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SELF-ASSOCIATION OF SOME ANTHOCYANINS IN NEUTRAL AQUEOUS SOLUTION*

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Key Word Index—Self-association of anthocyanins; hypsochromic shift; hypochromism; hydrophobic interaction; steric hindrance; malvin; hirsutin; 4′-*O*-methylmalvin; malvidin 3-glucoside; malvidin 5-glucoside.

Abstract—A good contrast in optical properties caused by self-association was found between malvin and cyanin quinonoidal bases. Circular dichroism measurements of the pigments in neutral aqueous solutions show that molecules of the malvin quinonoidal base self-associate quickly and the conformational orientation of the aggregates is opposite to those formed by cyanin quinonoidal bases. Hypsochromism and hypochromism in the visible absorption also occurred on the formation of malvin aggregates. CD comparisons of malvin, hirsutin and 4′-*O*-methylmalvin suggest that a methoxyl group in the B-ring of the anthocyanidin strongly suppresses self-association. It is proposed that the driving forces for self-association are mainly hydrophobic interactions among the aromatic nuclei stacked parallel to each other which are surrounded by the hydrophilic glucose moieties in a suitable spatial arrangement. Furthermore, the glucose moiety at the 5-position rather than that at the 3-position plays an important role in the self-association of these anthocyanidin 3,5-diglucosides. Addition of sodium chloride promotes self-association and the greater stability of the anthocyanins in solution.

INTRODUCTION

A variety of factors are involved in flower colour variation of higher plants [1]. Recently, the self-association of anthocyanin molecules has been reported [2–4], but little convincing evidence has so far been presented. To demonstrate self-association we have applied circular dichroism (CD), which is intrinsically most sensitive to molecular dissymmetry, and have found that CD is an effective probe for the conformational changes which occur during molecular association of anthocyanins [5, 6]. Thus, cyanin quinonoidal base self-associates in neutral aqueous solution to show large CD Cotton bands [6].

In this paper we report that the UV and CD spectra of malvin and its derivatives clearly indicate that the self-aggregates of malvin differ from those of cyanin quinonoidal base in both conformational arrangement and in colour tone. It is also reported that the intensity of the CD band progressively decreases with an increase in the number of methoxyl groups in the B-ring of anthocyanin nucleus; presumably the methyl group(s) reduces the amount of self-association by steric hindrance.

RESULTS AND DISCUSSION

Figure 1 shows the visible absorption spectra of malvin chloride at different concentrations measured immediately (within a few min) after dissolving in phosphate buffer (pH 7.0). At low anthocyanin concentration (5×10^{-5} M), where the interaction between anthocyanin molecules is minimal, the colour is blue. At a higher concentration (5×10^{-3} M), a red colour close to that of the flavylium ion was produced by stronger

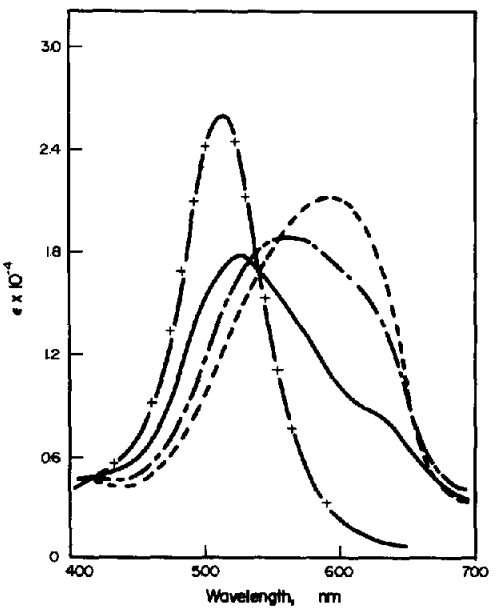


Fig. 1. Visible spectra of malvin chloride measured immediately after dissolving in Pi buffer, pH 7.0. – – – 5×10^{-5} M, – – – – 5×10^{-4} M, ——— 5×10^{-3} M; +——+ 5×10^{-4} M in N HCl.

* Part 2 in the series "Evidence for the Self-association of Anthocyanins". For Part 1 see Hoshino, T., Matsumoto, U. and Goto, T. (1980) *Tetrahedron Letters* 1751.

interactions between the molecules of the malvin quinonoidal base. Thus, self-association in the malvin quinonoidal base produces a remarkable hypsochromic shift; this contrasts with the bathochromic shift to blue colours shown by self-aggregation of the cyanin quinonoidal base [6].

