555. *The Flavonoid Glycosides of* Dahlia variabilis. *Part I. General Introduction. Cyanidin, Apigenin, and Luteolin Glycosides from the Variety "Dandy."*

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The sap-soluble pigments responsible for flower colour in *Dahlia variabilis* are reviewed. A method for identification of flavones and their glycosides on a micro-scale is described. When applied to the compounds extracted from the flowers of the blue dahlia "Dandy" the presence of apigenin, its 4′- and 7-monoglucosides and 7-rhamnoglucoside, of luteolin 5-monoglucoside and 7-diglucoside, of cyanidin arabinoglucoside, and of a few minor compounds has been demonstrated.

Introduction.—The flower colour of garden varieties of *Dahlia variabilis* has been shown to depend on the presence of varying proportions of sap-soluble pigments of flavonoid type. Those which have been isolated from dahlia flowers are apigenin (Schmidt and Waschkau, *Monatsh.*, 1928, **49**, 83), luteolin, diosmetin (Nakaoki, *J. Pharm. Soc. Japan*, 1938, **58**, 639), butein (Price, *J.*, 1939, 1017), 2′ : 4 : 4′-trihydroxychalkone (Bate-Smith and Swain, *J.*, 1953, 2185), and cyanin (Willstätter and Mallinson, *Annalen*, 1915, **408**, 147). Robinson and Robinson (*Nature*, 1931, **128**, 413) showed the presence of pelargonin; and the 3-monoglucosides of pelargonidin and cyanidin were shown to be present in certain genotypes by Lawrence and Scott-Moncrieff (*J. Genetics*, 1935, **30**, 155). On the basis of their own and earlier chemical work the last authors related the genetic and the chemical evidence and showed that pigment distribution in the species falls into two colour groups: although anthocyanins and anthoxanthins could be present separately, the presence of the "yellow flavone" (afterwards shown to be butein and 2′ : 4 : 4′-trihydroxychalkone) in orange or scarlet flowers was accompanied specifically by a pelargonidin derivative, while the "ivory flavone" (apigenin and luteolin) in magenta, purple, and chocolate varieties was always associated with a cyanidin derivative. The "ivory flavone" was not present in yellow flowers. Schmidt and his co-workers (*loc. cit.*; *Monatsh.*, 1932, **60**, 32; 1933, **62**, 123) stated that both ivory and yellow pigments are non-glycosidic and, although Price (*loc. cit.*) indicated the possibility that butein glycosides may occur in yellow varieties, the only evidence that flavone glycosides are present in dahlia was the isolation of a diosmetin 7-rhamnoglucoside by Nakaoki (*loc. cit.*). Examination of a large number of garden varieties of dahlia (Bate-Smith, Swain, and Nordström, unpublished) by paper chromatography, while supporting in the main the conclusion reached by Lawrence and Scott-Moncrieff with regard to the anthoxanthin aglycones, showed that the anthoxanthins were present mainly as glycosides. The separation and identification of these compounds will be described in the present series of papers.

Present Work.—Although the complex mixture of phenolic glycosides present in many petal and other plant extracts may readily be separated by paper chromatography (Bate-Smith, *Biochem. Symp.*, 1949, No. 3, 62) the individual compounds cannot usually be identified by R_F values and colour reactions alone (cf. Bradfield and Flood, *J.*, 1952, 4740) because there may be present a large number of glycosides related to a single aglycone (cf. Ice and Wender, *J. Amer. Chem. Soc.*, 1953, **75**, 50). Even direct measurement of ultraviolet absorption spectra on paper chromatograms (Bradfield and Flood, *loc. cit.*) does not permit differentiation between glycosides related to one aglycone (cf. Table 4). Attempts were made therefore to separate the mixture before examination of individual components. While this work was in progress Wender and his collaborators (references cited by Ice and Wender, *loc. cit.*) published a substantial contribution to the separation of flavonoid compounds present in various plant extracts, but, since they were concerned in the isolation of relatively large quantities of flavones for biological testing, the amount of original plant material used was very much greater than was available to us. Our preliminary experiments with partition columns gave poor results. We found, however, contrary to Gage and Wender (*Analyt. Chem.*, 1950, **22**, 708) and Bradfield and Flood (*loc. cit.*), that provided

