Phytochemistry, 1970, Vol. 9, pp. 619 to 627. Pergamon Press. Printed in England.

A STABLE BLUE NON-METALLIC CO-PIGMENT COMPLEX OF DELPHANIN AND *C*-GLYCOSYLFLAVONES IN PROF. BLAAUW IRIS

S. Asen, R. N. Stewart, K. H. Norris and D. R. Massie[*]

Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md. 20705, U.S.A.

(*Received* 20 *August* 1969)

Abstract—The pigment of Prof. Blaauw Iris is a stable blue non-metallic co-pigment complex of *C*-glycosylflavones and the anhydro-base of delphanin [delphinidin 3-(*p*-coumaroylrutinoside)-5-glucoside]. At a concentration comparable to that within the plant cell the reconstituted complex is stable and has an absorption spectrum matching that of the intact cell. The blue pigment is associated with a pectin and the co-pigment *C*-glycosylflavones are 6-*C*-β-D-glucopyranosylgenkwanin (swertisin), *O*-xylosylswertisin, 8-*C*-β-D-glucopyranosylapigenin (vitexin), 6-*C*-β-D-glucopyranosylluteolin (iso-orientin) and 6-*C*-β-D-glucopyranosyl-7-*O*-methylluteolin (swertiajaponin).

INTRODUCTION

The structural changes red anthocyanins undergo to form stable blue pigments have been considered to be *in vivo* modifications produced by co-pigments, metal complexing, or by a combination of these two factors. Each of the natural anthocyanin-based blue pigments isolated has contained the anthocyanin combined with metals and an organic substance(s).[1-3]

The concept that stable violet quinoid anhydro-bases of delphinidin glycosides contribute to flower color was advanced by Bayer *et al.*[4] They reported that the pigment of larkspur was a compound of a pectin-like polysaccharide and the neutral anhydro-base of delphinin, without metal ions. We now report the pigment from Prof. Blaauw Iris, a hybrid of *Iris tingitana* Boiss. & Reut. (also known commercially as "Blue Ribbon") is a stable, non-metallic, anhydro-base of delphinidin 3-(*p*-coumaroylrutinoside)-5-glucoside associated with a pectin and complexed with co-pigment *C*-glycosylflavones.

RESULTS

Blue Pigment

The blue pigment was expressed through three layers of cheese-cloth from a water slurry of the air-dried tissue (pH 5·3). It is non-dialysable, unstable in dilute solution (*ca.* 1 absorptivity unit/cm), and stable at higher concentrations. The blue pigments from *Centaurea cyanus* L.[1, 2] and *Commelina communis* L.[3] were crystallized by the addition of excess alcohol to water extracts. This procedure decolorized the iris blue pigment and pale blue floccules

---

[*] Physiologist, Crops Research Division; Horticulturist, Crops Research Division; Director, Instrumentation Research Laboratory, Market Quality Research Division; Agricultural Engineer, Market Quality Research Division, respectively.

[1] E. Bayer, *Chem. Ber.* **91**, 1115 (1958).

[2] K. Hayashi, N. Saito and S. Mitsui, *Proc. Japan Acad.* **37**, 393 (1961).

[3] S. Mitsui, K. Hayashi and S. Hattori, *Botan. Mag. Tokyo* **72**, 325 (1959).

[4] E. Bayer, H. Egeter, A. Fink, K. Nether and K. Wegmann, *Angew Chem. Intern. Ed. Engl.* **5**, 791 (1966).

were deposited after a short time. Acidification of the supernatant revealed the presence of a large amount of anthocyanin. The pale blue flocculent was separated by filtration, dissolved in H_2O and precipitated again with excess alcohol. Repeating this procedure eventually resulted in a flocculent devoid of color. The flocculent was characterized as a pectic substance by its positive reaction to carbazole[5] after de-esterification and acid hydrolysis and also by the enzymatic breakdown to galacturonic acid by pectinase and pectin esterase. The iris blue pigment, which is readily removed from the pectic substance, is probably only adsorbed and not chemically bound. After dialyzing the blue pigment in water for 41 hr at 5°, approximately 50 per cent of the total anthocyanin was removed.