CD spectra of the malvin quinonoidal base were measured within 60 sec after dissolving (Fig. 2). The increasing intensity of CD with hypsochromic shifts was observed as anthocyanin concentration increased; at a low anthocyanin concentration the solution showed little molecular ellipticity $[\theta]$, whereas at a higher concentration it was larger $[\theta]$. This suggests that monomeric anthocyanins show little circular dichroism whereas self-aggregated species have a large molecular dissymmetry in their fixed conformation in aqueous solutions.

Interesting results in CD were found according to the rate of self-association and to the orientation of the anthocyanin molecules in the complexes. The conformation for self-association of malvin quinonoidal base is rapidly determined after dissolving malvin flavylium salt in a neutral buffer solution, because characteristic CD appears immediately on dissolution. On the other hand, the degree of self-association in the cyanin quinonoidal base is time-dependent; the conformation for self-association is only taken up slowly and exhibits a larger θ with time elapsed after dissolving (ageing effect). In addition, the Cotton effects in malvin and in cyanin quinonoidal bases are of opposite sign, indicating that the orientation of malvin for self-association (negative) is opposite to that of cyanin (positive).

Figure 3 shows that the hydration process is slower in a more concentrated solution. This is additional evidence for the self-association of anthocyanins. The stability of the anthocyanin quinonoidal base in 5×10^{-3} M solution is much greater than in 5×10^{-4} or 5×10^{-5} M. This is significant in that the anthocyanin concentration in the cell sap of flower petals is generally higher than 10^{-2} M [1]. Thus, the self-association of anthocyanins in plant tissues may be partly responsible for the expression of flower colour. A slight decrease of maximal ε values due to self-association was observed (Fig. 1). By extrapolation of ε values to 0 min after dissolving, the decrease in ε values can be determined (Fig. 3). Thus, self-association of the malvin quinonoidal base causes hypochromism. Plots of the maximal $[\theta]$ of malvin quinonoidal base against time (Fig. 3) show that θ decreases with decolorization. This could be interpreted as follows: the self-associated malvin quinonoidal base with a fixed conformation gradually dissociates to produce the monomeric quinonoidal base, which is gradually converted via the flavylium ion to the colourless pseudobase [7], thus resulting in a decrease of θ. In the case of the cyanin quinonoidal base, aggregate formation and hydration of the monomeric quinonoidal base via the flavylium ion are competitive processes [6].

Asen *et al.* [2] reported anthocyanin self-association at pH 3.16. At this pH, malvin exists essentially as a mixture of the flavylium cation and the neutral carbinol base. The phenomenon reported here is quite different from that of Asen since the anthocyanin structures present at pH 7 are completely different from those present at pH 3.16. For malvin, at pH 7 and room temperature, there is an equilibrium between the quinone methide (A) and its ionized form (A^-). Whether A associates with A (homo-association) or A^- (hetero-association) is now under active investigation.

The self-association of malvin quinonoidal base was shown to be reversible as follows: (a) when a 5×10^{-3} M solution of malvin was rapidly diluted to 5×10^{-5} M, the resulting spectrum was identical to the initial spectrum of 5×10^{-5} M obtained without dilution, and (b) the absorption maximum of a 5×10^{-3} M solution very slowly changed towards the initial absorption maximum of a 2.5×10^{-4} M solution as decolorization proceeds, and similarly maximal absorbance of 5×10^{-4} M changed towards that of a 1.7×10^{-5} M solution. (At equilibrium the amount of pigment still present in the coloured form is about 3–5% of the analytical concentration.)

