Phytochemistry, 1972, Vol. 11, pp. 1139 to 1144. Pergamon Press. Printed in England.

CO-PIGMENTATION OF ANTHOCYANINS IN PLANT TISSUES AND ITS EFFECT ON COLOR

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(*Received* 8 *July* 1971)

Abstract—Glycosides of the 6 common anthocyanidins all formed co-pigment complexes with flavonoids and other compounds at pH's ranging from 2 to 5. The formation of co-pigment complexes resulted in a bathochromic shift in the visible λ_{max} of the anthocyanins and a large increase in extinction at pH 3 and higher. These complexes apparently formed with both the red flavylium salts and the purple anhydro bases. The increase in extinction at pH's 3 to 5 was attributed to the stabilizing effect co-pigmentation had on the anhydro bases. The degree of co-pigmentation was a function of the concentration of the anthocyanins and the molar ratio of co-pigments to anthocyanins. Co-pigmentation offers an explanation for the infinite color variations that occur in flowers in a pH range where anthocyanins alone are virtually colorless.

INTRODUCTION

ANTHOCYANINS are responsible for most pink, red, mauve and blue colors in higher plants. The degree of blueness is influenced by the hydroxylation/methoxylation status of the anthocyanin B-ring. However, the number of variations in these colors cannot be explained by the colors of the relatively few known anthocyanidins. It would also appear most improbable that anthocyanins alone could contribute significant color to those tissues whose pH is in the range of 4–6, since at this pH range and at the optical pathlength of colored cells most anthocyanins are virtually colorless. Co-pigmentation, the bluing of anthocyanins by flavones and related substances, was suggested as early as 1931.[1] The phenomenon of co-pigmentation has been shown to offer a suitable explanation for the color of 'Prof. Blaauw' iris,[2] 'Red Wing' azalea,[3] and 'Better Times' rose.[4]

The purpose of this investigation was to determine factors that effect co-pigmentation of anthocyanins in an attempt to explain the intense and infinite color variations that occur in plant tissues in a pH range where anthocyanins alone are virtually colorless.

RESULTS AND DISCUSSION

Co-pigmentation occurred with a glycoside of all 6 common anthocyanidins (Table 1). This is in contrast to metal chelation which occurs only with anthocyanins that have an *ortho*-dihydroxyl system.[5–7] Co-pigmentation not only caused a bathochromic shift in the

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visible λ_{max} of all the anthocyanins, which made them appear bluer, but also resulted in a large increase in absorptivity. Delphinidin 3-glucoside was the only anthocyanin that had an appreciable extinction at pH 3·32 without benefit of co-pigmentation, probably due to the greater stability of the anhydro base of this anthocyanin.[7]

Table 1. Co-pigment effect of quercitrin on the λ_{max} and absorbance of anthocyanins (pH 3·32)

Anthocyanins (2×10^{-3} M)	Quercitrin (6×10^{-3} M)			
	λ_{max}, nm		A/mm at λ_{max}	
	−	+	−	+
Pelargonidin 3,5-diglucoside	495	510	0·490	1·600
Cyanidin 3,5-diglucoside	508	527	0·500	1·588
Delphinidin 3-glucoside	510	530	2·903	3·371
Peonidin 3,5-diglucoside	512	533	0·486	1·608
Petunidin 3,5-diglucoside	515	538	0·648	1·868
Malvidin 3,5-diglucoside	519	549	0·300	1·990

Solutions containing equal molar concentrations (5×10^{-4} and 5×10^{-3}) of cyanidin 3,5-diglucoside and quercetin 3-rhamnoside (quercitrin) co-pigmented in a pH range of 2·12 to 5·10 (Table 2). Co-pigmentation previously was shown to occur at a pH as low as 1·2.[2] The bathochromic shift in the visible λ_{max} due to co-pigmentation, was influenced by concentration and not pH. In the pH range of 2·12 to 5·10 the $\Delta\lambda_{max}$ at 5×10^{-5}, 5×10^{-4}, and 5×10^{-3} M was 0, 6 and 14 nm, respectively. Absorbance increased only at pH's where the anthocyanin is converted via the unstable anhydro base into its colorless carbinol base.[8] Thus, co-pigmentation occurred both with the red flavylium salt as well as with the purple anhydro base and any increase in absorbance was probably due to the stabilization of the anhydro base.