Anthocyanin from Blue Pigment

The absorption spectrum of the purified anthocyanin had a λ_{max} (in 1% HCl–MeOH) of 538 nm, an absorption band at 300–310 nm, and lacked a distinct shoulder to the main absorption peak in the 410–440 nm region. This is characteristic of an acylated anthocyanin with a sugar residue at both the 3- and 5-OH positions.[6] Acid hydrolysis yielded an anthocyanidin which spectrally and chromatographically matched delphinidin (Table 1). The sugar moieties were glucose and rhamnose (*ca.* 2:1 ratio).

TABLE 1. PROPERTIES OF ANTHOCYANIDIN FROM ACID HYDROLYSIS OF BLUE PIGMENT ISOLATED FROM PROF. BLAAUW IRIS

Anthocyanidin	λ_{max}* MeOH–HCl (nm)	$AlCl_3$ $\Delta\lambda$ (nm)	R_f values (×100) in†		
			Forestal	Formic	BAW
Iris anthocyanidin	546	34	26	13	46
Delphinidin	546	34	27	13	46
Cyanidin	535	38	46	23	73
Petunidin	543	39	42	20	56

* Measured in methanolic 0·01% HCl.

† Determined by TLC on cellulose plates: Forestal, acetic acid–HCl–H_2O (30:3:10 v/v); Formic, formic acid–HCl–H_2O (5:2:3) v/v); BAW, 1-butanol–acetic acid–H_2O (6:1:2 v/v).

Controlled H_2O_2 oxidation permitted the specific identification of sugar groups at the C-3 position and yielded an intact disaccharide which was chromatographically identical to rutinose. A standard of rutinose was obtained by H_2O_2 oxidation of rutin [R_g 0·6 in 1-butanol–acetic acid–H_2O (6:1:2 v/v)]. Further confirmation was obtained by acid hydrolysis of the disaccharide which yielded rhamnose and glucose.

Alkaline hydrolysis yielded an acyl residue which spectrally and chromatographically matched *p*-coumaric acid. Acyl groups are attached to the sugar in the 3-position;[7] therefore the anthocyanin from the blue pigment of Prof. Blaauw Iris is delphinidin 3-(*p*-coumaroylrutinoside)-5-glucoside (delphanin). The anthocyanin from the blue pigment and the dialyzed anthocyanin are the same.

When the pH of a solution *ca.* 6×10^{-4} M of delphanin in 0·1 per cent HCl is adjusted to 5·1, the initial absorbance is *ca.* 12 absorptivity units/cm at a λ_{max} of 540 nm. After 30 min

---

[5] E. A. McCOMB and R. M. McCREADY, *Anal. Chem.* **24**, 1630 (1952).

[6] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, p. 17, Academic Press, New York (1967).

[7] J. B. HARBORNE, *Phytochem.* 3, 151 (1964).

the absorbance decreases to *ca.* 50 per cent of the initial value and the λ_{max} changes to 575 nm. Thereafter, for at least 15 hr, the solution is virtually stable.

C-glycosylflavones from Blue Pigment

Along with the anthocyanin and the pectic substance, eight flavonoids were isolated from the blue pigment. Five of these flavonoids (Compounds *1–5*) were present in amounts large enough for characterization. Chromatographic mapping[8] showed that Compound *2* and Compounds *1*, *3*, *4*, and *5* migrated to the areas assigned to *O*-xylosyl-*C*-glycosylflavones and simple *C*-glycosylflavones, respectively. The absorption spectra of these compounds indicated that they were either apigenin-based or luteolin-based *C*-glycosylflavones (Table 2).