Similar experiments were carried out using hirsutin (**2**) and 4′-*O*-methylmalvin (**3**) instead of malvin (**1**) in order

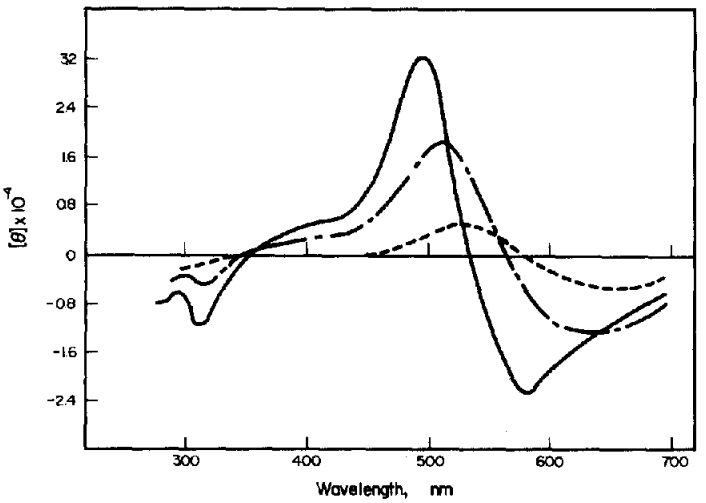


Fig. 2. CD spectra of malvin chloride in Pi buffer, pH 7.0. · · · 5×10^{-5} M (4000), ——— 5×10^{-4} M (18000), ——— 5×10^{-3} M (32000). The values in parentheses represent $[\theta]$, deg cm^2/dmol, at λ_{max} (values at second Cotton).

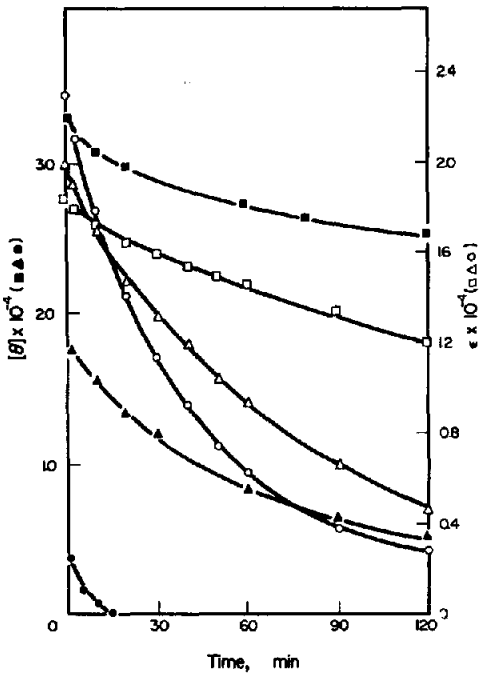
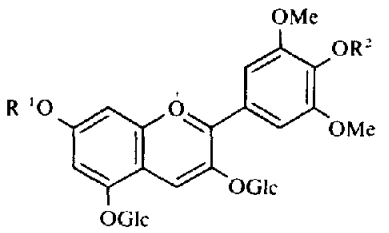


Fig. 3. ε_{max} and $[\theta]_{max}$ of malvin quinonoidal base against time. ○, ●: 5×10^{-5} M (22 800); △, ▲: 5×10^{-4} M (19 900); □, ■: 5×10^{-3} M (18 000). The values in parentheses represent extrapolated ε values to 0 min.

to obtain a better understanding of the self-association of the malvin quinonoidal base. 4′,7-Dimethylated malvin (4) does not form a quinonoidal base. The structures of hirsutin and 4′-*O*-methylmalvin quinonoidal bases must have the 4′- or 7-keto forms, respectively. The colours of hirsutin and the *O*-methylmalvin were blue and red, respectively, at a low anthocyanin concentration (5×10^{-5} M) (Fig. 4). The colours of malvin and hirsutin are the same, suggesting that the structure of the malvin quinonoidal base is also in the 4′-keto form (see also [8]). Large hypsochromic shifts, analogous to those of malvin, were observed in the visible spectra of hirsutin by increasing anthocyanin concentration (Fig. 4). The higher stability and larger θ with increase in anthocyanin concentration (Figs. 5 and 6) also prove that self-association occurs with the hirsutin quinonoidal base. In the case of 4′-*O*-methylmalvin, little change in visible absorption (Fig. 4) and stability (Fig. 5) occurred when anthocyanin concentration was increased. Thus self-association in 4′-*O*-methylmalvin is minimal and far less than in hirsutin. Indeed, the CD value of the 4′-methyl ether was extremely small even at a higher concentration (1×10^{-2} M) (Fig. 6).

