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CIRCULAR DICHROIC MEASUREMENTS ON ANTHOCYANINS IN INTACT FLOWER PETALS

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Key Word Index—CD of intact flower petals; intermolecular copigmentation; intramolecular copigmentation; metal complex; acylated anthocyanin; self-association; pH effect; quantitative effect; CD of flavylium ions; CD of acylated anthocyanins.

Abstract—Circular dichroism has been applied for the first time to living flower petals. Flowers of 11 from 30 species exhibited strong and characteristic circular dichroism. In flower plant tissues, the anthocyanin pigment chiroptically associates with itself or with other compounds such as flavone copigments. Circular dichroism spectra are discussed in relation to factors governing flower colour variation. Circular dichroism of flavylium salts and mono- or poly-acylated anthocyanins are given.

INTRODUCTION

Many new spectral procedures have been applied to the study of anthocyanin pigments in plants [1, 2], including electronic spectrophotometry with relaxation [3], FT-NMR [4], circular dichroism (CD) [5], fast atom bombardment (FAB) MS [6], and resonance Raman spectroscopy [1]. Some of these methods have been successfully applied to intact plant tissues containing anthocyanin pigments. The first *in situ* measurements of visible absorption spectra of flower petals were made by Saito [7], and similar reports appeared later [8]. Asen *et al.* have measured the exact pH of epidermal cells and also succeeded in reproducing the visible spectra of intact flower petals *in vitro* by controlling anthocyanin–copigment ratio, pigment concentration and pH [9, 10].

In earlier papers, I have demonstrated that CD is a sensitive probe for examining the molecular association of anthocyanin pigments [5, 11–13]. Considering that the pigment concentration in flowers may be about 10^{-2} M or higher, it might be anticipated that flower petals would show CD curves as a result of molecular association. We report here some CD results obtained from intact flower plant tissues.

RESULTS AND DISCUSSION

Figures 1 and 2 show the circular dichroism together with visible absorption spectra of living flower petals. Typical blue flowers, e.g. *Centaurea cyanus* and *Commelina communis*, exhibited characteristic CD around the visible absorption (Fig. 1), which consist of a pair of Cotton effects of negative and positive signs (called exciton coupled CD). The first Cotton effects of their flowers were negative. CD spectra of the intact petals were identical to those of the pure pigments, cyanocentaurin (protocyanin) [14–16] and commelinin [17, 18], in aqueous solutions. The blue colour of these pigments is due to both copigmentation with flavones and metal complexing. Blue flowers of *Parochetus communis* also showed typical split-CD, although the first Cotton effect was positive (Fig. 1). In our previous studies on the self-

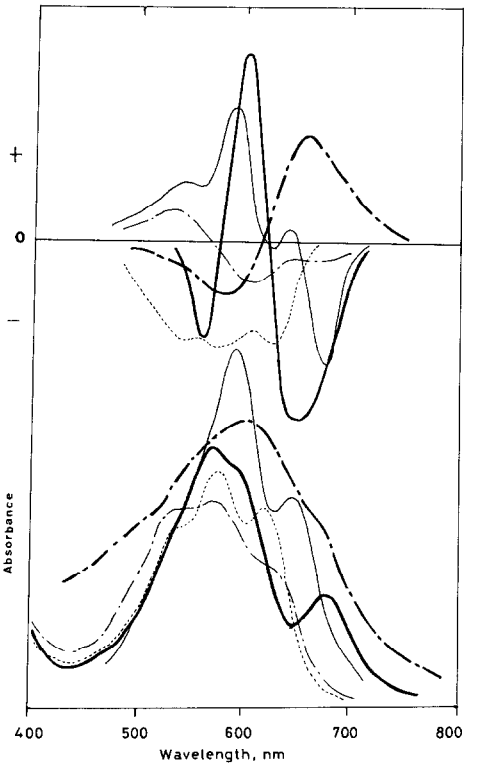

Fig. 1. CD (top) and visible spectra (bottom) of live flower petals with blue colour. *Centauria cyanus* L. ——; *Commelina communis* L. ——; *Parochetus communis* Buch-Ham. et D. Don — - —; *Saintpaulia ionantha* H. Wendl. — - —; *Senecio cruentus* DC. - - - - -. CD magnitudes and absorbances are arbitrary.

association of anthocyanins, we have reported that in the case of negative first Cotton effect anthocyanin chromophores stack in left handed helical conformation, while in the case of positive first Cotton effect anthocyanin molecules have the right handed helical stacking geometry [12, 13]. Results from blue petals suggest that the stacked pairs between anthocyanins and other flavonoids further associate in a right or left handed helical conformation [12, 13, 18]. It is significant that both stacking arrangements occur in nature.