TABLE 2. SPECTRAL PROPERTIES OF *C*-GLYCOSYLFLAVONES ISOLATED FROM BLUE PIGMENT OF PROF. BLAAUW IRIS

	λ_{max} (nm) in EtOH					
	Band I				Band II	
Compound	Alone	+NaOEt	+AlCl$_3$	+H$_3$BO$_3$ +NaOAc	Alone	+NaOAc
* Iris *C*-glycosylflavones:						
1	334	399	378	336	271	270
2	334	398	380	335	271	270
3	350	411	398	379	270 255	269
4	350	411	406	383	270 255	278
5	333	400	384	336	271	280
Authentic *C*-glycosylflavones:						
Swertisin	335	398	380	336	270	268
Vitexin	334	399	384	336	270	278
Iso-orientin	350	411	405	380	270 255	278

* Spectra before and after acid hydrolysis were identical.

Compound 1 was present in the largest amount. From 75 g of tissue approximately 25 mg of chromatographically pure material was isolated. The u.v. spectra and R_f values are the same as for swertisin (Tables 2, 3). The u.v. spectra are indicative of an apigenin-based compound and the spectral shifts with AlCl$_3$ and NaOEt showed that the OH groups in position 4′ and 5 were free.[9] With NaOAc the short-wavelength band does not shift, and with H$_3$BO$_3$–NaOAc the long-wavelength band does not shift, indicating the absence of a free 7-OH and the absence of an *ortho*-dihydroxyl group, respectively. Acid hydrolysis yielded no hydrolyzable sugar and did not alter the R_f values. NMR studies* of the hexa-acetate showed signals for the acetyl methyl groups at δ 2·49 (aromatic acetyl), 2·34 (aromatic acetyl), 2·07 (3″-acetyl), 2·07 (4″-acetyl), 2·03 (6″-acetyl) and 1·79 (2″-acetyl). This pattern of sugar acetyl signals is typical of 6-*C*-glycosylflavones and is distinct from that of 8-substituted compounds.[10] Singlets at δ 6·58 and 6·90 are assigned to H-3 and H-8, respectively.

---

* Work of R. M. Horowitz.

[8] M. K. SEIKEL, J. H. S. CHOW and L. FELDMAN, *Phytochem.* **5**, 439 (1966).

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

[10] B. GENTILI and R. M. HOROWITZ, *J. Org. Chem.* **33**, 1571 (1968).

The chemical shift of the latter proton indicates it is adjacent to a methoxyl group.[11] Doublets centered at δ 7·93 ($J = 9$ Hz) and 7·27 ($J = 9$ Hz) are assigned to the protons at positions 2′,6′ and 3′,5′, respectively. A doublet at δ4·94 (measured at 55°) is assigned to H-1″, its J value (10 Hz) indicates *trans*-diaxial coupling with H-2″. The chemical shifts observed showed a small, constant difference from those reported by Komatsu and Tomimori.[12] This difference is probably accounted for by instrumental calibration.

The NMR spectrum of the free *C*-glycosylflavone in deuteriopyridine at 100° was also determined. Signals at δ 7·90 ($J = 9$ Hz) for H-2′,6′; 7·22 ($J = 9$ Hz) for H-3′,5′; and 3·85 for methoxyl are observed. Signals at δ 6·68 and 6·78 can be ascribed to H-3 and H-8. The signal due to *trans*-diaxially coupled H-1″ of the glycosyl residue is seen very clearly at δ 5·51 ($J = 10$ Hz).

NMR, spectral, and chromatographic data identify Compound 1 as 6-*C*-β-D-glucopyranosylgenkwanin (swertisin).[12]

Spectral data for chromatographically pure solutions of Compound 2 also matched those of swertisin (Table 2) but the R_f values are different (Table 3). Acid hydrolysis yielded xylose and swertisin (Tables 2, 3) and thus Coupound 2 presumably is *O*-xylosylswertisin.