It is significant then that hirsutin is capable of self-association, although the pigment quinonoidal base lacks the phenolic OH group that might participate in hydrogen-bonding. Thus, the driving force for self-association would be the hydrophobic bonds present. Introduction of methyl group(s) into malvin weakens the association in the following order: malvin, hirsutin and 4′-*O*-methylmalvin, as is evident from the degree of enhancement of $[\theta]$ following an increase in anthocyanin concentration (compare Fig. 2 with 6). Methylation at the 4′-OH group hinders self-association much more than



Malvin (1) $R^1 = R^2 = H$
Hirsutin (2) $R^1 = Me$, $R^2 = H$
4′-*O*-Methylmalvin (3) $R^1 = H$, $R^2 = Me$
(4) $R^1 = R^2 = Me$

methylation at the 7-OH, i.e. substitution of OMe for OH in the B-ring makes the degree of self-association lower. Indeed, delphin has a higher degree of self-association than malvin, since the slope of $[\theta]$/anthocyanin concentration of delphin is larger than that of malvin. (Hoshino, T., unpublished results).

In order to determine the effect of the glucose moiety on the self-association of anthocyanidin 3,5-diglucosides, malvidin 3-(Mv-3G) and 5-monoglucoside (Mv-5G) were examined. Mv-3G showed little concentration dependence on its $[\theta]$ values and stability, suggesting that Mv-3G is less self-aggregated. Mv-5G, at a dilute concentration (5×10^{-5} M), decolorized within about 10 min and showed little CD in neutral solution. At a high concentration ($> 5 \times 10^{-4}$ M) red precipitates rapidly settled out of the neutral solution. The precipitates, when dissolved in water, showed characteristically large optical activity and considerable stability (Fig. 7); the ε value at 492 nm was reduced by less than 10% when the solution was left at room temperature for 1 hr. Thus, the

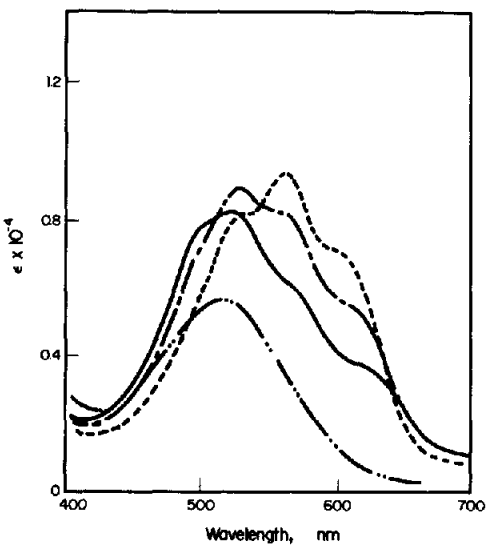


Fig. 4. Visible spectra of hirsutin and 4′-*O*-methylmalvin chlorides measured immediately after dissolution in Pi buffer, pH 7.0. Hirsutin: --- 5×10^{-5} M, ——— 5×10^{-4} M, —— 5×10^{-3} M. 4′-*O*-Methylmalvin: —··— 5×10^{-5}, 5×10^{-4} and 5×10^{-3} M (λ_{max} 518 nm). The λ_{max} of hirsutin and 4′-*O*-methylmalvin in 1% HCl–H_2O are 514 ($\varepsilon = 13\,600$) and 496 nm ($\varepsilon = 8000$), respectively.

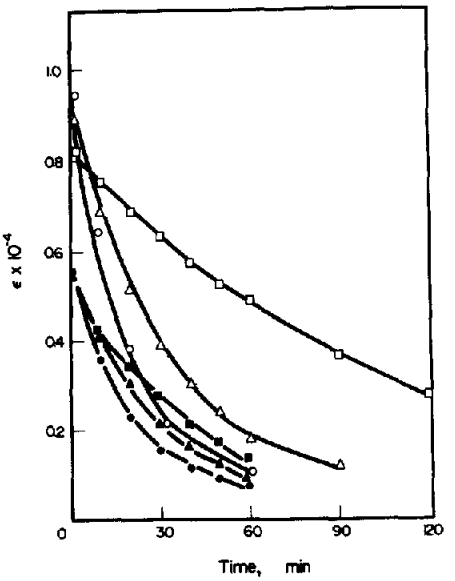


Fig. 5. Visible maximal absorbance of hirsutin (□, △, ○) and 4′-*O*-methylmalvin (■, ▲, ●) vs time. 5×10^{-3} M (□, ■), 5×10^{-4} M (△, ▲), 5×10^{-5} M (○, ●).