Table 2. Effect of pH on the co-pigmentation of equal molar solutions of quercitrin and cyanidin 3,5-diglucoside

	Concentration											
	5×10^{-5} M				5×10^{-4} M				5×10^{-3} M			
	λ_{max}		A/cm		λ_{max}		A/cm		λ_{max}		A/cm	
pH	−	+	−	+	−	+	−	+	−	+	−	+
2·12	506	506	1·07	1·03	505	511	10·60	10·56	501	515	110·0	110·3
3·16	508	508	0·16	0·14	508	514	1·71	1·85	506	520	34·0	45·3
4·15	510	510	0·03	0·02	512	518	0·28	0·37	515	529	4·6	12·3
5·10	518	518	0·02	0·02	523	529	0·14	0·25	526	540	2·1	10·0

− Solutions containing only cyanidin 3,5-diglucoside.
+ Solutions containing both cyanidin 3,5-diglucoside and quercitrin.

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The molar ratio of quercitrin to cyanidin 3,5-diglucoside determined the magnitude of the co-pigmentation effect. The absorbance of a 10^{-2} M solution cyanidin 3,5-diglucoside, at pH 4·38, increased 2·65/mm for every unit increment increase of quercitrin (Fig. 1). At concentrations of $7 \cdot 5 \times 10^{-3}$ and 5×10^{-3} M cyanidin 3,5-diglucoside this increase was 1·55 and 0·65/mm, respectively. The increase in absorbance for each unit increment of quercitrin was only 0·00056/mm at a concentration of 10^{-4} M cyanidin 3,5-diglucoside.

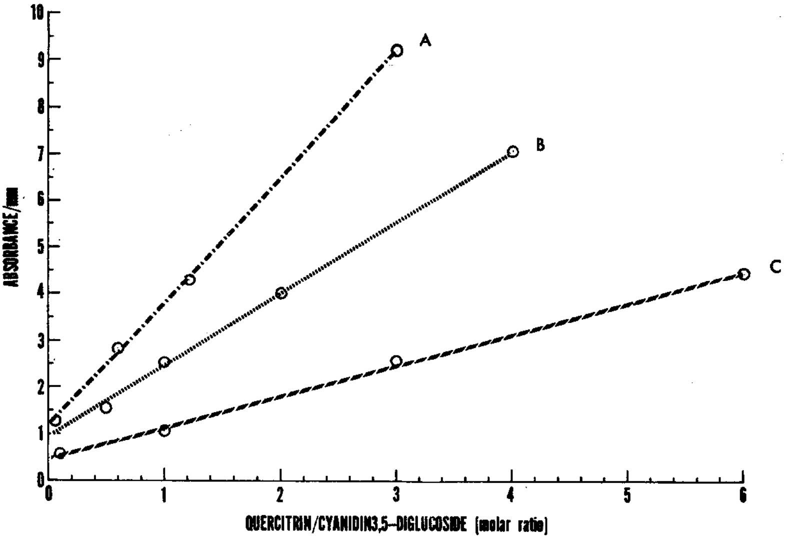

FIG. 1. INCREASE IN THE ABSORBANCE OF THE VISIBLE λ_{max} OF CYANIDIN 3,5-DIGLUCOSIDE DUE TO CO-PIGMENTATION WITH QUERCITRIN (pH 4·38).

Molarity of cyanidin 3,5-diglucoside: A—10^{-2} M; B—$7 \cdot 5 \times 10^{-3}$ M; C—5×10^{-3} M.

The magnitude of the bathochromic shift of the visible λ_{max} of cyanidin 3,5-diglucoside also was influenced both by the concentration of the pigment and the molar ratio of co-pigment to pigment (Table 3). At 10^{-2} M cyanidin 3,5-diglucoside, up to a 21 nm shift occurred when the molar ratio of quercitrin to cyanidin 3,5-diglucoside reached 3:1. At lower concentrations of cyanidin 3,5-diglucoside a shift of similar magnitude was obtained by increasing the concentration of quercitrin. An earlier observation[8] showed that there was no change in the visible λ_{max} of cyanidin 3-glucoside at $3 \cdot 5 \times 10^{-5}$ M from the addition of 1 to 10 molar equivalents of quercitrin at pH 3–6 and under these conditions quercitrin was involved in complex formation with the anthocyanin only in the presence of metal ions. We have now shown that quercitrin can co-pigment with anthocyanins without the presence of a metal ion. The co-pigmentation effect, as expressed by the change in the visible λ_{max} as well as an increase in absorbance, is a function of the anthocyanin concentration and the molar ratio of co-pigment to anthocyanin. Factors shown to effect the co-pigmentation of quercitrin and cyanidin 3,5-diglucoside also influenced the co-pigmentation of quercitrin and a glycoside of the other 5 common anthocyanidins.