TABLE 3. R_f VALUES OF *C*-GLYCOSYLFLAVONES ISOLATED FROM BLUE PIGMENT OF PROF. BLAAUW IRIS

	R_f (×100) in*				
Compound	20% HOAc	BAW	2-PW	2-PFW	AHH
Iris *C*-glycosylflavones:					
1	66	72	54	89	91
2	93	67	92	98	98
†*2* (hydrolyzed)	66	72	56	91	93
3	48	51	38	75	79
4	36	47	32	62	66
5	21	43	16	50	56
Authentic *C*-glycosylflavones and flavones:					
Swertisin	66	72	54	89	91
Iso-orientin	36	46	30	60	66
Orientin	15	24	11	30	39
Vitexin	20	42	15	48	55
Apigenin	9	96	11	46	42
Genkwanin	12	97	10	60	53
Luteolin	6	83	7	26	26

* Determined by TLC on cellulose plates in: 20% HOAc, 20% acetic acid; BAW, 1-butanol-acetic acid–H_2O (6:1:2 v/v); 2-PW, 2-propanol-H_2O (22:78 v/v); 2-PFW, 2-propanol–formic acid–H_2O (2:5:5 v/v); and AHH, acetic acid–HCl–H_2O (5:1:5 v/v).

† Compound 2 was the only *C*-glycosylflavone which on acid hydrolysis yielded a hydrolyzable sugar and altered R_f values.

In ethanol the u.v. spectrum (λ_{max} 256, 270, 350 nm) of a chromatographically pure solution of Compound 3 is indicative of a luteolin-based compound (Table 2). With NaOEt the intensity of the long-wavelength band increases, and it undergoes a pronounced bathochromic shift to 411 nm suggesting that the 4′-OH group is free. The $AlCl_3$ spectrum shows the presence of a free 5-OH group. With NaOAc the short-wavelength band does not shift;

---

[11] W. E. HILLIS and D. H. S. HORN, *Australian J. Chem.* **19**, 705 (1966).

[12] M. KOMATSU and T. TOMIMORI, *Tetrahedron Lett.* 1611 (1966).

and with H_3BO_3–NaOAc the long-wavelength band undergoes a bathochromic shift of 37 nm, indicating the absence of a free 7-OH and the presence of an *ortho*-dihydroxyl group, respectively. Acid hydrolysis yielded no hydrolyzable sugar or altered R_f values (Tables 2, 3) but did produce a minor spot which migrated behind the parent compound in all solvents in Table 3. This is due to 6-glycosly-8-glycosyl isomeration taking place via pyran ring opening in the acid solution.[8] On the basis of the spectral changes, Compound 3 is presumably 6-*C*-β-D-glucopyranosyl-7-*O*-methylluteolin (swertiajaponin).[12]

The u.v. spectra and the R_f values of chromatographically pure solutions of Compound 4 matched those for iso-orientin (Tables 2, 3). Spectral shifts, in the presence of diagnostic reagents, are the same as those shown for swertiajaponin except that in the presence of NaOAc the short-wavelength band undergoes a bathochromic shift of 9 nm, indicating the presence of a free 7-OH group. Acid hydrolysis yielded no hydrolyzable sugar or altered R_f values. This compound presumably is 6-*C*-β-D-glucopyranosylluteolin (iso-orientin).

The u.v. spectra and R_f values of chromatographically pure solutions of Compound 5 matched those for vitexin (Tables 2, 3). The u.v. spectra are indicative of an apigenin-based compound and the spectral shifts with $AlCl_3$ and NaOEt showed that the OH-groups in position 4′ and 5 were free. With NaOAc the short-wavelength band undergoes a bathochromic shift of 8 nm; and with H_3BO_3–NaOAc the long-wavelength band shows a minor change, indicating the presence of a free 7-OH group and the absence of an *ortho*-dihydroxyl group, respectively. Acid hydrolysis yielded no hydrolyzable sugar or altered R_f values. This compound presumably is 8-*C*-β-D-glycopyranosylapigenin (vitexin).