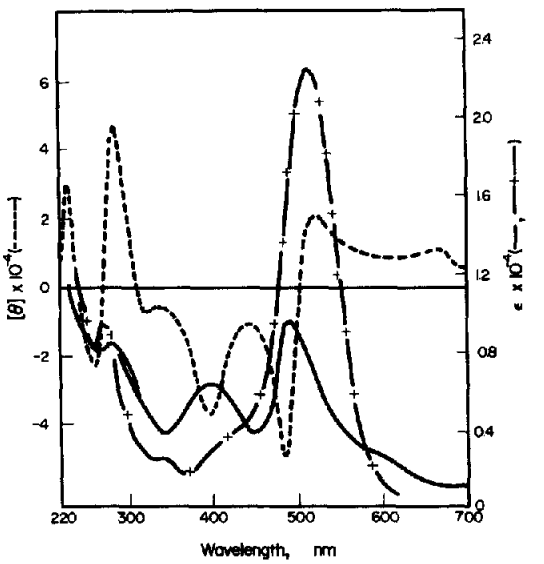


Fig. 7. UV-visible absorption (———) and CD spectra (– – –) of aggregated precipitates of malvidin 5-glucoside quinonoidal base (1×10^{-3} M) dissolved in water. + —— +, malvidin 5-glucoside (5×10^{-5} M) in N HCl. The visible λ_{max} values of the aggregated quinonoidal base and the flavylium ion are 492 and 516 nm, respectively.

precipitates must be highly self-associated aggregates. Therefore, the glucose moiety at the 5-position has an important role in the self-association of anthocyanidin 3,5-diglucosides. Self-aggregation in malvidin 5-glucoside also produces a remarkable decrease of the ε value (hypochromism) in the visible absorption (Fig. 7), if one assumes that the ε value of the quinonoidal bases is equal to [4] or slightly smaller than that of flavylium salts (see Figs. 1 and 3).

Attractive forces between anthocyanin molecules to form aggregates could be due to hydrogen bonds, hydrophobic interactions, etc. As shown in Figs. 8 and 9, addition of urea or DMSO produces a decrease in θ and a long wavelength shift in the visible spectra (compare Figs. 1 with 8, and Figs. 2 with 9); DMSO and urea presumably disrupt 'structured water' surrounding the aggregate to weaken the self-association. That self-association arises from hydrophobic interactions is in accord with the fact that hirsutin is capable of self-association.

We previously reported that concentrated solutions of some neutral salts such as 4 M NaCl and 4 M $MgCl_2$ strongly stabilize anthocyanin quinonoidal bases [9]. The stabilizing mechanism has remained unsolved. Addition of 4 M NaCl to malvin quinonoidal base gave larger θ, a hypsochromic shift (Figs. 8 and 9) and greater stability, suggesting that sodium chloride promotes self-association of anthocyanins. On the other hand, magnesium chloride may promote self-association

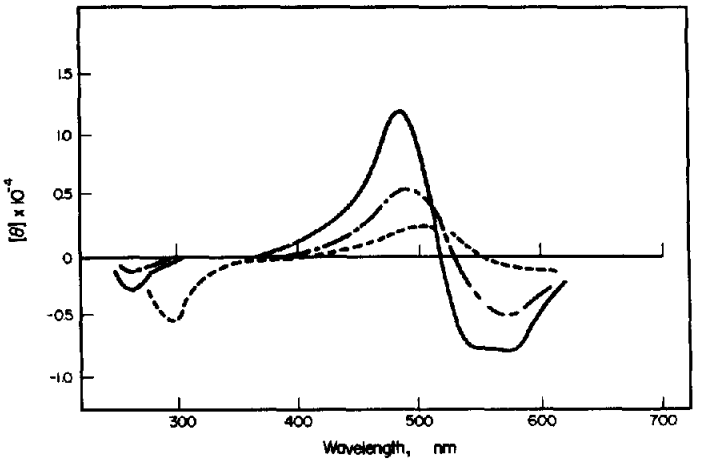


Fig. 6. CD spectra of hirsutin and 4′-*O*-methylmalvin in Pi buffer, pH 7.0. Hirsutin: ——— 5×10^{-3} M (11 600), — — — 5×10^{-4} M (6000). 4′-*O*-Methylmalvin: — — 1×10^{-2} M (2200). The values in parentheses represent the $[\theta]_{max}$ at second Cotton.

