Plant Polyphenols

3. FLAVONOIDS IN GENOTYPES OF *PRIMULA SINENSIS**

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It is now well established that the C_{15} skeleton of the flavonoid molecule is synthesized in plants by the condensation of three acetate units with phenylpyruvic acid (Watkin, Underhill & Neish, 1957). Much less is known about how this skeleton is then elaborated by hydroxylation, methylation and glycosidation. In order to obtain more information about these later stages in biosynthesis, the flavonoids present in several series of colour mutants of cultivated plants are being reinvestigated with recently developed methods of pigment analysis (see Harborne, 1958*a*, *b*, 1959). The results obtained for the diploid potato, *Solanum phureja*, have already been described (Harborne, 1960*a*). The present account is of a similar investigation of the phenolic pigments of *Primula sinensis*, of which many colour mutants of known genetic constitution are available for biochemical analysis (De Winton & Haldane, 1933; De Winton, 1936).

Scott-Moncrieff (1936) and Dayton (1954) have shown that in the inheritance of pigmentation in *P. sinensis*, gene *K* controls the production of malvidin 3-monoside in mauve flowers. Recessive genotypes (*kk*) have coral flowers and contain pelargonidin 3-monoside. Another gene, *Dz*, independently of *K*, also controls production of pelargonidin monoside, though its activity is obscured by complicated dosage and dominance relationships. *KDz* types contain mixtures of malvidin and pelargonidin glycosides and are various shades of red or bluish red; *kDz* types contain pelargonidin 3-monoside only and are bright orange. Gene *R* lowers the pH of the cell sap by 0.6 unit so that *KR* flowers are redder than *Kr* flowers. A gene *B* has a bluing effect on flower colour, by producing flavone copigments, provisionally identified as a mixture of kampferol glycosides. Other genes control pigment distribution; e.g. gene *D* inhibits colour in the flowers. Finally, genes whose main effect is on morphological characters (e.g. leaf shape) may sometimes have minor effects on pigment synthesis.

In spite of all the genetical information available, only one pigment in *P. sinensis* has previously been fully characterized. This is 'primulin', which

was isolated from mauve flowers by Scott-Moncrieff (1936) and identified by Bell & Robinson (1934) as malvidin 3-galactoside. That Scott-Moncrieff's pigment must, however, have been the 3-glucoside is shown by the present work, in which 21 glycosides have been identified. They are derived from ten known flavonoid aglycones and all contain glucose and not galactose as their only sugar.

MATERIALS AND METHODS

Plant material. The 43 genotypes of *Primula sinensis* have been grown under glasshouse conditions from self-seed of diploid lines of plants used in earlier investigations (Scott-Moncrieff, 1936; Dayton, 1954) but also include a few tetraploid lines and some commercial varieties.

Authentic pigments. Known anthocyanins and anthocyanidins were obtained as already described (Harborne, 1958*a*, 1960*a*). Crystalline specimens of primulin, isolated by Miss R. Scott-Moncrieff, were made available by Miss V. C. Sturgess. Kampferol 3-glucoside and 3-rhamnogalucoside were obtained from Professor L. Hörhammer, Munich. Dihydrokämpferol (aromadendrin) and its acetate were supplied by Dr R. A. Laidlaw. Kampferol 7-glucoside was prepared from naringenin 7-glucoside (Seikel, 1955) by treatment with alkaline hydrogen peroxide (Simpson & Whalley, 1955) and then with hot dilute acid.

Paper chromatography and spectroscopy. Conditions used for the paper chromatography of phenolic pigments have been described (Harborne, 1958*b*, 1959). The solvent mixtures used were: *A*, butan-1-ol-2*N*-HCl (1:1, v/v; upper phase); *B*, butan-1-ol-acetic acid-water (4:1:5, by vol.; upper phase); *C*, acetic acid-water (15:85, v/v); *D*, butan-1-ol-benzene-pyridine-water (5:1:3:3, by vol.); *E*, butan-1-ol-ethanol-water (4:1:5, by vol.); *F*, butan-1-ol-water (1:1, v/v; upper phase); *G*, water; *H*, water-conc. HCl (97:3, v/v); *I*, phenol saturated with water.

The spectra of phenolic pigments were determined by the methods of Geissman (1955), Jurd & Horowitz (1957) and Harborne (1958*a*). Measurements were made with a Unicam SP. 500 spectrophotometer.

Extraction of the anthocyanins. Fresh petals or foliar parts were homogenized in methanol containing 1% of conc. HCl. After centrifuging and re-extraction of the residue, the supernatants were combined and evaporated *in vacuo* to a small volume. The concentrate was filtered and the filtrate washed successively with light petroleum and ethyl acetate. When storage was necessary, the extract was first freeze-dried, the product being purified by solution in methanol, followed by precipitation with dry ether; it

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was kept in a desiccator and used within 3 months of its preparation.

