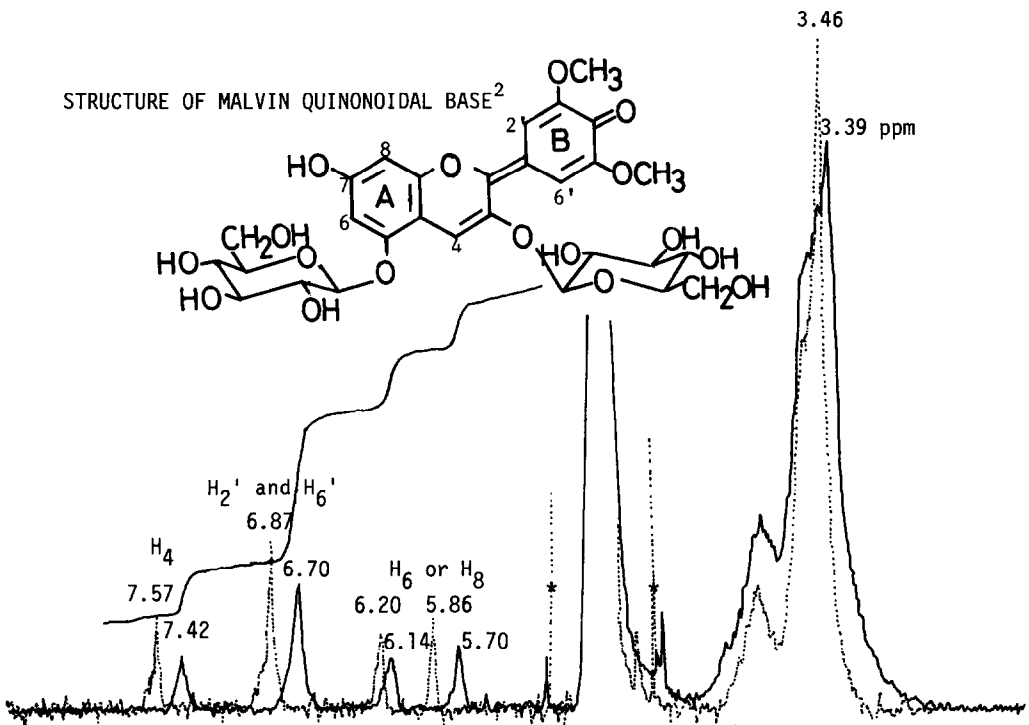

Fig. 1. PMR spectra of malvin quinonoidal base at different molar concentrations. solid line —— 6.2×10^{-2}M (50 scans), dotted line —— 1.0×10^{-2}M (100 scans). * side bands

From these PMR shift data, it may be promising to derive a stacking geometry, since aromatic protons are differently shielded by increasing anthocyanin concentration (Fig. 2). More shifted protons would be located more nearly over the adjacent anthocyanin nuclei. However, it is

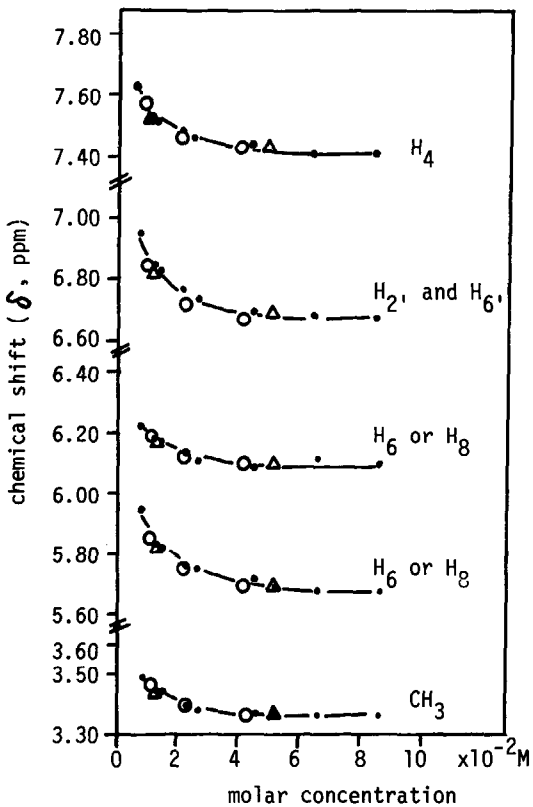

Fig. 2. Concentration dependence of proton chemical shifts of malvin quinonoidal base.

internal TSP: pH 7.0 (●), pH 6.0 (○).
external TSP: pH 7.0 (△).

