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ANTHOCYANIN, FLAVONOL COPIGMENTS, AND pH RESPONSIBLE FOR LARKSPUR FLOWER COLOR

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Key Word Index—*Consolida ambigua*, *Delphinium ajacis*, Ranunculaceae, anthocyanin, delphinidin 3-di(*p*-hydroxybenzoyl)glucosylglucoside, kaempferol glycosides, kaempferol 3-(caffeylgalactosylxyloside)-7-rhamnoside, epidermal pH, anthocyanin polymerization

Abstract—The anthocyanin and flavonol glycosides in Larkspur flowers (cv. Dark Blue Supreme) are delphinidin 3-di(*p*-hydroxybenzoyl)glucosylglucoside, kaempferol 3-robinobioside-7-rhamnoside (robinin), kaempferol 3-rutinoside, kaempferol 7-rhamnoside, and kaempferol 3-(caffeylgalactosylxyloside)-7-rhamnoside. As young flowers age the pH of epidermal tissue increases from 5·5 to 6·6 and the color of many of the cells changes from moderate reddish-purple to light purplish-blue. Many of the older cells also contain blue crystals. Visible absorption spectra of moderate reddish-purple and light purplish-blue cells were simulated with a solution of the anthocyanin (10^{-2} M) plus robinin (5×10^{-3} M) at pH 5·6 and 7·1, respectively. Changes in the absorption spectra of living tissue with heating or cooling and of concentrated solutions of the anthocyanin with dilution or moderate heat, indicate that in the natural state the pigment is present in an associated form.

INTRODUCTION

Of the several hundred *in vivo* absorption spectra of flower petals that we have recorded, several exhibited characteristics of special interest. Among these were the moderate reddish-purple flowers of larkspur cv. Dark Blue Supreme [*Consolida ambigua* (L.) P. W Ball & Heywood (*Delphinium ajacis* auct.)] because they show distinctive sharp absorption bands not apparent in others[1]. We now report the pH, anthocyanin and flavonol glycosides responsible for these sharp absorption bands.

RESULTS AND DISCUSSION

Anthocyanin

The absorption spectrum of the isolated anthocyanin in 1% HCl–MeOH showed a λ_{max} at 545 nm (log ϵ 4·36) and an unusual λ_{max} at 248 nm (log ϵ 4·37). The $E_{440}/E_{vis\,max}$ was clearly that of a 3-substituted anthocyanin. Typical R_f's (×100) were 15 (1% HCl); 11 (*n*-BuOH–HOAc–H_2O, 6:1:2); 15 (*n*-BuOH–2N HCl, 1:1); and 47 (HOAc–HCl–H_2O, 15:3:82). After base hydrolysis, R_f's in the same solvents were 12, 3, 2, and 36, respectively, and the anthocyanin now had a λ_{max} at 535 and 278 nm. Changes in R_f's and absorption spectrum after base hydrolysis established that the isolated anthocyanin was acylated.

The acyl moiety obtained from base hydrolysis was chromatographically and spectrally indistinguishable from an authentic sample of *p*-hydroxybenzoic acid. Typical R_f's (×100) were 21 (*iso*-PrOH–NH_4OH–H_2O, 8:1:1); 60 (C_6H_6–propionic acid–H_2O, 2:1:1); and 5 (toluene–HOAc–H_2O, 4:1:5). The R_f's of the *m*-isomer were distinctively different. The acyl moiety and *p*-hydroxybenzoic each became orange yellow when sprayed with diazotized sulphanilic acid[2] and the absorption spectrum of each in EtOH showed a λ_{max} at 250 nm, shifting to 278 nm with the addition of NaOEt.

The mass spectral fragmentation pattern of the acyl moiety and that of an authentic sample of *p*-hydroxybenzoic acid were identical. Each showed that the molecular ion was *m/e* 138 and principal fragments were observed at *m/e* 121 and 93 presumably due to loss of OH and CO_2H,

respectively. The structure was confirmed by reaction with diazoethane to give ethyl *p*-ethoxybenzoate, which possessed the expected mass spectral fragmentation pattern. The mass spectrum of the *p*-isomer could not be distinguished from that of the *m*-isomer, but the *o*-isomer showed a principal fragment at *m/e* 120 due to loss of H_2O from the molecular ion. There was no intense peak due to loss of CO_2H. Chromatography previously confirmed the acyl moiety as the *p*-isomer.