Details of the bonding of the anthocyanin to the co-pigment are not known. Hydrogen bonding is suggested from the facts that co-pigmentation is concentration dependent, and that the co-pigment complex is dissociated by heating or by the addition of alcohol or

TABLE 3. THE EFFECTS OF CONCENTRATION AND CO-PIGMENTATION WITH QUERCITRIN ON THE VISIBLE λ_{max} (nm) OF CYANIDIN 3,5-DIGLUCOSIDE AT pH 4·38

Molar ratio of quercitrin to cyanidin 3,5-diglucoside	Cyanidin 3,5-diglucoside (M) λ_{max} nm			
	10^{-2}	$7{\cdot}5 \times 10^{-3}$	5×10^{-3}	10^{-4}
0:1	518	518	518	515
0·1:1	522	521	520	515
0·6:1	528	526	526	515
1:1	532	531	530	515
2:1	536	534	534	515
3:1	539	537	537	515
4:1	*—	539	539	515
6:1	—	*—	542	515
10:1	—	—	*—	515
20:1	—	—	—	515
40:1	—	—	—	520
100:1	—	—	—	536

* Not determined due to solubility limitation.

dimethylformamide. There is also an indication that anthocyanins may undergo self-association in aqueous solutions. At pH 3·16 the visible λ_{max} of cyanidin 3,5-diglucoside varied from 507 to 502 nm by increasing the concentration from 10^{-4} to 10^{-2} M. For this same 100-fold increase in concentration the absorbance increased 300 times.

Flavonoid compounds formed co-pigment complexes with cyanidin 3,5-diglucoside (2×10^{-3} M) at a molar ratio of 3:1 (Table 4). The greatest shift in λ_{max} as well as increase in absorbance, occurred with an aurone, 6-*C*-glycosylflavones, and flavonols. Alkaloids such as caffeine and brucine also co-pigmented with cyanidin 3,5-diglucoside but basic, acidic, and neutral amino acids had little or no effect. The small co-pigment effect shown for benzoic acids probably could have been increased with higher molar ratios. Other compounds reported to act as co-pigments are *C*-glycosylxanthones,[9] pectin[7] and tannin.[10] The natural occurrence of the phenomenon of co-pigmentation has been demonstrated by recombining the isolated anthocyanins and co-pigments from iris,[2] azalea[3] and rose[4] flowers, at the proper pH and concentrations, to match the absorption spectra of the pigment from intact cells.

An explanation offered for many variations in flower colors is that all the hues between deep blue and red are produced by a mixture of blue metal chelates with red flavylium salts.[7] Examination of the absorption spectra of the anthocyanin pigments in intact cells from a great number of flowers (unpublished data) revealed little evidence of metal chelation except for blue hydrangeas. The pH of the pigmented cell sap of most flowers examined was 4·5–5·5. The optical pathlength of these cells varied from 25 to 50 μm and they had an absorbance of up to 3. Within this pH range and optical path length anthocyanins alone as concentrated as 10^{-2} M would be virtually colorless, being converted via the unstable anhydro base to the colorless carbinol base. Cyanidin, delphinidin, and petunidin glycosides can be stabilized with metals at this pH range. However, spectra of aluminum or iron chelates of these anthocyanins, at various concentrations and pH, showed a single λ_{max} with

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Table 4. Co-pigmentation of cyanidin 3,5-diglucoside (2×10^{-3} M) at pH 3·32