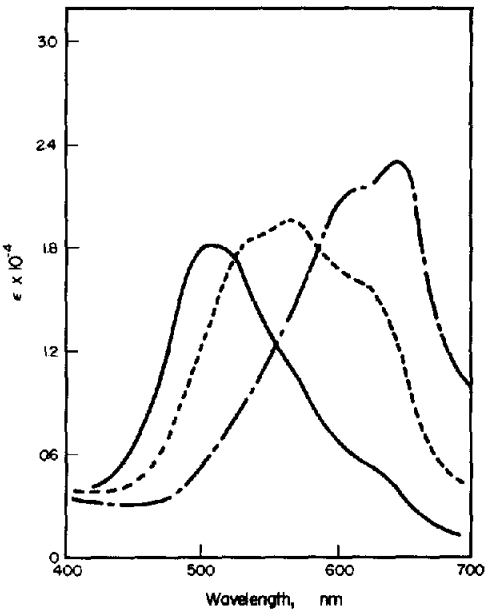


Fig. 8. Effect of DMSO, urea and sodium chloride in Pi buffer, pH 7.0. ——— containing 4 M NaCl (pigment concentration 5×10^{-4} M). Orange precipitates separated out about 60 min after dissolution. – – – and — — — contain 8 M urea and 50% DMSO, respectively, at 5×10^{-3} M of anthocyanin concentration.

through a different mechanism. When malvin quinonoidal base, freshly prepared according to ref. [10], was dissolved in 4 M $MgCl_2$, the θ was small and an increment of θ was not so concentration-dependent as that in the phosphate buffer solution. This suggests that the degree of self-association in 4 M $MgCl_2$ solution is smaller than that in the phosphate or NaCl solution, but 4 M $MgCl_2$ strongly stabilizes the quinonoidal base compared with 4 M NaCl. The reason is not clear, but may be attributable to the reduction of free water in the solution by the stronger hydration of Mg^{2+} ion compared to that of Na^+.

EXPERIMENTAL

Materials. Malvin chloride was purchased from Aldrich and used without further purification. Hirsutin and 4′-*O*-methylmalvin were synthesized from malvin. An ethereal soln of CH_2N_2 was added dropwise to the methanolic soln of malvin chloride and the reaction was followed by means of Avicel TLC with 1% HCl, in which the products hirsutin (R_f 0.21), 4′-*O*-methylmalvin (0.46) and dimethylated malvin (0.62) were clearly distinguished from starting material (0.11). The reaction mixture was treated with 10% HCl–MeOH in order to convert the colourless pseudobases to the red flavylium ions, evapd to dryness and chromatographed on an Avicel column with 1% HCl. The anthocyanins were purified by repeated chromatography. The structures of hirsutin and 4′-*O*-methylmalvin were determined by alkaline degradation [11]. Phloroglucinol and 3,4,5-trimethoxybenzoic acid from 4′-*O*-methylmalvin, and phloroglucinol monomethyl ether and syringic acid from hirsutin were identified by means of TLC (Si gel) or PC and detected by spraying 2,6-dibromoquinone-4-chloroimide in $CHCl_3$ followed by fuming with 2 M NH_4OH according to ref. [12]. Malvidin 3- and 5-monoglucosides were prepared by the partial hydrolysis of malvin with 2 N HCl at 80°. The reaction was monitored by TLC (Avicel) with H_2O–HOAc–HCl (87:10:3). The hydrolysates were chromatographed on an Avicel column with the same solvent as TLC to separate the mixture of Mv-3G and Mv-5G from the starting material and the aglycone. The fraction of the mixture of the 3- and 5-G was concd *in vacuo* to a small quantity and allowed to stand overnight, when only Mv-5G separated out. The supernatant was subjected to Avicel column chromatography with an eluant of *n*-BuOH–2N HCl (1:1). The fraction of Mv-3G was evapd to dryness. Mv-5G was finally purified by crystallization from 1% HCl. Mv-5G has the pink fluorescence, whereas Mv-3G lacks the fluorescence on Avicel TLC in UV light [13].