Table 1. Effect of urea on the chemical shifts of the aromatic protons of malvin quinonoidal base.

ppm / conc. of urea	H_4	$H_{2'}$ and $H_{6'}$	H_6 or H_8
0	7.42	6.70	6.14, 5.70
1.5 M	7.46	6.76	6.16, 5.76
3.0 M	7.49	6.83	6.21, 5.84
7.0 M	7.59	7.00	6.30, *

* The large signal of exchangeable protons interfered with this signal.

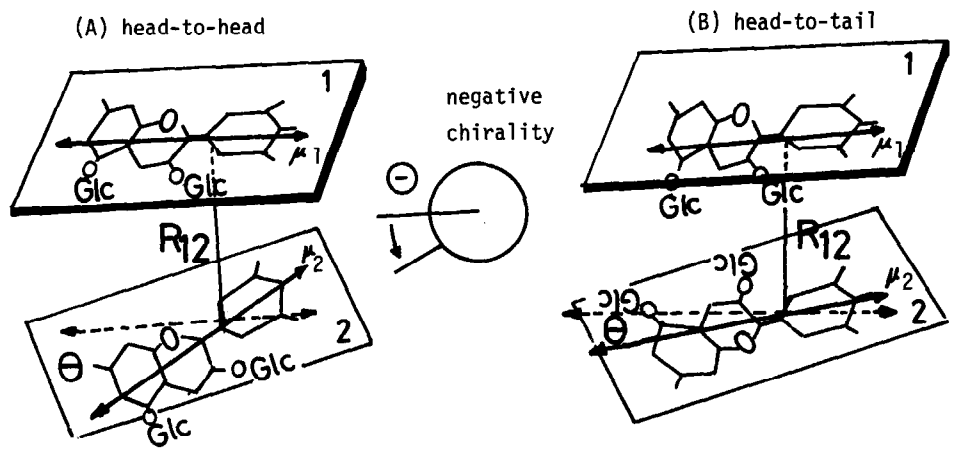

Fig. 3. Proposed geometry for the vertical stack of two planar anthocyanin molecules (presumably further association beyond dimer occurs, thus obtaining a helical conformation). R_{12} is perpendicular to the anthocyanidin rings and equal to the Van der Waals distance of closest approach (3.4 Å). θ shows the angle between μ_1 and μ_2 transition moments.

difficult to give a favorable geometry, because it would be expected that ring currents in A, B and heterocyclic rings are not equivalent. Possibly, one proton of either 6- or 8-position of A ring would be oriented more nearly over the neighboring aromatic ring than the alternative proton of A ring, since the shieldings are remarkably different. Previously we reported that malvin quinonoidal base self-associates to show exciton coupling CD of the first negative sign.[2,3] The orientation of $\pi \rightarrow \pi^*$ transition moments of anthocyanidin chromophore is shown in Fig. 3.[11] Qualitatively, the transition moments of malvin quinonoidal bases in the vertical stack could be arranged as depicted in Fig. 3, thus, showing negative chirality (left-handed screwness).[3,12] Theoretical calculation of the CD by the molecular exciton theory is under investigation.

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8) Protons of malvin quinonoidal base were assigned according to the ref. 11 a). They calculated π electron densities of cyanidin quinonoidal base. Unfortunately, electron density at 6-position was not reported. Thus, we cannot assign the protons at 6- or 8-position. Presumably, electron densities at 6- and 8-position would be nearly equal.
9) Chemical shifts were determined as the shifts from the internal reference of sodium trimethylsilylpropionate (TSP). There are some indications that the use of the internal TSP is unreliable in the presence of aromatic moiety, since hydrophobic interactions between the trimethylsilyl group and the aromatic moiety shift the TSP signal considerably upfield (Ref. 13). However, there are little differences of PMR shifts between the internal and external TSP (Fig. 2), indicating little interaction between anthocyanin molecules and TSP. No bulk susceptibility corrections were made. However, it was confidently estimated that these corrections are less than 0.02 ppm for the 0.01-0.062 M solute concentration.
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