Co-Pigmentation

The water-extracted blue pigment has absorption characteristics almost identical to those of the pigment within a typical intact epidermal cell (Fig. 1D, 1C). The absorption characteristics of the anhydro-base and the iron and aluminum chelates of delphanin are different from those of the intact cell (Figs. 1E, 1A, 1B).

At pH 1·2, swertisin co-pigmented with delphanin and caused a 40 nm bathochromic shift in the λ_{max} (Figs. 2A, 2B). When the pH is shifted to *ca.* 5 the absorption spectrum (Fig. 2C) is almost identical to that of the blue pigment within the cell (Fig. 1C) but different from the anhydro-base of delphanin alone (Fig. 2D). The addition of iron or aluminum to the co-pigment complex gave characteristic metal-chelate curves similar to those in Figs. 1A, 1B. Co-pigmentation and the formation of the blue pigment observed for swertisin (Fig. 2) are also evident for each of the other isolated *C*-glycosylflavones.

DISCUSSION

The anthocyanin from the blue pigment of Prof. Blaauw Iris is delphinidin-3-(*p*-coumaroylrutinoside)-5-glucoside and is the same as delphanin which was previously isolated from garden iris.[7] Because of the pronounced instability of both the flavylium salt and anhydro-base at higher pH, it has been considered most improbable that anthocyanin *per se* could contribute significant color to tissues whose pH was in the range 4–6·2.[13] This conclusion was drawn from observations on a relatively dilute concentration of anthocyanin. At *ca.* pH 6, a $3{\cdot}5 \times 10^{-5}$ M solution of delphinidin or cyanidin-3-glucoside becomes virtually colorless within 1 hr. We now find that at a pH of *ca.* 5, a 6×10^{-4} M solution of delphanin

---

[13] L. Jurd and S. Asen, *Phytochem.* **5**, 1263 (1966).

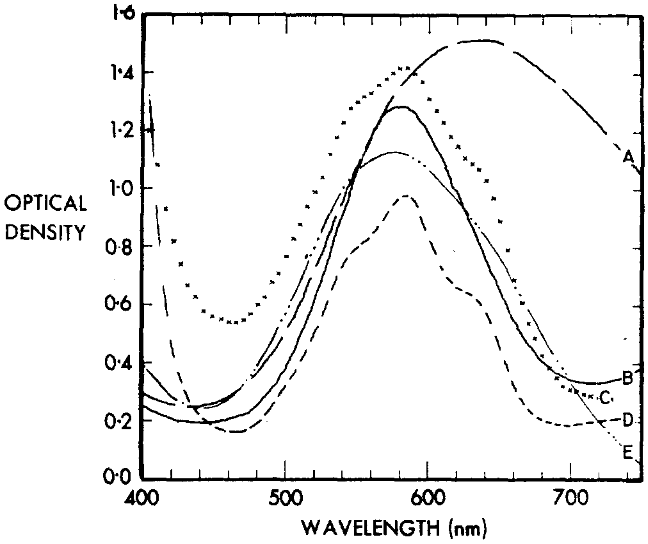

Fig. 1. Absorption spectra: (optical path length 2 mm except curve C *ca.* 25 μm).

(A) Delphinidin 3-(*p*-coumaroylrutinoside)-5-glucoside (delphanin) + $FeSO_4$ (pH 5·4).
(B) Delphanin + $AlCl_3$ (pH 5·2).
(C) Intact epidermal cell of Prof. Blaauw Iris.
(D) H_2O extract of Prof. Blaauw Iris (pH 5·2).
(E) Delphanin (pH 5·1).