CD measurements on flowers containing acylated anthocyanins were made. Recently polyacylated anthocyanins have been discovered in the flowers of *Senecio cruentus* [4, 19, 20], *Platycodon grandiflorum* [21, 22], *Ipomea tricolor* cv. Heavenly Blue [23, 24] and *Gentiana makinoi* [25]. Acylated anthocyanins with two or more acyl groups are stable in slightly acidic or neutral solutions and the pigments alone are responsible for the flower colours expressed *in vivo*. Their extraordinary stability is due to the stacking of anthocyanin nuclei with the aromatic residues of acyl groups [1, 25]. Live petals of blue and red cineraria displayed a chirality shown in Figs 1 and 2, respectively. The absorption spectra of intact petals were identical to those of pure cinerarin [19] and rubrocinerarin [20] reported by Yoshitama [26]. Other flowers, *Platycodon grandiflorum* and *Ipomea tricolor*, did not give characteristic CD around the visible region. Pure platyconin displays a chirality [27, 28]. The reason for the *in vivo* and *in vitro* differences is not clear. The pigment, isolated from deep violet petunia petals, also exhibited large Cotton effects shown in Fig. 3. Interestingly, concentration dependence for CD was not observed; the quinonoidal bases showed almost identical CD in solutions of both low ($\sim 10^{-5}$ M) and high concentration ($\sim 10^{-3}$ M). CD and visible spectra of the petunia petals were almost the same as those in the buffered solution of the pure pigment at pH 6.0 (Figs 2 and 3). The complete structure of this pigment has yet to be determined. However, the pigment is an acylated anthocyanin, presumably with two aromatic residues, because the value of $E_{acyl}/E_{vis\text{-}max}$ is 0.96 and the colour was more stable than that of monoacylated anthocyanins. The monoacylated anthocyanins, shisonin and tibouchinin, gave rise to a little CD at both low and high pigment concentrations in neutral aqueous solutions. On the other hand, awobanin quinonoidal bases showed large split-CD even in a dilute solution. Surprisingly, at high pigment concentrations, the magnitude of the CD spectrum became smaller (Fig. 4). The origin of the anomalous CD behaviour for these acylated anthocyanins with one or more aromatic acyl groups is not yet clear.

Exciton coupled CD was also found in the flowers with red colours, as in petunia and azalea petals (Fig. 2). The pigments of azalea flowers are cyanidin glycosides and the pH of the epidermal cells is reported to be 3.1–3.2 [9],

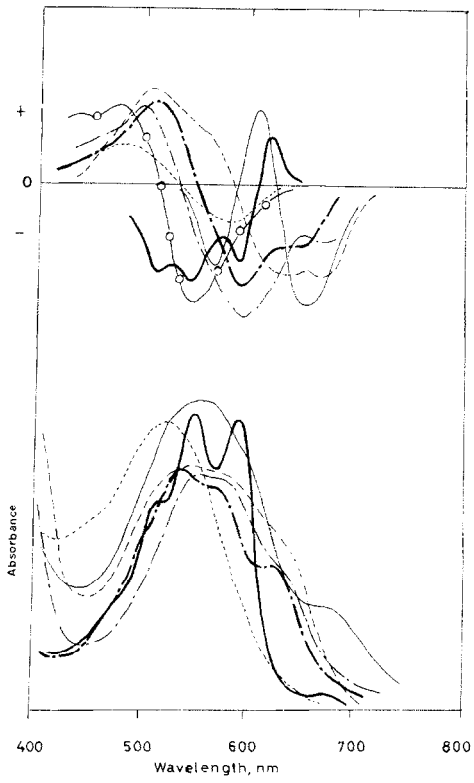

Fig. 2. CD (top) and visible absorption spectra (bottom) of the flower petals having colours of red to deep purple. *Senecio cruentus* DC. (red) ——; *Centauria cyanus* L. (reddish purple) ——; *Aquilegia flavellata* Sieb. et Zucc (deep purple) — - —; *Viola tricolor* L. (deep purple) — - —; *Petunia hybrida* (deep purple) — -- —; *Petunia hybrida* (red) - - - - - -; *Azalea* (red) O——O. Units of CD and absorbances are arbitrary.

