Phytochemistry, 1971, Vol. 10, pp. 171 to 175. Pergamon Press. Printed in England.

CO-PIGMENTATION EFFECT OF QUERCETIN GLYCOSIDES ON ABSORPTION CHARACTERISTICS OF CYANIDIN GLYCOSIDES AND COLOR OF RED WING AZALEA

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(*Received* 23 *March* 1970, *in revised form* 30 *April* 1970)

Abstract—A quercetin 5-methyl ether and five quercetin glycosides were isolated from flowers of Red Wing azalea[1] but were found only in trace amounts in an orange sport of this cultivar. The anthocyanins (cyanidin glycosides) extracted from the orange and red flowers were identical, even though the absorption spectra of the intact cells differed. The absorption spectrum of the orange sport was simulated with a 10^{-3} M aqueous solution of cyanidin 3,5-diglucoside at the pH of the tissue, 2·8. The absorption spectrum of Red Wing was matched with cyanidin 3,5-diglucoside at the same concentration and pH, co-pigmented with the 3-rhamnoside or galactoside of quercetin.

INTRODUCTION

A SPORT of Red Wing azalea (a *Rhododendron* cultivar) with orange rather than red petals was discovered at Beltsville. Anthocyanins extracted from the sport were cyanidin glycosides quantitatively and qualitatively the same as those previously reported for Red Wing.[2] The color difference could not be attributed to variation in anthocyanins; therefore, this study was initiated to determine the cause.

RESULTS

Flavonols from Flowers of Red Wing Azalea

Six flavonol glycosides were isolated from flowers of Red Wing azalea (Tables 1 and 2). Four of these were previously reported in *Rhododendron* species:[3,4] quercetin 3-arabinoside, 3-rhamnoside (quercitrin), 3-galactoside (hyperin), and the 3-rhamnoside of quercetin 5-methyl ether (azalein). A quercetin 3-rhamnoside chromatographically different from quercitrin and a second 3-arabinoside also were isolated. Only traces of each of the six flavonols were obtained from flowers of the mutant.

Absorption Spectra and pH *of Intact Cells*

The absorption spectra of a portion of the vacuole (20 μm dia.) of intact cells from epidermal peels (Fig. 1) showed the λ_{max} in the visible region at 507 and 518 nm for the sport and Red Wing, respectively. Compounds absorbing at *ca.* 350 nm (flavonols) were present in Red Wing and almost totally lacking in the sport. The pH of expressed sap as well as that of epidermal peels from either Red Wing or the sport was 2·8–3·0.

[1] L. L. BROOKS, *Plant Patent No.* 1159, U.S. Patent Office (1952).
[2] S. ASEN and P. S. BUDIN, *Phytochem.* **5**, 1257 (1966).
[3] J. B. HARBORNE, *Arch. Biochem. Biophys.* **96**, 171 (1962).
[4] R. DE LOOSE, *Phytochem.* **8**, 253 (1969).

TABLE 1. CHROMATOGRAPHIC PROPERTIES OF FLAVONOL GLYCOSIDES (CO-PIGMENTS) FROM RED WING AZALEA FLOWERS

Compound	R_f value (×100) in *				Products of acid hydrolysis
	BAW	PhOH	15% HOAc	H_2O	
Azalea flavonols					
1	99	98	66	26	Quercetin + rhamnose
2	86	68	50	18	Quercetin + rhamnose
3	97	98	45	12	Quercetin + arabinose
4	87	72	29	8	Quercetin + arabinose
5	71	67	34	9	Quercetin + galactose
6	68	80	55	19	Azaleatin + rhamnose
Authentic flavonols					
Quercitrin	86	68	50	17	Quercetin + rhamnose
Hyperin	71	66	33	9	Quercetin + galactose

* Determined by TLC on cellulose plates: BAW, 1-butanol–acetic acid–H_2O (6:1:2, v/v); PhOH, phenol–H_2O, (73:27, w/v); 15% HOAc, 15% aq. acetic acid.