Separation, purification and identification of the anthocyanins. The methods used have been described by Harborne (1958*a, b*, 1960*a*). The anthocyanin concentrates were separated by chromatography on Whatman no. 3 paper with solvent A. Successive chromatography in solvents A, B and C of the pigment bands thus obtained eventually gave solutions of pure anthocyanins. Fifteen chromatographically distinct pigments were thus obtained from the genotypes investigated; the best sources of these pigments are given in Table 1. When an anthocyanin was obtained from more than one genotype, identity was established by cochromatography, spectroscopic analysis and examination of the products of acid hydrolysis.

In view of the report of Bell & Robinson (1934) that the major anthocyanin of *P. sinensis* (A13, Table 1) contains galactose, special care was taken in identifying the monosaccharide produced on acid hydrolysis of the anthocyanins. The sugar specimens were inseparable from added glucose, but separated from added galactose on paper chromatograms developed in solvents B, D or E for 36–48 hr. The sugars were oxidized by notatin and also gave the same distinctive colour as glucose when treated with resorcinol in sulphuric acid (Harborne, 1960*a*).

Isolation and identification of the flavonol glucosides. Fresh white petals from plants of Alexandra Beetle *KKDD* genotype were extracted with boiling methanol for 0.5 hr. After filtration, the extract was concentrated *in vacuo* to a small volume and filtered. The pale-yellow extract was separated by chromatography on Whatman no. 3 paper in solvent B. The five bands (F1–5; Table 2) were purified by chromatography in solvents C, F and G. Eluates of the two major bands F2 and F4 gave pale-yellow, water-soluble, non-crystalline solids after evaporation to dryness. These were purified further by precipitation with light petroleum from moist butanol. Both compounds retained water of crystallization, even after they had been dried at 100° *in vacuo* for 10 hr. F2 had m.p. 204° (Found: C, 51.3; H, 5.4. $C_{27}H_{30}O_{16} \cdot 3H_2O$ requires C, 51.1; H, 5.7%). F4 had m.p. 234–236° (Found: C, 50.6; H, 5.9. $C_{33}H_{40}O_{21} \cdot H_2O$ requires C, 50.1; H, 5.4%). F2 and F4 had the same infrared spectra as known kampferol 3-glycosides (e.g. the 3-glucoside). The other three flavonol glucosides were not present in sufficient amounts for characterization in the above-described manner. The five compounds F1–5 and the aglycones and sugars they give on acid hydrolysis were identified by similar methods to those described (Nordström & Swain, 1953; Harborne, 1959). Sugar:aglycone ratios were determined as described by Harborne (1960*a*). The sugar was also determined by the resorcinol-sulphuric acid reagent (Devor, Conger & Gill, 1958), since the flavonol aglycones do not interfere with the colour produced in this reaction.

Determination of the position of sugar residues of the flavonols. Measurement of the absorption spectra of F1–5 in ethanol and then in ethanol containing inorganic ions (see Table 2) indicated that only the 3-position in these flavonol molecules contains a sugar substituent (see Jurd & Horowitz, 1957). This was confirmed in the following way. The five pigments were fully methylated and then hydrolysed with acid (Nordström & Swain, 1953). The products were then compared with the respective authentic, partially methylated flavonols. 5:7:4'-Trimethylkampferol and

7:4'-dimethylkampferol were prepared as described above from kampferol 3-glucoside and robinin (kampferol 3-rhamnosylgalactoside, 7-rhamnoside). The former substance and the products from F2 and F4 had λ_{max} in 95% ethanol at 257 and 351 m μ , in 95% ethanol-NaOH at 260 and 390 m μ and in ethanol-sodium acetate at 257 m μ ; the latter compound had λ_{max} in 95% ethanol at 265 and 338 m μ and in ethanol-sodium acetate at 270 m μ . R_F values and colour of these two compounds have been recorded (Harborne, 1959). The products from F3 and F5 were similarly found to be identical with 5:7:3':4'-tetramethylquercetin (Herrmann, 1958). The products from F1 and from myricitrin (myricetin 3-rhamnoside) were also identical.