the solvent is removed from developed paper chromatograms at room temperature, individual bands can be quantitatively eluted in 24—48 hr. with aqueous alcohol. The extracted bands may be then further purified if necessary by use of a second solvent. [When this work was completed Linstedt and Misiorny (*Svensk Papperstidning*, 1952, **55**, 602) and Williams and Wender (*J. Amer. Chem. Soc.*, 1952, **74**, 5919) described similar techniques for eluting flavonoid compounds although the elution time (2 hr.) used by the latter authors was possibly too short for complete extraction.] In this way it was possible in some cases to isolate sufficient material for both melting-point determination and analysis; but the nature of the compounds obtained has been established mainly by the methods indicated by Nordström *et al.* (*Chem. and Ind.*, 1953, 85) for identification of material in microgram quantities. Normally there are not more than two phenolic aglycones of any one type (*e.g.*, flavones, chalkones, etc.) represented as glycosides in the extracts from single plant varieties. These, whether occurring free or obtained from the glycosides on hydrolysis, can be identified with certainty by comparing their spectra in ethyl alcohol and 0·002M-sodium ethoxide (Mansfield, Swain, and Nordström, *Nature*, 1953, **172**, 23), R_F values in various solvents (Bate-Smith and Westall, *Biochim. Biophys. Acta*, 1950, **4**, 427; Gage, Douglass, and Wender, *Analyt. Chem.*, 1951, **23**, 1582) or on borate-impregnated filter paper (Wachmeister, *Acta Chem. Scand.*, 1951, **5**, 976; cf. Bate-Smith and Swain, *loc. cit.*), and their colour reactions (especially fluorescence) with synthetic standards. The sugars occurring in phenolic glycosides can be readily identified, after hydrolysis and removal of acid with a basic ion-exchange resin (cf. Piez, Tooper, and Fosdick, *J. Biol. Chem.*, 1952, **194**, 669), by R_F values and colour reactions (Partridge, *Biochem. J.*, 1948, **42**, 238; cf. Ice and Wender, *loc. cit.*).

The number of sugar groups attached to each aglycone in the purified glycosides can be tentatively deduced from the R_M value * (Bate-Smith and Westall, *loc. cit.*), but it can be determined more accurately after careful hydrolysis of each component (*ca.* 0·2 mg.) by estimation of the resulting aglycone spectrophotometrically and the sugar either by Somogyi's copper micro-method (*J. Biol. Chem.*, 1952, **195**, 19), or by the anthrone reagent (Morris, *Science*, 1948, **107**, 254), hydrolysis being unnecessary in the latter case. The anthrone method is especially useful for glycosides containing rhamnose since low concentrations of this sugar give erratic results with the Somogyi reagent. For diglycosides where the two sugars are different the concentration of each may be determined by either of the above methods after separation and elution from paper chromatograms. When our work was complete Ice and Wender (*loc. cit.*) described the determination of the sugar concentration in the hydrolysates of a quercetin diglucoside by the method of Fisher, Parsons, and Morrison (*Nature*, 1948, **161**, 764) but we had abandoned this technique since it gave unreliable results with our material.

The position of the carbohydrate moiety in the separated glycosides has been determined by methylation followed by hydrolysis, the partially methylated aglycone being identified by methods similar to those described above for the free flavones. In this connection spectra of the compounds in 0·002M-sodium ethoxide have been of great diagnostic value (Mansfield *et al.*, *loc. cit.*).

The spectral shifts and changes in intensity in bands I and II for 3′ : 4′ : 5- and 3′ : 5 : 7-tri-*O*-methyl-luteolin (Figure) are similar to those found for the corresponding apigenin derivatives. The corresponding 3′ : 4′ : 7-tri-*O*-methyl-luteolin behaves similarly.

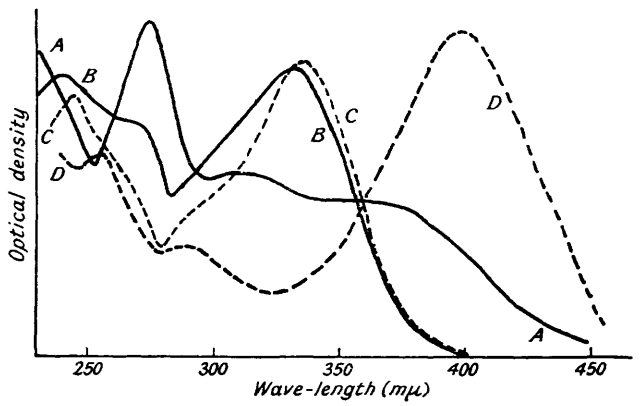
The order of monosaccharides in the case of biosides containing two different sugars was determined by separation and examination of the intermediate monoside resulting from partial hydrolysis (cf. Nordström *et al.*, *loc. cit.*). Application of all these methods of identification permits determination of the nature of the separated glycosides with only 1 mg. of each.