Spectral and CD measurements. CD spectra were determined with a Jasco J-500C spectropolarimeter equipped with a model DP-500 data processor for rapid measurements in the range of

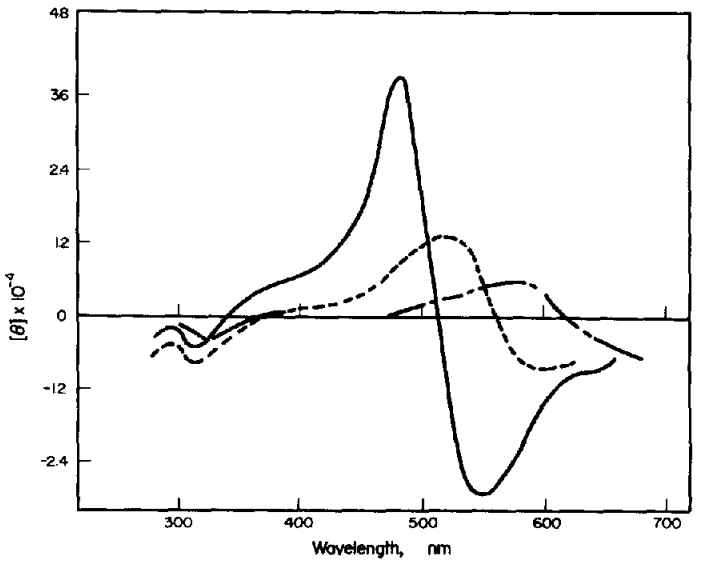


Fig. 9. CD spectra containing DMSO, urea and sodium chloride. Conditions the same as in Fig. 8.

200–700 nm within 60 sec. Variation of ε or θ with times after dissolution was determined. All anthocyanins to be investigated were dissolved in Na-Pi buffer (pH 7.0, 0.1 M). The spectra were determined at around 27° by using cells having a path length of either 0.1, 1 or 10 mm which were employed at 5×10^{-3}, 5×10^{-4} or 5×10^{-5} M of anthocyanin concn, respectively.

The spectra of Mv-5G quinonoidal base aggregates were determined as follows: ppts of the pigment were collected by centrifugation of the soln (2 ml of 5×10^{-4} or 1×10^{-3} M; dissolution of Mv-5G in 0.5 ml of 0.02 N HCl followed by addition of 1.5 ml of the Pi buffer, pH 7.0; the pH was 7.0) and left to stand for 2 hr. To the ppts was added 1 ml of water. The pH was determined to be 7. The anthocyanin concn of the supernatant was determined spectrophotometrically after dilution with 3 N HCl.

REFERENCES

1. Timberlake, C. F. and Bridle, P. (1975) in *The Flavonoids* (Harborne, J. B., Mabry, T. J. and Mabry, H., eds.) pp. 213–266. Chapman & Hall, London.
2. Asen, S., Stewart, R. N. and Norris, K. H. (1972) *Phytochemistry* **11**, 1139.
3. Asen, S., Stewart, R. N. and Norris, K. H. (1975) *Phytochemistry* **14**, 2677.
4. Scheffeldt, P. and Hrazdina, G. (1978) *J. Food Sci.* **43**, 517.
5. Goto, T., Hoshino, T. and Takase, S. (1979) *Tetrahedron Letters* 2905.
6. Hoshino, T., Matsumoto, U. and Goto, T. (1980) *Tetrahedron Letters* 1751.
7. Brouillard, R. and Dubois, J. E. (1977) *J. Am. Chem. Soc.* **99**, 1359.
8. Abe, K., Sakaino, Y., Kakinuma, J. and Kakisawa, H. (1977) *Nippon Kagaku Kaishi* 1197.
9. Goto, T., Hoshino, T. and Ohba, M. (1976) *Agric. Biol. Chem.* **40**, 1593.
10. Hoshino, T., Matsumoto, U. and Goto, T. (1980) *Phytochemistry* **19**, 663.
11. Karrer, P. and Widmer, R. (1927) *Helv. Chim. Acta* **10**, 5.
12. Yoshitama, K., Hayashi, K., Abe, K. and Kakisawa, H. (1975) *Bot. Mag. Tkyo* **88**, 213.
13. Abe, Y. and Hayashi, K. (1956) *Bot. Mag. Tokyo* **69**, 577.

NOTE ADDED IN PROOF

In conclusion, our unpublished results indicate that delphin and peonin behave similarly in solution to malvin and hirsutin, whereas pelargonin behaves similarly to cyanin. The origin of the anomalous CD behaviour shown by the latter two pigments is under investigation.