Isolation and identification of sinensin. Fresh orange flowers of Dazzler (*kkDzDz*) genotype were extracted with methanol containing 1% of conc. HCl. After filtration, the extract was evaporated *in vacuo* to an aqueous residue. Alternatively, dried powdered petals were extracted with 0.3N-HCl. In either case, the aqueous solutions were repeatedly extracted with small volumes of ethyl acetate. Evaporation of the ethyl acetate extracts gave a hygroscopic white powder which was purified by chromatography in solvent G on Whatman no. 3 paper to give sinensin (R_F 0.72) and an oxidation product (R_F 0.02). After precipitation from a cold methanolic solution with light petroleum, sinensin had m.p. 182° (decomp.). On acid hydrolysis it gave glucose, and an aglycone which, after three recrystallizations from water, separated as colourless needles, m.p. 238–240° (decomp.) and mixed m.p. with dihydrokampferol 236–238° (decomp.) (Found: C, 61.8; H, 4.3. Calc. for $C_{15}H_{18}O_6$: C, 62.5; H, 4.2%). The aglycone and dihydrokampferol had the same R_F values and ultraviolet spectra (see Table 2), and both gave pelargonidin on reductive acetylation (Pacheco, 1957). The structure of sinensin was confirmed by identifying its oxidation product (isolated as described above or from aqueous solutions of sinensin which had been boiled for 4 hr.) as kampferol 7-glucoside (Table 2).

Provisional identification of a derived disaccharide. Chromatograms of the acid hydrolysates of pigments that contained more than one glucose residue (notably A2, F2 and F4) showed the presence, after spraying with aniline hydrogen phthalate, of glucose and a single disaccharide. The same disaccharide was produced by heating the pigments for 4 hr. in 10% (v/v) acetic acid, as was used for preparing rutinose from rutin (Arakawa, 1956). This disaccharide (R_G 0.27, 0.43) was chromatographically identical with gentiobiose in solvents D and B and separated from added cellobiose (R_G 0.44, 0.58) and maltose (R_G 0.52, 0.58). It was different from the disaccharide (R_G 0.43, 0.61) produced during the acid hydrolysis of pigments isomeric with A2 and A5 which were isolated from *Papaver rhoeas* and related species (see Results).

Survey of the flavonoids. Petal and leaf extracts of all the colour genotypes were examined by one- and two-dimensional paper chromatography. The presence or absence of the 21 major pigments was determined by analysis of R_F values and colours. Acid hydrolysates of all the above-mentioned extracts were similarly examined for the presence or absence of the ten known flavonoid aglycones.

Measurement of pigment concentrations. The centres of the flowers (which contain plastid pigments) were removed by means of a 12 mm. cork borer from fresh corollas (ten per

sample) of the genotype under examination. The samples were weighed within 20 min. of picking and then extracted by crushing them in known volumes of methanol-conc. HCl (97:3, v/v). The extracts were stored for 24 hr. in the dark at 0°. Pigment concentrations were then estimated in the filtered petal extracts by spectrophotometric measurements at the appropriate absorption maxima; the results were compared with similar measurements made on solutions containing known amounts of pure pigment. Total anthocyanin concentration was thus measured at 507 m μ for *kk* types and at 532 m μ for *KK* types. No other pigments were present to interfere with anthocyanin spectra in the visible region. Total flavonol glucoside concentration was measured at the maxima in the 400–420 m μ region after addition of alkali to the petal extract. Interfering substances such as cinnamic acids are virtually absent from *P. sinensis* petals; an alkaline solution of sinensin shows no absorption in this region. Sinensin concentration was measured at its λ_{max} at 287 m μ after it had been separated from other pigments by chromatography in solvent *B* and subsequent elution. Results are given in Tables 3 and 6.

Measurements were also made of the relative amounts of the various pigments on eluates of bands cut from chromatograms developed with solvent *A* or *B*, of fresh extracts, or, in a few cases, of fresh extracts after acid hydrolysis. Results are given in Tables 4 and 5.

RESULTS

Identification of the anthocyanins

Fifteen anthocyanins have been found in flowers and leaves of *Primula sinensis* and some of their properties are listed in Table 1. They all give one of the six common anthocyanidins and glucose on acid hydrolysis. No galactose was detected, in spite of the earlier identification of the major malvidin pigment (A13 in Table 1) as the 3-galactoside (Bell & Robinson, 1934). A specimen of 'primulin', isolated by Scott-Moncrieff (1936), gave malvidin, mixed with petunidin (about 15%), and glucose on acid hydrolysis.

Quantitative acid hydrolyses of pigments A1–3 give pelargonidin and one, two and three molecular proportions of glucose respectively. The spectra and colour of these three pigments are identical and are the same as those of authentic pelargonidin 3-glucoside, but differ from those of known 3:5- and 3:7-glucosides. Furthermore, A3 gives only two intermediate glucosides (A2 and A1) during acid hydrolysis; A2 gives only one (A1) and A1 gives none. Therefore the glucose residues of all three pigments are attached to only one of the four hydroxyl groups of pelargonidin, the one in the 3-position. Similar hydrolysis experiments establish that the remaining pigments are 3-glucosides, 3-glucosylglucosides (3-diglucosides) or 3-glucosylglucosylglucosides (3-triglucosides) of the other five anthocyanidins. Lack of material has prevented the measurement of sugar:aglycone ratios in all 15 substances. However, their chromato-

graphic behaviour (Table 1) is strong evidence that they all have the number of glucose residues assigned to them. All but three out of the 18 possible combinations of six anthocyanidins with three states of glucosidation have been identified.