Co-pigments (6×10^{-3} M)	λ_{max} (nm)	$\Delta\lambda_{max}$ (nm)	A/mm at λ_{max}	% A increase at λ_{max}
None	508	—	0·500	—
Aurone				
*Aureusidin	540	32	2·135	327
Alkaloids				
Caffeine	513	5	0·590	18
Brucine	512	4	1·110	122
Amino acids				
Alanine	508	0	0·525	5
Arginine	508	0	0·600	20
Aspartic acid	508	0	0·515	3
Glutamic acid	508	0	0·530	6
Glycine	508	0	0·545	9
Histidine	508	0	0·595	19
Proline	508	0	0·625	25
Benzoic acids				
Benzoic acid	509	1	0·590	18
o-Hydroxybenzoic acid	509	1	0·545	9
p-Hydroxybenzoic acid	510	2	0·595	19
Protocatechuic acid	510	2	0·615	23
Coumarin				
Esculin	514	6	0·830	66
Cinnamic acids				
m-Hydroxycinnamic acid	513	5	0·720	44
p-Hydroxycinnamic acid	513	5	0·660	32
Caffeic acid	515	7	0·780	56
Ferulic acid	517	9	0·800	60
Sinapic acid	519	11	1·085	117
Chlorogenic acid	513	5	0·875	75
Dihydrochalcone				
Phloridzin	517	9	1·005	101
Flavan-3-ols				
(+)-Catechin	514	6	0·890	78
Flavone				
*Apigenin 7-glucoside	517	9	0·840	68
C-glycosyl Flavone				
8-*C*-Glucosylapigenin (vitexin)	517	9	1·690	238
6-*C*-Glucosylapigenin (isovitexin)	537	29	1·705	241
6-*C*-Glucosylgenkwanin (swertisin)	541	33	2·835	467
Flavonones				
Hesperidin	521	13	1·095	119
Naringin	518	10	0·985	97
Flavonols				
Kaempferol 3-glucoside	530	22	1·693	239
Kaempferol 3-robinobioside-7-rhamnoside (robinin)	524	16	1·423	185
Quercetin 3-glucoside (isoquercitrin)	527	19	1·440	188
Quercetin 3-rhamnoside (quercitrin)	527	19	1·588	217
Quercetin 3-galactoside (hyperin)	531	23	1·910	282
Quercetin 3-rutinoside (rutin)	528	20	1·643	228
Quercetin 7-glucoside (quercimeritrin)	518	10	1·363	173
7-*O*-Methylquercetin-3-rhamnoside (xanthorhamnin)	530	22	1·576	215

* Formed a slight precipitate.

too high an absorption on the long wavelength side of the λ_{max} when compared to the absorption spectra of intact cells. Although metals have been shown to be present in isolated blue pigments[7,11–13] their importance in flower color has yet to be established.

By manipulating pH, anthocyanin concentration, and the molar ratio of anthocyanin to co-pigment, the absorption spectra of most flowers can be simulated. Therefore, the phenomenon of co-pigmentation offers a more logical explanation of the infinite variation in red to blue flower colors that exist in a pH range where anthocyanins alone are virtually colorless. It is also important to recognize that by varying the factors that effect co-pigmentation it is possible to obtain similar absorption spectra with different anthocyanins.[14,15] Thus, the color and the absorption spectra of 'Potomac Rose' snapdragon (pelargonidin glycoside) and 'Briarcliff' rose (cyanidin glycoside) are similar. Other factors that also can influence color variation are mixtures of different anthocyanins in tissue, background effects from chlorophyll or carotenoids, anatomical modifications and possibly the binding of anthocyanins to macromolecules.

EXPERIMENTAL

Isolation of anthocyanins. Cyanidin 3,5-diglucoside was isolated from 'Forever Yours' roses, delphinidin 3-glucoside from 'Merveille' hydrangeas, and pelargonidin 3,5-diglucoside from an unnamed geranium seedling. The petals or sepals were dried at 50°in a forced-air oven and then ground to pass a 40-mesh screen. The anthocyanins were extracted by forming a slurry of the tissue with hot (70°) citrate–phosphate buffer pH 3·0 and expressing the extract through cheese cloth. The extract was filtered with the aid of celite. Cyanidin 3,5-diglucoside and pelargonidin 3,5-diglucoside were absorbed separately on 25 × 400 mm columns of purified insoluble polyvinylpryrrolidone.[16] Delphinidin 3-glucoside was absorbed on the same size column of polyamide. The anthocyanins were eluted with 30% aq. MeOH containing 1 ml of 2 N HCl/l. and crystallized.[17]

Anthocyanin-co-pigment complex. Co-pigments were obtained from plants[18] or commercial sources. Stock solutions of anthocyanins or co-pigments, containing twice the required normality, were prepared in citrate–phosphate buffer pH 2·24 and 0·08 N NaOH, respectively. Stock solutions of amino acids were prepared in 0·1 N HCl. Each stock solution was immersed briefly into boiling water and then an equal volume (0·25 ml for high concentrations and 1·5 ml for low concentrations) of each co-pigment was thoroughly mixed with an equal volume of anthocyanin. The pH of each solution of anthocyanin and co-pigment was measured with a combination pH microelectrode and was adjusted to the desired pH with either solid fused-anhydrous NaOAc or oxalic acid. Absorption spectra were determined (*ca.* 15 min after mixing) with a Cary 15 spectrophotometer using quartz cells having a pathlength of either 0·062, 1 or 10 mm.

Acknowledgements—We thank Dr. G. Hrazdina for samples of peonidin, petunidin, and malvidin 3,5-diglucosides and Dr. H. W. Siegelman for supplying some of the co-pigments.

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Key Word Index—Flower color variation; copigmentation; anthocyanins; flavonoid glycosides.