In 1% HCl–MeOH, solutions which contained molar ratios of delphinidin 3-glucoside to *p*-hydroxybenzoic acid of 1:0, 1:1, 1:2, and 1:3 exhibited E_{UVmax}/E_{vismax} of 0·30, 0·74, 1·18, and 1·63 respectively. In the same solvent the E_{UVmax}/E_{vismax} of the isolated anthocyanin was 1·04, thus indicating that the ratio was 1:2.

Complete hydrolysis with acid yielded delphinidin and glucose whereas partial hydrolysis yielded delphinidin 3-glucoside as the only intermediate. Typical R_f's (×100) for an authentic sample of delphinidin 3-glucoside and the intermediate compound were 18 (HOAc–HCl–H_2O, 3:1:8) and 13 (*n*-BuOH–HCl–H_2O, 7:2:5).

All the above criteria indicate that the isolated anthocyanin was delphinidin 3-di(*p*-hydroxybenzoyl) glucosylglucoside. The location of the acyl groups and the glucose–glucose linkage have yet to be established.

There are a number of conflicting reports as to the anthocyanin present in "violet" larkspur flowers. Willstäter and Mieg[3] isolated delphinin from "violet" larkspur flowers (*Delphinium consolida* L.), the first described acylated anthocyanin, and reported that this pigment consisted of 1 mol of delphinidin and 2 mol each of glucose and *p*-hydroxybenzoic acid. Harborne [4], who reported great difficulty with purification, isolated an anthocyanin from a "dark-blue" form of *Delphinium consolida* and concluded that the pigment was probably delphinidin 3,5-diglucoside. No *p*-hydroxybenzoic acid was detected in the alkaline or acid hydrolysis and H_2O_2 oxidation also failed to yield *p*-hydroxybenzoic acid glucose ester. Shibata and Yoshitama[5] isolated an anthocyanin from "violet" flowers of larkspur (*Delphinium consolida* L.) which they postulated was *p*-hydroxybenzoylcaffeyldelphinidin 3-diglucoside. Although the anthocyanin we report was obtained from moderate reddish-purple *Consolida ambigua* (*Delphinium ajacis*) flowers the results agree with those reported by Willstater and Mieg[3] Discrepancies reported for the anthocyanin in "violet" larkspur are most likely due to use of different plant material, since the various annual larkspurs are known to be of complex hybrid origin

Kaempferol glycoside copigments

The major flavonoid copigments were kaempferol glycosides. They were separated into 4 fractions by column chromatography on MN-Polyamide-SC6 and the compounds in each fraction were resolved by preparative TLC.

Fraction 1 contained the anthocyanin and compound 1. Spectral data for compound **1** were those of a 3,7-disubstituted kaempferol (Table 1) Complete hydrolysis with acid yielded kaempferol, galactose and rhamnose whereas controlled acid hydrolysis yielded 4 intermediate kaempferol glycosides which were chromatographically indistinguishable from those obtained from an authentic sample of kaempferol 3-robinobioside-7-rhamnoside (robinin) The intermediate glycosides formed were kaempferol 3-galactoside, kaempferol 7-rhamnoside, kaempferol 3-galactoside-7-rhamnoside, and kaempferol 3-robinobioside[6] Spectral and chromatographic criteria indicated that compound 1 was robinin.

Fraction 2 only contained compound **2**. Although complete hydrolysis with acid yielded kaempferol, galactose and rhamnose, spectral data (Table 1) were not indicative of a kaempferol glycoside. The λ_{max} at 337 nm was lower than that expected although tiliroside, originally thought to be kaempferol 7-*p*-coumaryl-3-glucoside[7] but subsequently identified as the 3-*p*-coumarylglucoside[8], has a λ_{max} at 317 nm Compound **2** also showed an 18 nm borate shift which indicated the presence of an *o*-dihydroxyl group Base hydrolysis of compound **2** changed its R_f (Table 1) and yielded a compound chromatographically and spectrally indistinguishable from an authentic sample of caffeic acid. Both the acyl moiety and caffeic acid showed the characteristic orange brown color when treated with diazotized sulfanilic acid and