The identity of pigments A1, A4, A6, A9, A10 and A13 was confirmed by direct comparison with authentic specimens of the six anthocyanidin 3-glucosides. Pigments A2, A5, A7, A11 and A14 are the 3-diglucosides of pelargonidin, cyanidin, peonidin, petunidin and malvidin respectively. Since A2 and A5 are chromatographically different from the pelargonidin and cyanidin 3-diglucosides, known to occur in various *Papaver* spp. (Robinson & Robinson, 1931; Harborne & Sherratt, 1957), these five pigments are new compounds. Acid hydrolysis of A2 and A5 yields, besides glucose, a small quantity of a disaccharide, which appears to be gentiobiose. As the isomeric cyanidin pigment (mecocyanin) from poppy is supposed to be the 3-gentiobioside (Grove, Inubuse & Robinson, 1934), a re-examination of the two poppy anthocyanins was carried out. On hydrolysis they give glucose and a disaccharide, different in its chromatographic behaviour from gentiobiose but similar to cellobiose.

The remaining four pigments, A3, A8, A12 and A15, are the 3-triglucosides of pelargonidin, peonidin, petunidin and malvidin and are also new pigments. The related cyanidin 3-triglucoside has been identified as occurring in acylated form in red cabbage (Stroh, 1959), so that only the delphinidin glycoside of this structure remains to be found.

Identification of the flavonol glucosides and of sinensin

Five main flavonol glucosides occur in flowers of *P. sinensis* and at least three of them are also present in the leaves. Their properties are given in Table 2. They have been identified as the 3-diglucoside (F2) and 3-triglucoside (F4) of kampferol, the 3-diglucoside (F3) and 3-triglucoside (F5) of quercetin and the 3-glucoside (F1) of myricetin. Only the last compound (cannibiscitrin) has been thoroughly described before (see Karrer, 1958): it occurs in the flowers of *Hibiscus cannabinus*, in leaves of tea (*Camellia*) and in the seed-coat of *Phaseolus vulgaris*. The 3-triglucosides of kampferol and quercetin were thought to occur in the leaves of tea (Oshima & Nakabayashi, 1954), but reinvestigation by Roberts, Cartwright & Wood (1956) did not reveal them.

Sinensin (Harborne & Sherratt, 1958) is present in nearly all genotypes of *P. sinensis*, but occurs in quantity in flowers of only a few, e.g. the Dazzler mutant. It has been identified as dihydrokampferol 7-glucoside. Its properties closely resemble those of dihydrokampferol 7-methyl ether (Chopin &

Table 1. Sources and properties of the anthocyanins of *Primula sinensis*

Pigment	Colour in visible (and in ultraviolet) light	Principal source	Visible spectra		R_F			Products of complete hydrolysis	Intermediate products of hydrolysis	Ratios: aglycone glucose
			$\lambda_{\max}$ in methanol-0.03N-HCl (m μ)	E'_{440} m μ / $E'_{\max}$ pigment (as %)	Solvent A	Solvent B	Solvent H			
A1 } A2 } A3 }	Orange-red (dull red)	'Dazzler' flowers (kkbbRRDzDz)	508 507 510	39 39 36	0.43 0.25 0.18	0.48 0.32 0.26	0.12 0.21 0.36	Pelargonidin glucose	{None A1 A1 and A2	1:1.10 1:1.82 1:3.28
A4 } A5 } A6 } A7 } A8 }	Magenta (dull magenta)	{ 'Dazzler' flowers 'Dazzler' leaves 'Dazzler' flowers 'Dazzler' leaves 'Dazzler' leaves	520* 521* 518 519 518	29 29 29 33 35	0.14 0.10 0.16 0.10 0.09	0.26 0.20 0.33 0.25 0.10	0.07 0.14 0.09 0.19 0.36	Cyanidin glucose Peonidin glucose	{None A4 None A6 A6 and A7	— — — — —
A9 }		'Duchess Fern Leaf' flowers (KKbbRR)	534*	18	0.11	0.15	0.03	Delphinidin glucose	None	—
A10 }		Standard 'stellata' flowers (KKbRRR)	534*	20	0.14	0.23	0.03	Petunidin glucose	{None A10 A10 and A11	1:1.01 — 1:2.78
A11 } A12 }	Dull mauve (dull mauve)	{ 'Beetle' leaves (KKDD) 'Beetle' leaves	531* 533*	23 24	0.07 0.05	0.16 0.11	0.12 0.29		{None A13 A13 and A14	1:1.06 — 1:2.79
A13 }		Standard 'stellata' flowers	533	26	0.16	0.33	0.04	Malvidin glucose		
A14 } A15 }		{ 'Beetle' leaves 'Beetle' leaves	532 532	25 22	0.10 0.06	0.19 0.14	0.15 0.31			