TABLE 2. SPECTRAL PROPERTIES OF FLAVONOL GLYCOSIDES (CO-PIGMENTS) FROM RED WING AZALEA FLOWERS

Compound	λ_{max} (nm) in EtOH					
	Band I				Band II	
	Alone	+NaOEt	NaOAc +H_3BO_3	+$AlCl_3$	Alone	+NaOAc
Azalea flavonols						
1 or 2	350	398	372	415	256	270
3 or 4	356	404	379	412	256	273
5	362	415	385	404	256	267
6	339	379	359	340 427*	250	269
Authentic flavonols						
Quercitrin	350	398	372	415	256	270
Hyperin	362	415	385	404	256	267

* Due to contamination with the aglycone.

Influence of co-pigments on Color

Of the six flavonols and five anthocyanins isolated from flowers of Red Wing azalea only quercitrin, hyperin, and cyanidin 3,5-diglucoside were available for co-pigmentation studies. In aq. solution (pH 2·80) cyanidin 3,5-diglucoside at 10^{-3} M interacted with quercitrin to produce measurable absorption changes for quercitrin concentrations greater than 5×10^{-5} M. (Table 3). Increasing the mole equivalent of quercitrin resulted in a bathochromic shift of as much as 15 nm in the λ_{max} of the anthocyanin spectrum and a change in color from orange to red. When the mole equivalent was 1:1 or greater, absorbance also increased. Diluting cyanidin 3,5-diglucoside to 10^{-4} M reduced the co-pigmentation effect

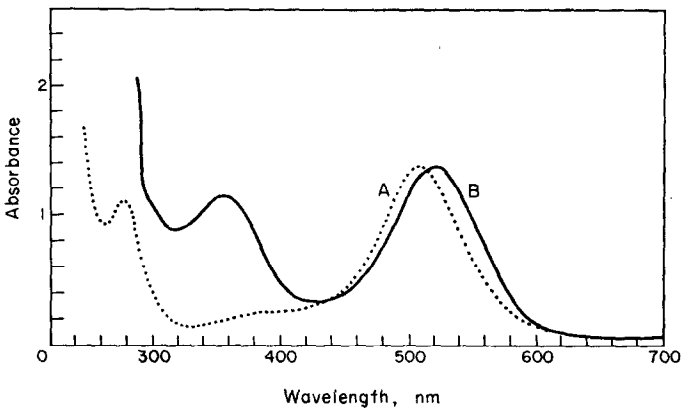

FIG. 1. ABSORPTION SPECTRA OF VACUOLE (AREA MEASURED *ca.* 20 μm dia.) FROM INTACT EPIDERMAL CELL.
(A) orange sport of Red Wing azalea
(B) Red Wing azalea

so that only a 5 nm shift was obtained at 1:5 mole equivalent of anthocyanin to quercitrin. Hyperin produced the same co-pigmentation effect as quercitrin.

TABLE 3. EFFECT OF QUERCITRIN ON THE WAVELENGTH OF MAXIMUM ABSORPTION AND THE ABSORBANCE OF CYANIDIN 3,5-DIGLUCOSIDE (IN AQUEOUS SOLUTION pH 2·80)

Mole/L			
Cyanidin 3,5-diglucoside	Quercitrin	λ_{max} ± 0·3 (nm)	Absorbance/nm at λ_{max}
10^{-3}	—	507·5	0·54
10^{-3}	10^{-5}	507·5	0·55
10^{-3}	2×10^{-5}	507·6	0·54
10^{-3}	5×10^{-5}	508·0	0·57
10^{-3}	10^{-4}	508·5	0·58
10^{-3}	2×10^{-4}	509·5	0·55
10^{-3}	5×10^{-4}	511·0	0·58
10^{-3}	10^{-3}	513·8	0·64
10^{-3}	2×10^{-3}	517·8	0·79
10^{-3}	5×10^{-3}	522·7	1·15

DISCUSSION

The phenomenon of co-pigmentation, the blueing of anthocyanins by flavonoids and related substances was observed as early as 1931.[5] Compounds reported as co-pigments have been identified as kaempferol and quercetin glycosides,[6] *C*-glycosylxanthone (mangiferin),[7] and

[5] G. M. ROBINSON and R. ROBINSON, *Biochem. J.* **25B**, 1687 (1931).
[6] J. B. HARBORNE, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. GOODWIN), p. 261, Academic Press, New York (1965).
[7] E. C. BATE-SMITH and J. B. HARBORNE, *Nature* **198**, 1307 (1963).

