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ANTHOCYANIN AND pH INVOLVED IN THE COLOR OF 'HEAVENLY BLUE' MORNING GLORY

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Key Word Index—*Ipomoea tricolor*; Convolvulaceae; anthocyanin; peonidin 3-(dicaffeylsophoroside)-5-glucoside; quercetin 3-rutinoside; quercetin 3-glucoside; epidermal pH.

Abstract—The major anthocyanin in blue morning glory flowers, peonidin 3-(dicaffeylsophoroside)-5-glucoside, is stable in a neutral aqueous solution and is solely responsible for the color of the flowers. Co-ocurring flavonols based on quercetin at the pH's of epidermal cells have no effect on the color of the anthocyanin. Deep or strong reddish-purple buds change to moderate or light blue open flowers within a 4 hr period, and during this time the pH of epidermal tissue increases from *ca* 6.5 to 7.5.

INTRODUCTION

Among the several hundred *in vivo* absorption spectra of flower petals we have recorded, those of blue morning glory (*Ipomoea tricolor* Cav cv Heavenly Blue) exhibited characteristics of special interest. The visible λ_{max} of fully opened light blue [1] flowers is 600–605 nm and preliminary analyses indicated that the principal anthocyanin was a peonidin glycoside. Most blue or purplish–blue flowers are reported to contain glycosides or acylated glycosides of delphinidin. We now report on the pigment system responsible for the color of these flowers.

RESULTS

The major isolated anthocyanin is peonidin 3-(dicaffeylsophoroside)-5-glucoside, the same pigment as has been isolated from *Ipomoea batatas* [2] and *I. congesta* [3]. A cyanidin glycoside is also present in trace amounts. Quercetin 3-rutinoside (rutin) is the major flavonol glycoside and comprises *ca* 2% of the weight of the dry tissue. Also present is quercetin 3-glucoside (isoquercitrin) and a salt of an acylated isoquercitrin, which was not fully identified because of lack of material. Similar results on the major pigments of morning glory flowers have recently been reported by Ishikura and Shimizu [4].

Strong reddish-purple buds change to light blue open flowers within 4 hr. During this maturation period, the pH of epidermal tissue increases from *ca* 6.5 to 7.5. The color of a 10^{-3} M solution of the isolated anthocyanin intensifies and became bluer with increases in pH due to a bathochromic shift in the visible λ_{max} and an increase in log ε. At pH 6.5 the visible λ_{max} is 547 nm and the log ε 4.06; at pH 7.4, 592 nm and 4.13; at pH 7.8, 603 nm and 4.25; and at pH 8.3, 607 nm and 4.35.

The shape and magnitude of the visible absorption spectrum of a 5×10^{-3} M solution of the isolated anthocyanin at pH 6.6 and with an optical pathlength of 0.062 mm (*ca* that of 2 epidermal petal cells), closely matches the spectrum of typical intact, deep reddish-purple petals. At pH 7.5, the visible absorption spectrum of a 5×10^{-3} M solution of the isolated anthocyanin closely matches that of typical intact light blue petals from opened flowers.

At pH 2.7, a 5×10^{-3} M solution of the isolated anthocyanin has a λ_{max} at 528 nm and, with the addition of an equal concentration of rutin, the λ_{max} shifted to 535 nm. No such copigment effect was evident at pH 6.6 or 7.9. The extinction of the visible λ_{max} of either a 1.2×10^{-3} M or a 6×10^{-5} M solution (pH 7.9) of the isolated anthocyanin was reduced only 2% when left standing for 1 hr at 27° in diffused light. Under similar conditions, the extinction of the visible λ_{max} of a 1.2×10^{-3} M solution of peonidin 3,5-diglucoside was reduced 31%.

DISCUSSION

As the number of hydroxyl groups in the B-ring of anthocyanidin molecules increases, they absorb at longer wavelengths which causes them to appear bluer so that pink, scarlet and orange-red flowers generally contain pelargonidin, crimson and magenta flowers contain cyanidin, and mauve and blue flowers contain delphinidin. However, this generalization ignores the modifying effects on color in plant tissue of glycosylation, acylation, pH, copigmentation, self-association and metal chelation. The majority of the blue-flowered species surveyed by Forsyth and Simmonds [5] and Gascoigne *et al.* [6] contained glycosides of delphinidin although a few contained glycosides of malvidin, cyanidin or petunidin. With the identification of peonidin 3-(dicaffeylsophoroside)-5-glucoside from 'Heavenly Blue' morning glory flowers, [see also refs 4 and 7] glycosides of five of the six common anthocyanidins have now been reported in blue flowers. The visible absorption spectrum of a 5×10^{-3} M solution of the isolated anthocyanin was not altered nor was the stability enhanced by the addition of an equal molar concentration of rutin or isoquercitrin. At pH 6.6 and higher, the isolated anthocyanin occurred

as the ionized anhydro base and, in this form did not exhibit copigment effects with those flavonols. Thus, the color of 'Heavenly Blue. morning glory flowers is due solely to peonidin 3-(dicaffeylsophoroside)-5-glucoside Color changes associated with flower maturation are caused by the effect of increases in pH on the anthocyanin. The manifestation of bluish color flowers by anthocyanin *per se* has previously been suggested by Yoshitama and Hayashi [8] for garden cineraria and by Saito *et al.* [9] for Chinese Bellflower; both these flowers contain delphinidin glycosides acylated with caffeic acid.