* Only these pigments had their spectra shifted to the blue region by the addition of ethanolic AlCl₃.

Table 2. *Properties of the flavonol glucosides, sinensin and its derivatives*

The aglycones were: F1, myricetin; F2, F4, kampferol; F3, F5, quercetin.

Pigment	$\lambda_{\max}$ (m μ)					Intermediate products of hydrolysis	Ratio: aglycone: glucose
	95% Ethanol	95% Ethanol with 2N-NaOH (2 drops/3 ml.)	95% Ethanol with 5% of AlCl ₃ (2 drops/3 ml.)	95% Ethanol satd. with sodium acetate	95% Ethanol satd. with H ₃ BO ₃ and sodium acetate		
F1	258, 305, 363	Decomp.	365, 398	264	262, 305, 385	None	1:0.96
F2	267, 298, 349	275, 398	351, 395	274	268, 305, 353	None	1:1.96
F3	258, 305, 360	Decomp.	367, 388	272	265, —, 385	None	1:2.09
F4	268, 298, 350	275, 397	275, 393	274	267, 298, 350	F ₂	1:3.35
F5	257, 305, 361	Decomp.	337, 410	273	265, —, 385	F ₃	1:2.86
Sinensin	215, 287, ~325	288, 365	309, ~370	—	—	None	1:0.95
Sinensin acetate	271, ~305	—	—	—	—	None	1:0.88
Oxidation product of sinensin	265, 325, 366	410 (slow decomp.)	—	264	—	—	—
Kampferol 7-glucoside	215, 292, ~330	243, 318	314, ~365	—	—	—	—
Sinensin aglycone							
Dihydrokampferol							

Pigment	R_f		Solvent		Colour in ultraviolet light	
	Solvent B	Solvent G	Solvent I		In air	In NH ₃ vapour
F1	0.47	0.05	0.32		Ochre	Yellow-brown
F2	0.43	0.27	0.55		Ochre	Yellow-green
F3	0.37	0.19	0.36		Ochre	Yellow
F4	0.31	0.33	0.45		Ochre	Yellow-green
F5	0.23	0.18	0.26		Ochre	Yellow
Sinensin	0.57	0.64	0.76		Dull purple	Light yellow
Oxidation product of sinensin	0.54	0.02	0.76		Bright yellow	Bright yellow
Kampferol 7-glucoside	0.92	0.30	0.77		Dull purple	Dull purple
Sinensin aglycone						
Dihydrokampferol						

Pacheco, 1958) but differ from those of dihydro-kampferol itself. This dihydroflavonol typically occurs in the free state or as a 3-glycoside in heartwood of trees (Karrer, 1958) so that its presence as the 7-glucoside in a herbaceous plant is of interest.

Distribution of flavonoids

Hydroxylation of the flavonoids. *KK* genotypes, which have coloured flowers (red, maroon, mauve or blue), contain varying amounts of the 3-glucosides of malvidin, petunidin and delphinidin. Myricetin, quercetin and kampferol glucosides are also present and are the only pigments found in white flowers of *KKDD* genotype. By contrast, *kk* types lack pigments with the 3':4':5'-trihydroxylation pattern: in them the anthocyanidins are pelargonidin, peonidin and cyanidin and the flavonols are quercetin and kampferol. Gene *K* therefore controls a simple step in biosynthesis, the introduction of a 5'-hydroxyl group into a precursor common to both anthocyanidin and flavonol pigments. Unlike other known genes with similar effect in other plants (e.g. *P* in *Solanum phureja*, Harborne, 1960*a*), it is incompletely dominant in certain genetic backgrounds (see below and Dayton, 1954).