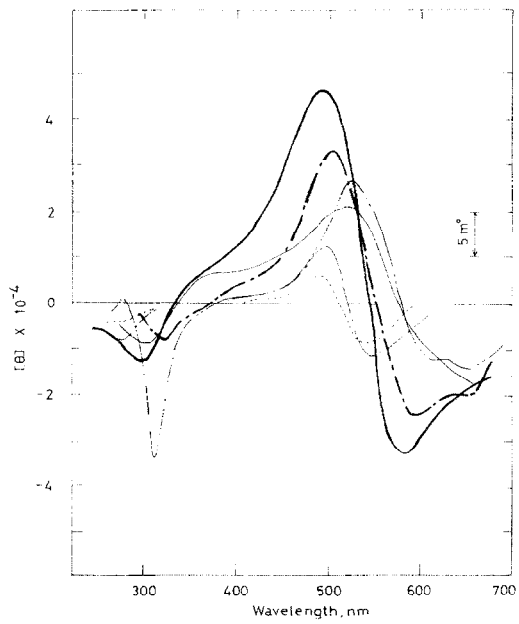

Fig. 3. CD spectra of some anthocyanins in aqueous solutions. Cyanin at 5×10^{-4} M in N HCl - - - - -; petunin 5×10^{-4} M in N HCl — - —; petunin at 5×10^{-4} M in pH 7.0 of phosphate buffer — - —; the pigment from deep purple *Petunia* petals, at the concentration of 3.40 mg per 10 ml (pH 6.5) — - - - (cell length 1 mm. CD scale is shown in the figure); violanin in a buffered medium of pH 6.0: 2.5×10^{-3} M ——, 5×10^{-5} M ——. CD measurements of quinonoidal bases were within 1 min after dissolution.

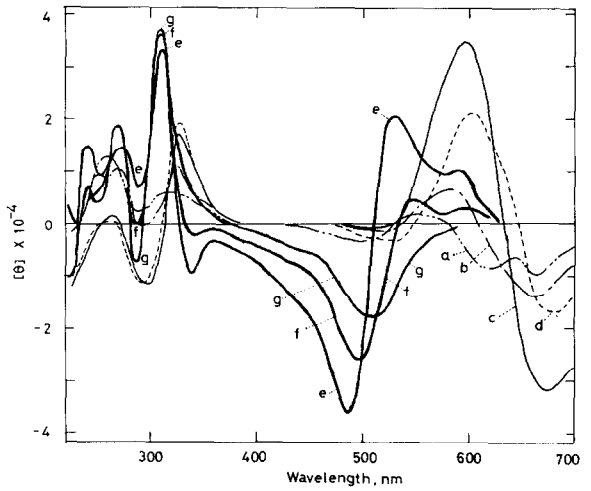

Fig. 4. CD spectra of awobanin at different concentrations in buffered media of neutral and strongly acidic pH. The quinonoidal bases are shown in a, b, c and d at the pH of 7.0. The flavylium ions are represented by e, f and g in a solution of N HCl. Pigment concentrations are as follows: a and e, 5×10^{-3} M; b and f, 5×10^{-4} M; c and g, 5×10^{-5} M; d, 1×10^{-5} M. CD measurements of quinonoidal bases were within 1 min after dissolving.