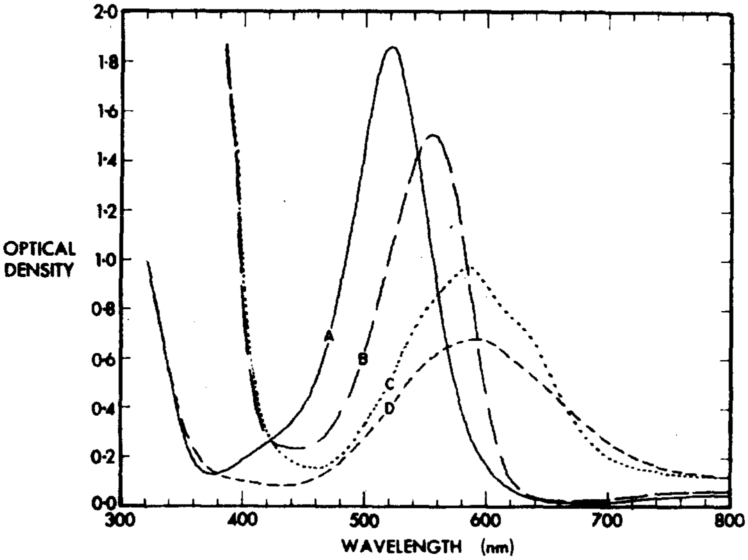

Fig. 2. Absorption spectra: (optical path length 2 mm).

(A) Delphinidin 3-(*p*-coumaroylrutinoside)-5-glucoside (delphanin) (pH 1·3).
(B) Delphanin + 6-*C*-β-D-glucopyranosylgenkwanin (swertisin) (pH 1·2).
(C) Delphanin + swertisin (pH 4·9).
(D) Delphanin (pH 4·9).

is much more stable. Since the color is presumably from an anhydro-base, the initial absorbance loss and change in λ_{max} may be due to establishment of an equilibrium with the flavylium salt and the colorless carbinol base.

The optical path through epidermal cells of Prof. Blaauw Iris is *ca.* 25 μm and their absorptivity at the λ_{max} varies from 0·5 to 1·0. Presumably the *E* value for the Prof. Blaauw Iris pigment and that for delphanin are similar, therefore the concentration of the pigment in the plant cells would approach 10^{-2} M and, at this concentration, would presumably be stable.

Co-pigments isolated from rhizomatus bearded garden iris are either mangiferin, a *C*-glycosylxanthone, or *C*-glycosylflavones similar to, if not identical with, orientin and vitexin.[14] The co-pigments associated with the blue pigment from the bulbous iris Prof. Blaauw are *C*-glycosylflavones with only two fundamental flavonoid nuclei, namely, apigenin or luteolin. Characteristic of *C*-glycosylflavones, all the isolated co-pigments yielded two compounds when hydrolyzed in hot HCl due to the 8-glycosyl-6-glycosyl isomeration. The most abundant co-pigment is swertisin, previously isolated from *Swertia japonica* (Roem. and Schult.) Makino.[12] The 4′ glucoside of swertisin, named "flavocommelinin",[15] was also found to be an essential component of a crystalline blue pigment isolated from flowers of *Commelina communis.* Another crystalline blue pigment isolated from *Centaurea cyanus* also contained a co-pigment not fully characterized but thought to be a biflavonyl of genkwanin and swertisin.[16] Whether or not *C*-glycosylflavonoids are usually associated with blue pigments remains to be established.

In aqueous acid the spectral change (bathochromic shift) caused by flavones or gallotannin, when co-pigmented with malvidin or cyanidin glycosides, is reported to be in order of 5 nm.[17] In contrast, at pH 1·2, the *C*-glycosylflavones co-pigmented with delphanin and caused a bathochromic shift of up to 40 nm. Increasing pH to *ca.* 5 (pH of the blue pigment extracted from Prof. Blaauw Iris) resulted in an absorption spectrum which approximated that of the intact cell (Fig. 2). The addition of iron or aluminum changed the characteristic absorption spectrum so it no longer matched that of the intact cell. Presumably, then, the blue pigment is a stable non-metallic co-pigment complex of *C*-glycosylflavones and delphanin and has a λ_{max} at *ca.* 585 nm with shoulders in the regions of 540 and 630 nm. The absorbers producing these shoulders could also be present in the anhydro-base of delphanin but they are not clearly defined. Co-pigmentation with *C*-glycosylflavones accentuates these shoulders, possibly by reducing the band pass of each of the absorbers.