Anthocyanin concentration: the Dazzler factor. *KKDzDz* plants normally have scarlet flowers and contain the anthocyanins of *KK* forms, together with those of *kk* forms. Plants of constitution *kkDzDz* (orange flowers) differ from *kkdzzd* forms (coral flowers) only by containing more of the same

anthocyanins. The concentrations of the three types of pigments of flowers of *kkDzDz* and *kkdzzd* genotypes have been measured and the results are shown in Table 3. Whereas the amount of flavonol glucoside and sinensin present is relatively constant, there is a threefold increase in the amount of pelargonidin glucosides on passing from the recessive to the dominant genotype. The Dazzler gene thus increases the total concentration of anthocyanins, and only anthocyanins, in the flowers. The production in *KKDzDz* plants of pelargonidin, cyanidin and peonidin glucosides, anthocyanins typical of the recessive *kk* forms (not, as might be expected, more malvidin and petunidin 3-glucosides), must be due to the fact that the amount of enzyme available in *KK* types for 5'-hydroxylation is strictly limited.

Methylation of the anthocyanins. There are noticeable differences in the degree of methylation between different genotypes and between different tissues of the same genotype. The proportions of 3-glucosides of delphinidin, petunidin and malvidin in the flowers of a series of *KK* genotypes are recorded in Table 4. The amount of methylation is greatest in the wild-type plant and in six other *BB* forms examined (measurements on only one typical example are given in Table 4). Genotypes recessive for the *b* or copigment gene have 10 % less malvidin present. Methylation is further decreased in some morphological mutants, being, indeed, absent from the 'Duchess Fern Leaf' form. These results suggest that there are genes controlling

Table 3. Amounts of different pigments in *DzDz* and *dzzd* genotypes

Results given are the averages of three determinations and are expressed as mg. of pigment/g. fresh wt. of flower petal.

Pigment type	Estimated as	Concentration in		
		<i>kkDzDz</i> (orange 'Dazzler')	Pale coral mutant*	<i>kkdzzd</i> (coral 'Pink Star')
Anthocyanin	Pelargonidin 3-diglucoside	3.2	1.1	1.0
Flavonol glucoside	Kampferol 3-diglucoside	0.67	0.60	1.7
Dihydroflavonol	Sinensin	7.2	6.1	7.2

* The genotype of this mutant, which is morphologically closer to 'Dazzler' than 'Pink Star', is not known.

Table 4. Amounts of 3-glucosides of malvidin, petunidin and delphinidin in the flowers of *KK* genotypes

	Constitution	Flower colour	Percentage of total anthocyanin as		
			Malvidin 3-glucoside	Petunidin 3-glucoside	Delphinidin 3-glucoside
<i>BB</i>	Standard wild-type	Mauve	75 (±2)	25	<1
<i>B</i>	37 tetraploid	Mauve	73	24	4
<i>bb</i>	Oak tongue	Maroon	65	29	5
<i>bbbb</i>	36 tetraploid	Maroon	60	30	7
<i>bb</i>	Fasciated	Maroon	63	28	9
<i>bb</i>	'Sirdar'	Pink	48	52	<1
<i>bb</i>	'Reading Pink'	Pink	13	51	37
<i>bb</i>	'Duchess Fern Leaf'	Blue	0	0	100

methylation in *P. sinensis* and that they interact with other genes.

The expected correlation between an increase in methylation and a reddening effect on flower colour (cf. Scott-Moncrieff, 1936) is not apparent from the data of Table 4. Evidently, methylation as a determinant of colour is masked by other factors, such as copigmentation. The 'methylating genes' are less effective in their action in leaves than in flowers: for example, petunidin is the major anthocyanidin in the leaves of *KK* types, of which the flowers contain 60–75% of malvidin; again, cyanidin, which is largely masked as a flower anthocyanidin by overwhelming amounts of peonidin and pelargonidin, is the major leaf pigment aglycone in *kk* forms.

Table 5. *Distribution of flavonoid 3-glucosides in petals and leaves of Primula sinensis*

Unless indicated otherwise, values are for 3-monoglucoside in petals and for mono-, di- and tri-glucosides in leaves. Results are expressed as percentage of total anthocyanins or flavonol glucosides.

Glucosides of	<i>KK</i> genotypes		<i>kk</i> genotypes	
	Petals	Leaves	Petals	Leaves
Malvidin	75*	22†	—	—
Petunidin	25*	78†	—	—
Delphinidin	<1	<1‡	—	—
Peonidin	—	—	9	31§
Cyanidin	—	—	<1	69§
Pelargonidin	—	—	—	—
3-Glucoside	—	—	54	<1¶
3-Diglucoside	—	—	9	—
3-Triglucoside	—	—	28	—¶
Kampferol**	88	25	70††	30††
Quercetin**	9	72	30††	70††
Myricetin	3	3	—	—

* Traces of 3-triglucoside are present in two maroon genotypes ('fasciated' and 'oak tongue').

† 80–85% of total pigment is present as 3-triglucoside.

‡ Monoglucoside.