Table 1 Properties of kaempferol glycosides isolated from Dark Blue Supreme Larkspur flowers

Isolated kaempferol glycosides	λ_{max} in EtOH, nm Band I				Band II		$R_f \times 100$ *Solvents				†Products of acid hydrolysis	Identification
	Alone	+NaOEt	+$AlCl_3$	+H_3BO_3 +NaOAc	Alone	+NaOAc	1	2	3	4		
1	350	399	400	353	266	266	50	54	82	74	Km, Gal, Rha	Km 3-robinobioside 7-rhamnoside (robinin)
2	337	392	400	355	266	266	49	40	74	62	Km, Gal, Xyl, Rha, ‡Caf	Km 3-(caffeylgalactosylxyloside) 7-rhamnoside
§2A	351	400	400	352	266	266	39	57	86	56	Km, Gal, Xyl, Rha	Km 3-(galactosylxyloside) 7-rhamnoside
‖2B	335	398	395	357	266	275	63	32	64	68	Km, Gal, Xyl	Km 3-(caffeylgalactosylxyloside)
3	350	405	398	354	266	275	62	31	64	69	Km, Glc, Rha	Km 3-rutinoside
4	367	430	429	369	266	264	89	4	14	83	Km, Rha	Km 7-rhamnoside

* Determined by TLC on plates with a 250 μm layer of microcrystalline cellulose (Avicel) in 1, *n*-BuOH–HOAc–H_2O (6 1 2); 2, H_2O, 3, 15% HOAc, 4, Phenol–H_2O (73:27 w/w) † Abbreviations. Km = Kaempferol, Glc = glucose, Gal = galactose, Rha = rhamnose, Xyl = xylose, Caf = caffeic acid ‡ Product of base hydrolysis § Obtained from base hydrolysis of 2 ‖ Obtained from α-L-rhamnosidase hydrolysis of 2.

each had λ_{max}^{EtOH} 244, 295 (infl.), 326 nm; λ_{max}^{NaOEt} 252, 302, 345 nm (dec.), $\lambda_{max}^{EtOH-AlCl_3}$ 250 (infl.), 310 (infl.), 336; and $\lambda_{max}^{EtOH-H_3BO_3}$ 295, 334 nm. Typical R_f's (×100) were 56 (Phenol–H_2O, 73/27 w/w); 18 (C_6H_6–propionic acid–H_2O, 2:2:1); 0 (Toluene–HOAc–H_2O, 4:1:5) and 7 (C_6H_6–HOAc–H_2O, 6:7:3).

Compound **2** was hydrolyzed by α-L-rhamnosidase to yield rhamnose and a new kaempferol glycoside substituted only in the 3-position and still acylated (Table 1). Base hydrolysis yielded caffeic acid and complete hydrolysis with acid yielded kaempferol, galactose and xylose. Controlled acid hydrolysis yielded only one intermediate glycoside which was chromatographically indistinguishable from kaempferol 3-xyloside. Typical R_f's (×100) were 88 (*n*-BuOH–HOAc–H_2O, 6:1:2); 11 (H_2O); 35 (15% HOAc); and 77 (Phenol–H_2O 73:27 w/w). The new kaempferol glycoside which resulted from hydrolysis of compound **2** with α-L-rhamnosidase was kaempferol 3-caffeylgalactosylxyloside. In 95% EtOH, solutions which contain molar ratios of robinin to caffeic acid of 1:0, 1:1, and 1:2 exhibit $E_{266}/E_{UV\,max}$ of 0·64, 0·85 and 1·09, respectively. In the same solvent the $E_{266}/E_{UV\,max}$ of compound **2** was 0·81 which was indicative that 1 mol of caffeic acid was present. Therefore, compound **2** is kaempferol 3-(caffeylgalactosylxyloside)-7-rhamnoside. The galactose–xylose linkage and the location of the acyl group have yet to be established. Compound **2** was also present in flowers of larkspur cv. White Supreme.