The ethanolic hydrochloric acid extracts of the petals of the purple-blue garden dahlia "Dandy," when examined in this way, showed the presence of six components after separation on pre-washed paper by butanol–acetic acid–water. Three of the components could be further separated in either butanol–water or *iso*propanol–2N-hydrochloric acid

* $R_M = \log [(1/R_F) - 1]$. It varies directly with the number of functional groups.

(Table 1). The ethyl acetate-soluble portion of the hydrolysed extract was separated into two flavones. The flavone present in larger amount had the same colour reactions and R_F values (Table 2) as apigenin (I; R = R′ = H), and was isolated on a larger scale by paper chromatography and identified by analysis and its mixed melting point. The flavone present in minor amount was not isolated in sufficient quantity for a melting-point determination but from its R_F values and colour reactions (Table 2) was identified as luteolin (II; R = R′ = H). The presence of both compounds in one variety of dahlia had been shown previously by Nakaoki (*loc. cit.*), but unlike the Japanese worker we have not yet found even a trace of diosmetin or its derivatives in any dahlia we have examined.

Thus the main flavonoid components in the unhydrolysed extract (Table 1) were expected to be related to apigenin and luteolin, and this has been borne out by the results. Band I was shown to be apigenin itself (Tables 2 and 4), which was isolated on a larger scale and identified. Its concentration is higher in the extract examined than in extracts from fresh petals, presumably owing to hydrolysis during storage (6 months at 0°).



A, 3′ : 4′ : 5-*Tri*-O-*methyl-luteolin in* 0·002M-*sodium ethoxide.*
B, 3′ : 4′ : 5-*Tri*-O-*methyl-luteolin in ethanol.*
C, 3′ : 5 : 7-*Tri*-O-*methyl-luteolin in ethanol.*
D, 3′ : 5 : 7-*Tri*-O-*methyl-luteolin in* 0·002M-*sodium ethoxide.*

Band 2 was shown to be a glycoside since on treatment with acid it showed the presence of luteolin (Tables 2 and 4) and glucose (Table 3), and its R_F value in ethyl acetate–water was low (Table 2) (cf. Swain, *Biochem. J.*, 1953, **53**, 200). From its R_M value it was obviously a monoside and this was proved by quantitative analysis (Table 5). On

TABLE 1. *Separation, identification, and relative concentration of the flavonoid glycosides.*

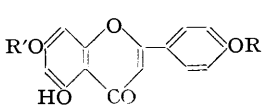
Band no.	R_F in: BuOH–AcOH–H_2O (6 : 1 : 2)	BuOH–H_2O	Pr^{i}OH–2N-HCl (1 : 1)	Identified as	Relative concn.
1	0·89	0·88	—	Apigenin	26
2	0·75	0·60	—	Luteolin 5-glucoside	8
3·1	0·62	0·59	—	Apigenin 4′- and 7-glucoside	40
3·2		0·12	—	Apigenin 4′- and 7-glucoside & naringenin glucoside	12
4	0·49	0·27	—	Apigenin 7-rhamnoglucoside	100 [a]
5·1	0·23	0·09	—	Unknown diglucoside	—
5·2		0·05		Luteolin 7-diglucoside	27
6·1	0·18	—	0·34	Pelargonidin glycoside	—
6·2			0·24	Cyanidin 3 : 5(?)-arabinoglucoside	8 [b]

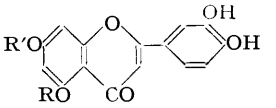
[a] Actual concn. 1·3 mg. per ml. of extract. [b] As cyanin.

methylation with methyl sulphate and potassium carbonate in acetone and subsequent hydrolysis a compound was obtained which, since it gave a negative reaction with ferric chloride, was presumably tetra-*O*-methyl-luteolin, and it was obvious that hydrolysis of the glucoside followed by methylation of the free hydroxyl group had occurred. Since glycosides in which the sugar is attached to the 4′- or the 7-hydroxyl group in flavones are not affected under these conditions (see below) it appeared probable that band 2 was due

to a 5-glucoside. This was proved by methylation with diazomethane to a product giving a negative ferric chloride reaction (unlike the luteolin 7-diglucoside, band 5·2, see below). On hydrolysis with acid, the sugar-free methyl ether gave a dark brown colour with ferric chloride, thus showing the presence of a free 5-hydroxyl group. The spectrum of the product was similar to those of 3′ : 4′ : 7-tri-*O*-methyl-luteolin, but examination on a paper chromatogram showed that tetra-*O*-methyl-luteolin was also present (Tables 6 and 7). However the spectrum of band 2 itself in sodium ethoxide solution (Table 4) indicates the presence of both a free 7- and a free 4′-hydroxyl group (cf. 3′ : 4′ : 5- and 3′ : 5 : 7-tri-*O*-methyl-luteolin, Table 7) and taken in conjunction with the positive ferric reaction shows that this band is due to luteolin 5-glucoside (II; R = glucosyl; R′ = H).