Acylated anthocyanins have been identified from many blue flowers. Yoshitama and Hayashi [8] observed that delphinidin glycosides acylated with *p*-coumaric acid were purple and unstable in a neutral aqueous solution; however, cinerarin, a delphinidin glucoside acylated with 2 mol of caffeic acid, was blue and stable. The peonidin glucoside in 'Heavenly Blue' morning glory flowers is acylated with caffeic acid, and like cinerarin, is stable. Yoshitama and Hayashi [8] suggested that the *o*-dihydroxyl groups in the B-ring of delphinidin and in the caffeyl residue attached to the 3-*O*-glucosyl residue(s) may be associated, and that the caffeic acid moiety in the molecule may be essential to the stability of the blue color of cinerarin. This explanation for stability would not apply to the present anthocyanin because the B-ring of peonidin has no *o*-dihydroxyl group. Although the role of the acyl moiety still remains speculative, the vicinal hydroxyl grouping of caffeic acid apparently is important to the stability of anthocyanins.

EXPERIMENTAL

Plant material. 'Heavenly Blue' morning glory seeds were purchased from George W. Parks Seed Co. Inc., Greenwood, South Carolina 29647, U.S.A. Plants were grown locally in a greenhouse under standard conditions.

Anthocyanin. Anthocyanin from the blue portion of open flowers was isolated by column chromatography on purified insoluble PVP [10,11]. The anthocyanin was further purified on a 40 × 500 mm cellulose column developed with HOAc-HCl–H_2O (15:3:82), by precipitation with Et_2O, and finally through a 25 × 400 mm column of Sephadex G-50 with H_2O containing 5 ml of 2N HCl/L. The isolated anthocyanin was hydrolyzed under usual conditions and the products identified by standard means [12]. The λ_{max} in 1% HCl–MeOH were 529 nm (log ε 4.37), 321 nm (log ε 4.37), and 292 nm (log ε 4.44). $E_{440}/E_{vis\ max}$ was 0.13. R_f's were: 0.12 (1% HCl), 0.30 (HOAc–HCl–H_2O 15:3:82), 0.21 (*n*-BuOH–HOAc–H_2O 6:1:2), and 0.30 (*n*-BuOH–2N HCl 1.1). After alkaline hydrolysis the λ_{max} and R_f's, in the same solvents, were 520 and 276 nm, and 0.70, 0.82, 0.17 and 0.10, respectively. Complete acid hydrolysis of the deacylated anthocyanin yielded peonidin and glucose, whereas partial acid hydrolysis yielded 4 intermediate peonidin glucosides. Three of the intermediate peonidin glucosides were indistinguishable from peonidin 3,5-diglucoside and the products of its partial acid hydrolysis, peonidin 3-glucoside and peonidin 5-glucoside. For comparable R_f's see Harborne [13]. R_f's for the fourth intermediate peonidin glucoside were: 0.35 (1% HCl), 0.65 (HOAc–HCl–H_2O 15:3:82), 0.29 (*n*-BuOH–HOAc–H_2O 6:1:2), 0.25 (*n*-BuOH–2N HCl 1:1), and 0.20 (EtOAc–HCOOH–H_2O 10:2:3). Partial acid hydrolysis yielded only peonidin 3-glucoside. The fourth intermediate was peonidin 3-sophoroside. The glucose-glucose linkage was not established, but R_f's indicated a sophoroside (β, 1 → 2) and not a gentiobioside (β, 1 → 6) linkage [13]. The acyl moiety obtained from alkaline hydrolysis was chromatographically (in 4 solvents) and spectrally indistinguishable from caffeic acid. Solutions in 0.5% HCl–MeOH that contained molar ratios of caffeic acid to peonidin 3,5-diglucoside of 0:1, 1:1, 2:1 and 3:1 exhibited E_{322}/E_{522} of 0.09, 0.51, 0.95, and 1.40, respectively. In the same solvent this absorbance ratio for the isolated anthocyanin was 1.01 which indicated that the molar ratio of caffeic acid to anthocyanin was 2:1. The two caffeyl moieties are presumably attached to the sugars in the C-3 position [14].

Flavonols. Flavonols in the light blue and in the white portions of the corolla were chromatographically the same, and only compounds extracted from the white portion were isolated by column chromatography on purified insoluble PVP. Elution with 50% aqueous MeOH gave 3 flavonols, exhibiting similar absorption spectra, λ_{max} in EtOH: 257 and 363 nm; with NaOEt, 272, 334, and 415 nm; with NaOAc, 273, 325, and 382 nm; with $AlCl_3$, 274 and 420 nm; and with H_3BO_3–NaOAc, 264 and 387 nm.

The first flavonol was soluble in H_2O, but insoluble in pyridine, which indicated it was a salt. R_f's were: 0.87 (H_2O), 0.59 (*n*-BuOH–HOAc–H_2O 6:1:2), 0.41 (15% HOAc) and 0.27 (PhOH–H_2O 73:27 w/w). After alkaline hydrolysis, R_f's in the same solvents were 0.10, 0.69. 0.32 and 0.41; none of the common hydroxy-cinnamic acids could be detected in the hydrolysate The deacylated compound yielded quercetin and glucose when completely hydrolyzed with acid or β-glucosidase. Partial acid hydrolysis yielded no intermediate glucoside and the compound was indistinguishable from isoquercitrin. Sufficient amounts were not available for complete identification. The other two flavonols were identified as isoquercitrin and rutin by standard procedures [13].

Tissue pH measurement. The pH of epidermal peels (*ca* 6 sq mm) from flowers was determined spectrophotometrically [15].

Intact-tissue spectra. Spectral absorption curves of entire fresh petals (1 × 4 mm aperture) or individual epidermal cells (20 μm aperture) were measured with a spectrophotometer developed in one of our laboratories [16].

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