Fraction 3 contained both compounds **2** and **3**. Compound **3** had the spectral characteristics of a 3-substituted kaempferol (Table 1). Complete hydrolysis with acid yielded kaempferol, glucose and rhamnose whereas controlled acid hydrolysis yielded only one intermediate glycoside which was chromatographically the same as kaempferol 3-glucoside. Typical R_f's (×100) were 65 (*n*-BuOH–HOAc–H_2O, 6:1:2); 13 (H_2O); 38 (15% HOAc) and 72 (Phenol–H_2O, 73:27 w/w). Compound **2** was chromatographically and spectrally indistinguishable from an authentic sample of kaempferol 3-rhamnosylglucoside. Also present in this fraction was a kaempferol 3,7-diglycoside acylated with caffeic acid but chromatographically distinct from compound **2**. Sufficient amounts were not available for complete identification.

Fraction 4 contained only compound **4**. Spectral data (Table 1) and its yellow fluorescence under UV radiation were characteristic of a 7-substituted kaempferol. Complete hydrolysis with acid yielded kaempferol and rhamnose whereas controlled acid hydrolysis yielded no

intermediate glycoside. Compound **4** was spectrally and chromatographically indistinguishable from an authentic sample of kaempferol 7-rhamnoside.

Simulating in vivo *absorption spectra*

The pH of epidermal peels from young moderate reddish-purple larkspur flowers was 5·5. Cells in this tissue were uniformly moderate reddish-purple. As flowers aged the pH increased to 6·6 and the color changed to light purplish-blue. Epidermal peels from aging flowers contained appreciable numbers of cells ranging in color from moderate reddish-purple through light purplish-blue to light blue. Many of the older cells contained strong blue crystals in vacuoles whose cell sap color ranged from moderate reddish-purple to light blue. Since this tissue exhibited each such a wide range of colors, the pH of the bluer cells presumably was higher than 6·6 and indicated a great variability in pH from cell to cell in aging tissue. The absorption spectra A) of a moderate reddish-purple cell, B) the light purplish-blue vacuolar sap of an adjacent cell, and C) an area in the same light purplish-blue cell including the strong blue crystals are shown in Fig. 1.

The visible absorption maxima in nm and absorbance of a typical young moderate reddish-purple cell (Fig. 1A) were: $\lambda_1$537 (0·49), $\lambda_2$568 (0·40) and $\lambda_3$626 (0·27). The shape of the visible absorption spectrum of a 10^{-2} M solution of delphinidin 3-di(*p*-hydroxybenzoyl) glucosylglucoside in 0·1 M Pi buffer pH 5·6 approximated that of a typical young moderate reddish-purple cell. This solution lacked stability and the visible absorption maxima were at slightly shorter wavelengths. The addition of kaempferol glycoside copigments resulted in a stable complex, a slight bathochromic shift, and a sharpening of the visible absorption maxima The absorption spectrum of a solution of delphinidin 3-di(*p*-hydroxybenzoyl)glucosylglucoside (10^{-2} M) plus kaempferol 3-(caffeylgalactosylxyloside)-7-rhamnoside or robinin (5×10^{-3} M) in 0·1 M Pi buffer pH 5·6 closely matched that of a typical moderate reddish-purple cell. In a 0·062 μm cuvette, *ca* 1·4 x the pathlength of a typical epidermal cell, the absorption maxima in nm and absorbance were. $\lambda_1$538 (0·68), $\lambda_2$569 (0·58) and $\lambda_3$625 (0·40).

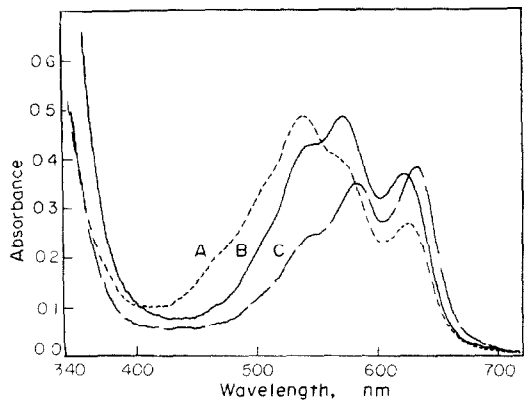

Fig 1 Absorption spectra of two adjacent cells in epidermal peels from aging Dark Blue Supreme Larkspur flowers A moderate reddish-purple cell with no apparent crystals B Crystal free region of light purplish blue cell containing a large cluster of strong blue crystals C Same cell as B but in the region containing the cluster of crystals