§ 6, 19 and 75% of total pigment are present as mono-, di- and tri-glucoside respectively.

|| Appreciable amounts are present in certain genotypes, e.g. *KKDzDz*.

¶ Increased amounts (up to 10%) of these pigments are present in *kkDzDz* forms.

** Di- and tri-glucoside.

†† Approximate values obtained by visual estimation of the intensity of aglycone spot on chromatograms.

Glucosidation of the flavonoids. All but one of the flavonols and anthocyanidins occur as 3-mono-, di- or tri-glucoside in relative amounts that vary with the situation in the plant (Table 5): generally a higher degree of glucosidation of the anthocyanidins occurs in leaves than in flowers. In flowers of the *KK* series, 3-glucosides predominate; 3-triglucosides of petunidin and malvidin occur exceptionally in traces in flowers of two out of the 15 *KK* genotypes available for examination. By contrast, leaves of *KK* forms contain mainly triglucosides. In *kk* genotypes the flowers contain 63% of the total anthocyanin as 3-glucoside, whereas the leaves contain 75% as triglucoside.

Copigmentation. Gene *B*, which has a bluing effect on flower colour, was originally thought (Scott-Moncrieff, 1936) to control the production of specific flavone copigments. Examination of the flavonol glucosides of a variety of *BB* and *bb* genotypes by paper chromatography shows, however, that qualitative differences in pigmentation are not involved. Measurements were therefore made of the amounts of anthocyanin and flavonol glucoside in flowers of plants of two families, 36 and 37, which were known to be segregating for the *B* factor (Table 6). Because of variations in the amounts of both pigments in the flowers of individual plants, the results are expressed as the ratio of the flavonol glucoside concentration to the total anthocyanin concentration. This ratio in mauve flowers is consistently higher than in maroon flowers. Copigmentation causes a shift of 4–5 m μ in the visible maximum of the anthocyanin (mainly malvidin 3-glucoside), when measured on fresh aqueous extracts. Similar shifts were produced when the purified kampferol 3-diglucoside and 3-triglucoside were added separately to an aqueous acid solution of malvidin 3-glucoside. It is therefore a reasonable assumption that these flavonol glucosides act as copigments, although other factors (e.g. metal-ion concentration) may determine their effectiveness in this respect.

Flavonoids and Primula taxonomy. Taxonomists place *P. sinensis* in the section *Sinenses* of this genus. The chemical results are consistent with this classification. A striking similarity between all *Primula* species that have been examined is the

Table 6. *Copigmentation in Primula sinensis*

Family number	Genotype*	Colour	No. of plants examined	$\lambda_{\max}$ in 0.3N-HCl (m μ)	Average ratio: flavonol glucoside/anthocyanin
36	<i>Kbdzch</i>	Maroon	5	516	1.1
	<i>KBdzch</i>	Mauve	3	520	2.8
37	<i>KbdzCh</i>	Maroon	4	516	1.1
	<i>KBdzCh</i>	Mauve	4	521	4.9

* These are tetraploids: for simplicity, the full complement of gene symbols are omitted, as the plants are homozygous for pigment genes. The *Ch* gene controls a morphological effect in *P. sinensis*.

constant occurrence of anthocyanins which contain glucose as their only sugar. Thus malvidin 3-glucoside has been found in *P. polyanthus* (Scott-Moncrieff, 1930), malvidin 3:5-diglucoside in *P. viscosa* and in *P. integrifolia* (Karrer & Widmer, 1927), malvidin, petunidin and delphinidin 3:5-diglucoside in *P. obconica* (J. B. Harborne, unpublished work) and hirsutidin 3:5-diglucoside in *P. hirsuta* (Karrer & Widmer, 1927). *P. sinensis* also shares with at least two other species its ability to add trisaccharide residues to the 3-hydroxyl group of the flavonols: kampferol and quercetin 3-triglucoside have been found in flowers and leaves respectively of *P. obconica* and the former pigment occurs in flowers of *P. rosea* (J. B. Harborne, unpublished work). There are, however, at least three ways in which *P. sinensis* differs from other members of the Primulaceae. It apparently lacks the ability that plants such as *P. hirsuta* and *P. rosea* possess of both methylating the 7-hydroxyl group and also glucosidating the 5-hydroxyl group of its anthocyanins. And, unlike the other species, it can synthesize a dihydroflavonol 7-glucoside: sinensin appears to be a characteristic pigment of *P. sinensis*.