C-glycosylflavones[8,9] (swertisin, swertiajaponin, *o*-xylosylswertisin, vitexin, iso-orientin and flavocommelin). Co-pigmentation has been demonstrated by recombining the anthocyanin and C-glycosylflavones isolated from Prof. Blaauw iris, so that the absorption spectrum of the reconstituted pigment matched that of the intact cells.[8] The change in absorption spectrum (Table 3) demonstrates the co-pigmentation effect when quercetrin is added to cyanidin 3,5-diglucoside. Presumably the other flavonols and anthocyanins isolated from flowers of Red Wing azalea also could behave in the same manner. The spectrum obtained from an aqueous solution of 10^{-3} M cyanidin 3,5-diglucoside at pH 2·8 matched that of individual cells from the orange sport. The addition of quercitrin (2×10^{-3} M) modified the spectrum to match that of individual cells of Red Wing.

The concentration of anthocyanin and the molecular equivalent of anthocyanin to quercetin glycoside greatly influenced co-pigmentation. With a molecular equivalent of 1:5 the co-pigment effect caused a λ_{max} shift of 15 nm at a concentration of cyanidin 3,5-diglucoside of 10^{-3} M, and only 5 nm at 10^{-4} M. The effect of concentration on the absorption spectra suggests the formation of dimers. This may explain an earlier observation in which cyanidin 3-glucoside at $3{\cdot}5 \times 10^{-5}$ M and quercitrin at 1:10 molecular equivalents showed little or no co-pigment effect.[10] The optical path through epidermal cells of Red Wing azalea is *ca.* 50 μm and the absorbance at the λ_{max} is *ca.* 1. Assuming an ϵ value of 30,000–40,000 for the anthocyanins, the concentration of the pigment in the cell would approach 5×10^{-3} M. An equivalent or greater concentration of flavonoids in the individual cells is suggested by the absorbance at 350 nm (Fig. 1B). The difference of 11 nm in the λ_{max} between Red Wing and the orange sport could readily be accounted for by co-pigmentation at the estimated concentrations of anthocyanins and flavonols in the cell.

Comparison of absorption spectra of anthocyanin solutions with intact tissue requires a knowledge of pH.[10] The pH of expressed sap as well as that of epidermal peels from either Red Wing or the sport azalea was 2·8–3·0. This may or may not be the exact pH of the contents of the vacuole where the anthocyanins are present but presumably the value would not be higher. The λ_{max} of a 10^{-3} M solution of cyanidin 3,5-diglucoside at pH 2·8 was the same (within experimental error, 507–507·5 nm) as that of the intact cells of the orange sport. This supports the pH determination of 2·8–3·0 for the orange sport since at a higher pH cyanidin glycosides have a λ_{max} at longer wavelengths.[10]

The orange sport from the Red Wing azalea is apparently the result of a spontaneous mutation in the epidermal layer of cells. The mutation suppresses synthesis of quercetin and quercetin 5-methyl ether glycosides that are co-pigments in the flowers of Red Wing. This material presented a unique opportunity to study the natural state of cyanidin glycosides in two tissues of identical genotype except for a single mutant locus suppressing the synthesis of six closely related flavonols.

The orange color of the mutant is due to cyanidin glycosides whereas the color of Red Wing azalea is due to the same cyanidin glycosides co-pigmented with flavonol glycosides. Thus, when breeding for color the inheritance of co-pigments is as important as the inheritance of anthocyanins. Absorption spectra of intact cells obtained with the microspectrophotometer were valid both in the u.v. and visible portions of the spectrum and offered an excellent means for studying anthocyanins in their natural state.

[8] S. ASEN, R. N. STEWART, K. H. NORRIS and D. R. MASSIE, *Phytochem.* **9**, 619 (1970).
[9] K. TAKEDA and S. MITSUI, *Botan. Mag. Tokyo* **79**, 578 (1966).
[10] L. JURD and S. ASEN, *Phytochem.* **5**, 1263 (1966).