The absorption maxima in nm and absorbance of a typical sensecent light purplish-blue cell were: $\lambda_1$539 (0·42), $\lambda_2$570 (0·48), and $\lambda_3$622 (0·37). The shape and the absorption maxima of senescent light purplish-blue cells could not be matched by the absorption curves of a 10^{-2} M solution of delphinidin 3-di(*p*-hydroxybenzoyl)glucosylglucoside in a pH range of 6·6–7·2 The anthocyanin solutions were unstable. Although increasing pH did result in a bathochromic shift in the λ_{max} of the three visible bands, they were always at a shorter wavelength than those in the cells and the shape of the absorption curves more closely resembled those of young moderate reddish-purple cells. At the higher pH's the addition of kaempferol glycoside copigments to the anthocyanin solutions resulted in a stable complex, a bathochromic shift, a sharpening of the absorption maxima, and a slight increase in absorbance. The most pronounced effect was a change in the absorbance ratios of λ_1, λ_2 and λ_3 so that the shape of the absorption curves now resembled those of senescent light purplish-blue cells. The absorption spectrum of a solution of delphinidin 3 - di(*p* - hydroxybenzoyl)glucosylglucoside (10^{-2} M) plus kaempferol 3-(caffeylgalactosylxyloside)-7-rhamnoside or robinin (5×10^{-3} M) in 0·1 M Pi buffer pH 7·1 closely matched

that of a typical senescent light purplish–blue cell. In a 0·062 μm cuvette, the absorption maxima in nm and absorbance were $\lambda_1$540 (0·67), $\lambda_2$571 (0·73) and $\lambda_3$624 (0·58). Spectrophotometric measurements of tissue extracts indicated that there were no significant differences in the concentrations of anthocyanin or kaempferol glycoside copigments between young or senescing flowers. Therefore, color differences between moderate reddish–purple young cells and light purplish–blue senescent cells were primarily due to differences in pH.

A change in color and shape of the visible absorption spectrum occurred when a concentrated solution of the delphinidin derivative was diluted or heated without hydrolysis. Diluting an 8×10^{-3} M solution to 8×10^{-5} M (pH 6·6) resulted in a change in the absorbance ratio of λ_3/λ_1 from 0·58 to 1·10 and a much bluer solution. Raising the temperature from 11° to 52° of a 5×10^{-3} M solution (pH 6·5) changed the absorbance ratio of λ_3/λ_1 from 0·58 to 0·85 (Fig. 2). These changes indicated that the anthocyanin was changing from an associated to an unassociated form by diluting or heating. The concentration of the anthocyanin in larkspur epidermal cells was *ca* 10^{-2} M and the anthocyanin presumably was present in an associated form. This was substantiated by changes in the absorbance ratio of λ_3/λ_1 when flower petals were subjected to various temperatures. The absorbance ratios of λ_3/λ_1 of a young moderate reddish–purple petal at 10°, 30°, 60°, were 0·55, 0·62, and 0·69, respectively. Raising the temperature resulted in a larger ratio and a bluer color due to less association. Examination of individual cells from these young petals showed no signs of crystal formation.

The absorbance ratios of λ_3/λ_1 of an old light purplish–blue petal at 10°, 36°, and 55° were 0·79, 0·70, and 0·70, respectively, the opposite temperature effect of young petals. At the lowest temperature the formation of strong blue crystals was observed in many cells which resulted in a higher λ_3/λ_1 absorbance ratio for those cells and a general bluer petal color. The portion of the vacuole without crystals was also bluer due to the dilution effect from crystallization and a lesser degree of association in the solution. At the higher temperature the crystals dissolved and the solution color was not as blue.