Many anthocyanin-like pigments extracted from plants cannot be dialzyed and must therefore have high molecular weights. Macro-molecular components have been identified as polysaccharides, peptides, or pectins.[4, 18] Pectin has been shown to increase the extinction of delphinin.[4] We observed little effect from the pectin isolated from the Prof. Blaauw Iris pigment other than the prevention of the precipitation of iron-anthocyanin chelates. The role of pectin and the other macro-molecular components remains obscure.

Although a great deal of effort has been devoted to the identification of anthocyanins, little is known about how they exist in their natural state in plant cells. Spectral studies of pigments *in situ* at the cellular level, plus the known chemistry of these compounds, should help resolve some of the speculation about flower color.

---

[14] E. C. BATE-SMITH and J. B. HARBORNE, *Nature* **198**, 1307 (1963).

[15] K. TAKEDA, S. MITSUI and K. HAYASHI, *Bot. Mag. Tokyo* **79**, 578 (1966).

[16] S. ASEN and L. JURD, *Phytochem.* **6**, 577 (1967).

[17] J. B. HARBORNE, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. GOODWIN), p. 261, Academic Press, New York (1965).

[18] N. SAITO, S. MITSUI and K. HAYASHI, *Proc. Jap. Acad.* **37**, 485 (1961).

EXPERIMENTAL

Isolation of Blue Pigment

Fresh Prof. Blaauw Iris flowers were soaked in Me_2CO for 15 min, separated by filtration, and air-dried. The air-dried tissue was ground to pass a 40-mesh screen and then thoroughly extracted with light petroleum (boiling range 38–49°) to remove carotenoids and other soluble pigments. The blue pigment was extracted by adding 500 ml H_2O to 75 g of tissue and then squeezing the slurry through three layers of cheese cloth. This process was repeated using 250 ml and 200 ml H_2O, respectively. The total volume was reduced to 250 ml under reduced pressure at 40° and then filtered through celite.

Isolation, Purification, and Characterization of Anthocyanin

An aliquot of the blue pigment which was dialyzed in H_2O at 5° for 41 hr, was acidified (pH 2) and purified as previously described.[19] Further purification was obtained by preparative TLC on 2-mm layers of Avicel microcrystalline cellulose. The solvents used were 15% acetic acid; 2-propanol–H_2O–HCl (22:78:1 v/v) repeated three times; and acetic acid–HCl–H_2O (5:1:5 v/v). The anthocyanin was finally passed through a column of Sephadex LH-20 (15 × 300 mm) with 1% HCl–MeOH at a flow rate of 1 ml per min and collected in 6-ml fractions. Each fraction was examined spectrophotometrically for purity.

Methods for identification of the anthocyanidin and sugar moieties have been described previously.[19]

The purified anthocyanin was dissolved in MeOH and oxidized with H_2O_2 for identification of sugars at the C-3 position by the procedure of Chandler and Harper.[20]

Controlled acid hydrolysis for the determination of intermediate anthocyanins formed during hydrolysis was performed by the procedure of Abe and Hayashi.[21]

The presence of an acyl group was determined by treating the anthocyanin with 2 N NaOH for 15 min at room temperature (*ca.* 22°) in an atmosphere of N_2. After acidification (pH 2) the acyl group was extracted with Et_2O and purified by TLC with benzene–acetic acid–H_2O (125:72:3 v/v).