EXPERIMENTAL

Anthocyanin

Methods for identification of anthocyanidin and sugar moeties have been described previously.[2]

Flavonol co-pigments

Fresh flowers were dried at 50° in a forced-draft oven and then ground to pass a 40-mesh screen. The ground tissue was extracted with boiling MeOH and the flavonol glycosides were isolated and purified by PLC on 2 mm-layers of avicel microcrystalline cellulose. The solvents used were: for compound 1, 1-butanol–acetic acid–H_2O (6:1:2, v/v), 15% acetic acid and 30% acetic acid; for compounds 2 and 4, 1-butanol–acetic acid–H_2O (6:1:2, v/v) repeated twice, and 15% acetic acid; for compound 3, 1-butanol–acetic acid–H_2O (6:1:2, v/v) and 15% acetic acid repeated twice; for compounds 5 and 6, 1-butanol–acetic acid–H_2O (6:1:2, v/v), and 15% acetic acid.

Prior to acid hydrolysis each compound was passed through a column of Sephadex LH-20 with MeOH to eliminate any contamination from the cellulose plates. Each isolated compound was hydrolyzed by refluxing for 1 hr with IN HCl. The aglycones were extracted with ethyl acetate and the sugar moiety in the aqueous residue was examined by methods previously described.[2]

Flavonols and flavonol glycosides were characterized by spectrophotometric chromatographic methods. Absorption spectra were determined in EtOH with a Cary 15 spectrophotometer. Diagnostic shifts[11] were obtained by adding to solutions in cuvettes (*ca.* 2·5 ml): (1) 5 drops of 1% $AlCl_3$ in EtOH; (2) excess of fused anhydrous NaOAc; (3) 2 drops of 0·3% NaOEt; and (4) excess of fused anhydrous NaOAc plus five drops of a saturated solution of H_3BO_3 in EtOH.

Co-pigment Spectra

Standard solutions of cyanidin 3,5-diglucoside (3×10^{-3} M) and quercitrin or hyperin ($7{\cdot}5 \times 10^{-3}$ M) were prepared in citrate–phosphate buffer pH 2·21 and 0·02 N NaOH, respectively. To determine co-pigmentation effect, 0·2 ml of the standard anthocyanin solution was added to 0·4 ml of the properly diluted quercitrin or hyperin standard solution, and the spectrum measured immediately in a spectrophotometer developed in one of our laboratories.* This spectrophotometer consists of a Cary 14 monochromator operated as a single-beam instrument with our own photometer and interface coupled directly into a digital computer. The sample optical arrangement is the same as previously described.[12] The computer corrects for baseline, permits data analysis and provides a corrected absorbance curve. The samples were measured in a 10-mm dia. flat-bottom vial with sufficient sample to provide an optical path length of 2 mm.

Intact Cell Spectra

Upper epidermal tissue was stripped from petals and mounted on quartz microscope slides in a 5 per cent sucrose solution, so that the cells remained alive while the absorption spectra were being recorded. The individual cells were *ca.* 80 × 100 μm in surface dimension and 50 μm thick. Over 90% of the volume of the cells was taken up by a large central vacuole in which the pigment was dissolved.

Spectral absorption curves of individual cells were measured with the same spectrophotometer used for co-pigment spectra but with the addition of a microscope. The microscope attachment with u.v. reflecting optics for the Shimadzu spectrophotometer was mounted above the sample compartment and the monochromatic beam from the monochromator was reflected into the microscope. The measuring aperture of 20 μm-dia. was centered over the cell to be measured. A colorless cell having no absorption bands in the 300–700 nm region was used as a reference to establish the baseline.

pH Measurements

The pH of epidermal peels, expressed juice and solutions were determined with an Instrumentation Laboratory Catalog No. 14045 combination pH microelectrode. The sensitive area of the electrode was pressed on the epidermal peels and the minimum reading recorded. Juice was expressed from a petal by squeezing between the fingers and a drop measured on a teardrop slide.

Acknowledgements—We express appreciation to Dr. N. W. Stuart for bringing to our attention the sport of Red Wing azalea, to Dr. L. Jurd for a sample of azaleatin, and to Miss P. S. Budin for her competent technical assistance.

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[11] L. JURD, *The Chemistry of Flavonoid Compounds* (edited by T. A. GEISSMAN), p. 107, Pergamon Press, Oxford (1962).

[12] K. H. NORRIS and W. L. BUTLER, *IRE Trans. Bio-Med. Electronics BMS*-8, 153 (1961).