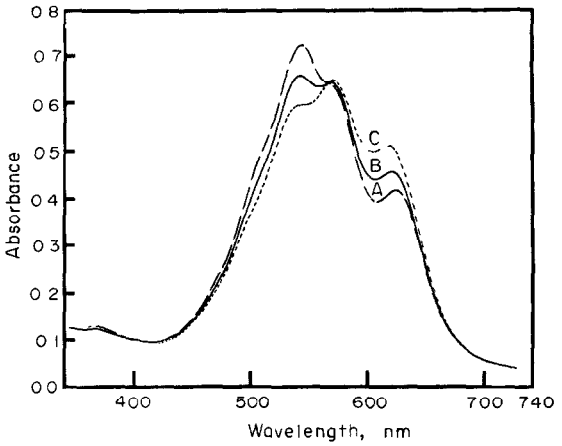

Fig 2 Effect of temperature on the visible absorption spectra (*ca* 100 μm pathlength) of a 5×10^{-3} M solution of delphinidin 3-di(*p*-hydroxybenzoyl) glucosylglucoside in Pi buffer pH 6 5. (A) 11°, (B) 30°, (C) 52°

Cells at the higher temperatures, even though the anthocyanin was in a lesser degree of association, were not as blue as those which had formed crystals at the lowest temperature. Formation of the strong blue crystals had a dramatic effect on the blueing of cells and this phenomenon explains the appearance of light blue streaks in older petals. The formation of crystals is an important factor which previously has not been considered with respect to color changes in senescing tissues.

EXPERIMENTAL

Plant material Seeds of annual larkspur cv Dark Blue Supreme and cv White Supreme were purchased from George J Ball, Inc , West Chicago, Illinois 60185 The Ball catalog lists all their annual larkspurs as *Delphinium ajacis* and specimens key out to *Consolida ambigua* (L) P W Ball & Heywood (*Delphinium ajacis* auct) A typical specimen is on deposit in the Herbarium of the U S National Arboretum, Washington, D C Plants were grown locally in a greenhouse under standard conditions

Anthocyanin and kaempferol glycosides. Flowers were harvested, dried at 40° in a forced-draft oven and then ground to pass a 40-mesh screen. Ground tissue was thoroughly extracted with 1% HCl–MeOH then with boiling meOH Combined extracts were reduced to min vol under red pres at 40°. The syrupy residue was taken up in citrate–Pi buffer pH 5, pH adjusted to 3 with 2N NaOH and filtered. Anthocyanin and kaempferol glycosides were separated into 4 fractions by column chromatography on MN–Polyamide SC-6 The column was eluted with 30 aq MeOH plus 4 ml of 2N HCl/l until free of anthocyanin and then with MeOH plus 4 ml of 2N HCl/l Compounds in each fraction were isolated by PLC on 2-mm-layers of avicel microcrystalline cellulose.

The final purification for the kaempferol glycosides was by column chromatography on Sephadex LH-20 eluted with MeOH Identification of the isolated compounds was by co-chromatography with authentic samples, UV spectra in EtOH using diagnostic reagents[9] and MS fragmentation patterns The various pigments were hydrolysed under usual conditions and the products identified by standard means[10].

Concentration of anthocyanin and kaempferol glycoside copigments Ten 4-mm discs were cut from flower petals with a cork borer and they were extracted in 3 ml 1% HCl–MeOH for 24 hr in the dark Absorbance at 545 and 350 nm was used to determine the concentration of anthocyanin and kaempferol glycoside copigments, respectively

Tissue pH measurement The pH of epidermal peels (*ca* 6 sq mm) from flowers was determined spectrophotometrically[1].

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[11] Controlled temperature measurements of the absorption properties were made with petals or solutions between two quartz microscope slides spaced *ca* 100 μm apart The slides were held between 2 metal plates with thermoelectric elements mounted on the plates to heat or cool under the control of a variable power supply The metal plates had slits (1 × 4 mm) in alignment with the light beam to permit light transmission through the quartz slides The temperature of the metal plates in contact with the slides was monitored with a thermistor-element electronic thermometer

Visible absorption maxima of individual cells were obtained with a microspectrophotometer previously described[1] Epidermal peels from either young moderate reddish-purple or aging light purplish-blue petals were placed in 0 3 M mannitol in a perfusion chamber and temperature changes were obtained by placing dry ice or the light beam from a prefocused projector lamp on the metal portion of the chamber which was in contact with the glass cover slips The temperature in the perfusion chamber was measured by a thermistor in a hypodermic needle which was inserted through a latex gasket between the two cover slips of the chamber.

Color notation All color notations except those in quotation marks are according to Kelly[12]

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