DISCUSSION

The original observation of Scott-Moncrieff (1936) that gene *K* in *Primula sinensis* controls the production of malvidin, in place of pelargonidin, does not appear to fit the 'one gene—one enzyme' hypothesis, since at least three biochemical processes separate the two pigments. The present study helps to resolve this difficulty. Traces of delphinidin and cyanidin found respectively in *KK* and *kk* genotypes (as glucosides) show that *K* has, in fact, only one biochemical effect on pigment production. It controls the introduction of a 5'-hydroxyl group into a precursor common to both the anthocyanidins and the flavonols. It is thus parallel in its action to *P* in *Solanum phureja* (Harborne, 1960*a*), *O* in *Streptocarpus* (Lawrence & Sturgess, 1957) and *L* in *Impatiens balsamina* (Alston & Hagen, 1958; Clevenger, 1958).

A feature of flower pigmentation in *P. sinensis* is the predominance of compounds containing the 4'-hydroxyl grouping (e.g. pelargonidin, kampferol, dihydrokampferol) or the 3':4':5'-trihydroxyl grouping (e.g. malvidin, petunidin). Cyanidin and quercetin, i.e. compounds with the 3':4'-dihydroxyl grouping, are relatively unimportant. This result fits the thesis of Lawrence & Sturgess (1957) that cyanidin (and quercetin) are most readily produced in the plant and appear in the flowers of more highly evolved plants, such as *Streptocarpus*, only as 'biosynthetic residues'.

The discovery of *KK* genotypes, in which there is

variation in the degree of methylation of the petal anthocyanins, indicates that there are genes present for the control of this process. Though yet unidentified in *P. sinensis*, a parallel study has revealed the presence of two such genes controlling the methylation of delphinidin in *P. obconica* (Harborne, 1960*c*).

As in a number of other plants (Harborne, 1960*c*), there is a close biogenetic relationship between the flavonols and anthocyanidins present in *P. sinensis* and their glycosidation pattern. Not only are the sugars attached to the same hydroxyl group of the anthocyanidins and the flavonols, but also parallel mono-, di- and tri-glucosides have been isolated in both series. In contrast, there appears to be no corresponding link between the flavonols and the dihydroflavonol. Indeed, the production of dihydrokampferol, which occurs as the 7-glucoside (sinensin) by direct reduction of kampferol, present as the 3-di- or tri-glucoside, seems unlikely in view of the difference in the glucosidation pattern of the two classes of pigment. Furthermore, there is no evidence that such a reduction occurs earlier in biosynthesis since the dihydroflavonols related to quercetin and myricetin have not been detected. The inheritance of sinensin is independent of the known genes (e.g. *K*) involved in pigment synthesis. Sinensin appears to be present in all the genotypes in traces; only its concentration is affected by the activity of pigment genes.

The presence in the same plant of related flavonoid 3-glucosides, 3-diglucosides and 3-tri-glucosides suggests that the glucose residues are attached separately one at a time, rather than that preformed di- and tri-saccharides are attached directly in a single step to the pigment molecules. The isolation from wheat germ (Cardini & Yamaha, 1960) of two enzymes which catalyse the synthesis of phenolic mono- and di-glucosides respectively supports this hypothesis. Since the same disaccharide is produced when the anthocyanidin and flavonol 3-di- and 3-tri-glucosides are hydrolysed, it is possible that the same or similar enzymes control the attachment of glucose residues to both flavonols and anthocyanidins. It is generally considered that in most plants anthocyanins and flavones compete directly for a common precursor, so that increased visual pigmentation is normally correlated with decrease of flavone production and vice versa. Indeed, there is evidence that the genes *K* and *B* interact by competing for this common precursor. The Dazzler gene is different in that it increases anthocyanin concentration without affecting the concentration of other flavonoids present. The only other plant known to contain genes with a similar action to *Dz* is *Impatiens balsamina* (Hagen, 1959). The biochemical action of

Dz may be interpreted by assuming that, as Lawrence & Sturgess (1957) have suggested, pigment genes can stimulate increased production of a common precursor; alternatively, it is possible that there is an unlimited amount of precursor available but that the enzyme controlling anthocyanin formation is limiting.

SUMMARY

1. Fifteen anthocyanins derived from six anthocyanidins have been found in flowers or leaves of *Primula sinensis* colour genotypes. They are 3-glucosides, 3-glucosylglucosides or 3-glucosylglucosylglucosides.

2. Five flavonol glucosides have also been found. They are myricetin 3-glucoside and quercetin and kampferol 3-glucosylglucoside and 3-glucosylglucosylglucoside.

3. The only uncommon pigment present, sinensin, has been identified as dihydrokampferol 7-glucoside.

4. The qualitative and quantitative distribution of these pigments in genotypes of *P. sinensis* is described.

5. These results provide information about the genetic control of the hydroxylation, methylation and glucosidation of the flavonoid pigments. The biochemical effects of factors controlling co-pigmentation and anthocyanin concentration are discussed.

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