where the predominant species is the flavylium ion [1]. At these pHs, however, large amounts of the corresponding pseudobase coexists [1]. Previously, we have reported that the colourless pseudobase does not show a CD curve [11]. It was examined *in vitro* whether anthocyanin pigments associate in strongly acidified media (pH < 1) to show the exciton split CD. Figure 3 shows CD spectra of the flavylium cations for petunin and cyanin at the pigment concentration of 5×10^{-4} M. In a dilute solution (5×10^{-5} M), flavylium salts of anthocyanidin 3,5-diglucosides show little CD. At a higher concentration of 5×10^{-3} M, which is close to the pigment concentration in flower petals, exciton coupled CD was observed with first negative and second positive signs for all six common anthocyanidin 3,5-diglucosides. The CD intensity was small compared to the self-association of quinonoidal bases (Fig. 3). The self-association phenomenon of flavylium ions was also found in the colour expression of grape skins [29]. The concentration of anthocyanin can affect flower colour [30]; anthocyanin content in a deep red rose petal is greater than that of a deep pink rose [31]. My attempts to measure CD of deep red petals were unsuccessful because the thickness of the petals causes poor light transmittance or light scattering. In the deep red flowers, cyanin pigments might self-associate to show the exciton coupled CD. For awobanin flavylium ions, in a dilute solution (5×10^{-5} M), the CD showed only a simple Cotton effect, but at higher concentrations exciton coupled CD was found (Fig. 4), indicating that positively charged delphinidin nuclei stack with each other. This finding suggests that intermolecular association of anthocyanin chromophores does occur in the case of monoacylated anthocyanins. Of interest is that the Cotton effects of the flavylium ions are opposite of those of the quinonoidal bases (Fig. 4). The pigment, violanin, from deep violet flowers of pansy, also self-associates to form a chiral complex; increasing concentration of the quinonoidal bases produced larger magnitudes of exciton split-CD (Fig. 3). The pH of the vacuoles is reported to be 5.9 [9]. CD and visible spectra of the live petals were the same as those in the solution of buffered medium (pH 6.0) (Figs 2 and 3). The deacylviolanin was unstable, compared to violanin; deacylviolanin became almost colourless within 30 min after dissolution even in a self-associated solution of 5×10^{-3} M, while the acylated pigment, violanin, retained about half its absorbance after 2 hr in a dilute solution (5×10^{-5} M). The increase in violanin concentration meant that the neutral solution was more stable to light. Therefore, acylation (intramolecular association) and self-association of delphinidin nucleus (intermolecular association) both provide flower colour *in vivo*. Both stabilizing effects may have contributed to the successful isolation of the genuine anthocyanin from deep violet pansy [32]. The crystallization of the genuine anthocyanin from *Fuchsia* petals can be ascribed to the self-association phenomenon of malvin quinonoidal bases [33].

In conclusion, CD measurements on living flowers may help in understanding how anthocyanin pigments are present in nature, especially with respect to the elucidation of the associated forms or conformational structures in the complex media of flowers.

EXPERIMENTAL

CD measurements of flowers. CD spectra were measured with a Jasco J-500C spectropolarimeter. Freshly picked flower petals were placed in the cell holder and set in the nearest position to the light source of a Xe lamp. CD measurements were repeated at least three times by rotating flower petals around the axis of light emerged from the instrument, and then uniformity of base line in CD was corrected or minimized. Electronic spectra of flowers were obtained using a Hitachi multipurpose spectrophotometer, 557.

Materials. The procedures used for the isolation of pigments were as follows: as in ref. [14] for cyanocentaurin or protocyanin; ref. [17] for commelinin; ref. [34] for awobanin and tibouchinin; ref. [5] for shisonin and cyanin; ref. [35] for violanin. Violanin was deacylated by standard procedures. The purification of the pigment from deep purple petunia was as follows: adsorption on polyamide column, elution with slightly acidified MeOH and then CC on Avicel microcrystalline cellulose with HOAc–HCl–H_2O (10:1:89) as eluent. Final purification was carried out with MeOH–Et_2O. The isolated pigment (1% HCl–MeOH) absorbed in the region of 300–310 nm due to acylation and lacked a distinct shoulder at 410–440 nm ($E_{440}/E_{\text{vis-max}} = 0.13$) [36]. Values of $E_{\text{acyl}}/E_{\text{vis-max}}$ is 0.96, which is close to those observed in poly acylated anthocyanins [4, 22, 25].

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