Isolation, Purification, and Characterization of C-*glycosylflavone Co-Pigments*

The blue pigment extracted from 75 g of tissue was dialyzed for 41 hr in 3 l. of H_2O at 5° then acidified (pH 2) and the flavonoids extracted in a liquid/liquid extractor with ether for 1 week followed by ethyl acetate for 24 hr. Five *C*-glycosylflavones were isolated and purified by TLC on 2-mm layers of Avicel microcrystalline cellulose. The solvents used were: for Compound *1*, 15% acetic acid; for Compound *2*, 15% acetic acid, and *n*-BuOH–acetic acid–H_2O (6:1:2 v/v) repeated twice; for Compound *3*, 15% acetic acid, 2-propanol–formic acid–H_2O (2:5:5 v/v) repeated three times, and ethyl acetate–acetic acid–H_2O (10:2:3 v/v upper phase); for Compound *4*, 15% acetic acid, 2-propanol–formic acid–H_2O (2:5:5 v/v) repeated twice, and ethyl acetate–acetic acid–H_2O (10:2:3 v/v upper phase); for Compound *5*, 15% acetic acid, and 2-propanol–formic acid–H_2O (2:5:5 v/v) repeated twice.

Each isolated compound was hydrolyzed by refluxing for 2 hr in EtOH and 2 N HCl in the ratio of 1 ml of each solvent per mg of sample. EtOH was evaporated from the solution by a stream of N_2 and the aglycone was extracted with *n*-BuOH saturated with H_2O. The sugar moiety in the aqueous residue was examined by methods previously described.[19]

Pectin

Pectin substance was analyzed colorimetrically (carbazole reaction) by the procedure of McComb and McCready.[5]

Ultra-violet Spectra

Ultra-violet spectra were determined in ethanol. Diagnostic shifts were determined by adding to solutions in cuvettes (*ca.* 2·5 ml): (1) 5 drops of 1% $AlCl_3.6H_2O$ in ethanol; (2) excess of fused anhydrous NaOAc; (3) 2 drops of 0·3% Na OEt; and (4) excess fused anhydrous NaOAc plus 5 drops of a saturated solution of H_3BO_3 in ethanol.

Co-Pigment Spectra

The anthocyanin and the *C*-glycosylflavones were dissolved in 0·01 N HCl and 0·002 N NaOH, respectively. The anthocyanin at a concentration of *ca.* 10 absorptivity units/cm was added to each *C*-glycosylflavone so that the long-wavelength absorption of the anthocyanin was *ca* 20% of the long-wavelength absorption of the co-pigment. The solution was immediately acidified, with 1 drop of HCl, to pH 1·3 and the absorption spectrum was recorded. The pH of the solution was then changed with solid NaOAc to *ca.* 5 and the absorption spectrum was recorded again. Spectra were measured with a multi-purpose spectrophotometer[22] with the sample thickness adjusted to 2 mm.

---

[19] S. ASEN and P. S. BUDIN, *Phytochem.* **5**, 1257 (1966).

[20] B. V. CHANDLER and K. A. HARPER, *Australian J. Chem.* **14**, 586 (1961).

[21] Y. ABE and K. HAYASHI, *Bot. Mag. Tokyo* **69**, 577 (1956).

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

Intact Cell Spectra

Upper epidermal tissue was stripped from petals and mounted on standard 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.* 30 × 50 μm in surface area and 25 μm thick. Over 90 per cent 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 epidermal cells were measured with a microspectrophotometer to be described in detail elsewhere. We converted a Zeiss Ultraphot II microscope into a microspectrophotometer by replacing the camera with a wedge-interference filter monochromator photometer unit. Absorptivity was measured at 5-nm intervals, scanning the 370 to 720 nm region in 2 min. Corrections for system response were made by subtracting the absorptivity of a non-pigmented cell at each wavelength. An image of the cell to be measured was focused on the entrance slit of the monochromator. This slit provided an effective aperture of 4 × 8 μm at the magnification used.

Nuclear Magnetic Resonance Spectra

The NMR spectra of swertisin and its hexaacetate were measured in deuteriopyridine and in deuteriochloroform, respectively.

Acknowledgements—We express appreciation to Dr. R. M. Horowitz whose NMR study confirmed the identification of swertisin, to Miss P. S. Budin for her competent technical assistance, and to Drs. L. Jurd and M. Shimokoriyama for samples of swertisin and orientin. This research was supported in part by the New York Florists Club.