Luteolin 5-glucoside (galuteolin) has been isolated previously from *Galega officinalis* (Barger and White, *Biochem. J.*, 1923, **17**, 836) and *Equisetum arvense* (Nakamura and Hukuti, *J. Pharm. Soc. Japan*, 1940, **60**, 449).

(I) 



(II)

Band 3 was due to a mixture, which gave components 3·1 and 3·2 by separation in butanol–water (Table 1). The faster-running component (3·1) gave apigenin and glucose only on hydrolysis (Tables 2 and 3), and quantitative examination showed it to be an apigenin monoglucoside (Table 5). On methylation and hydrolysis however it gave a product which was separable in butanol–water on borate- or phosphate-impregnated filter paper into two aglycones having R_F values and colour reactions identical with those of 4′ : 5- and 5 : 7-di-*O*-methylapigenin (Table 6). The easy separation of these two ethers on alkaline-buffered paper is presumably due to a difference in acidity, the 4′ : 5-di-*O*-methyl ether being, as expected, the more acidic (Table 6). Similar differences in acidity of 4′- and 7-hydroxy-groups have been disclosed by partial methylation of 4′ : 7-dihydroxyflavone (Beton and Simpson, unpublished results; cf. Narasimhachari and Seshadri, *Proc. Indian Acad. Sci.*, 1950, **32**, *A*, 256). The spectra of the methylated product in 0·002M-sodium ethoxide (Table 7) also indicated the presence of these two compounds. Thus, although the original monoglucoside component (3·1) could not be separated in any solvent system tried, it is obviously a mixture of apigenin 7- (I; R = H, R′ = glucosyl) and 4′-glucoside (I; R = glucosyl, R′ = H). Apigenin 7-glucoside (cosmetin) has been isolated from *Cosmos bipinatus* (Nakaoki, *J. Pham. Soc. Japan*, 1935, **55**, 173); when obtained by partial hydrolysis of apiin (cf. Nordström *et al.*, *loc. cit.*) it had the same R_F values (Table 2) as the mixture (3·1) in all solvents tried. Apigenin 4′-glucoside has not been reported previously; indeed except for the two genistein 4′-glycosides isolated from *Sophora japonica* (Bognàr, *Magyar Kem. hapja*, 1949, **4**, 519; *Chem. Abs.*, 1952, **46**, 8104) no other flavonoid 4′-glycoside has been reported.

The second component causing band 3 (3·2) was also a mixture, since it yielded apigenin and small quantities of naringenin (Table 2), together with glucose (Table 3), on hydrolysis. The spectra of the mixture of aglycones (Table 4) produced also indicated the presence of both compounds. Methylation and subsequent hydrolysis of 3·2 yielded a product, the R_F values and colour reactions of which indicated the presence of both 4′ : 5- and 5 : 7-di-*O*-methylapigenin (Table 6). The $\lambda_{max.}$ in the spectra of the product confirmed this although owing to the low concentration and presence of impurities the relative intensities could not be determined accurately. The apigenin monoglucosides present in band (3·2) therefore are the same as those in band (3·1) and the separation of these bands may be due to the formation of a molecular complex between the apigenin components of (3·2) and the naringenin derivative present. Attempts to separate further either band (3·1) or (3·2) met with no success.

Band 4, as can be seen from Table 1, is the major compound in the extract. It was shown to be a rhamnoglucoside of apigenin (Tables 2, 3, and 4) and quantitative examination gave an apigenin–sugar ratio of 1 : 2·2 (Table 5). Methylation followed by hydrolysis

gave a product identical with 4′ : 5-di-*O*-methylapigenin (Tables 6 and 7). On partial hydrolysis apigenin 7-glucoside was obtained (Tables 2 and 3), and band 4 is therefore apigenin 7-rhamnosylglucoside (I; R = H, R′ = rhamnosylglucosyl). A compound of this structure has been isolated from *Rhus succedanea* (Hattori and Matsuda, *Arch. Biochim. Biophys.*, 1952, **37**, 85) and assumed to be a rutinoside. Sufficient material was isolated from band 4 for melting-point determination, recrystallisation and analysis, and was identical with rhoifolin kindly supplied by Professor Hattori. The R_F values, colour reactions, and spectra of rhoifolin were the same as for band 4 (Tables 2 and 4).

Band 5 was shown to contain two components separable in butanol–water. The major component (5·2) was a luteolin diglucoside (Tables 2—5), and methylation followed by hydrolysis showed that the sugar was attached to the 7-position. Since it was found that band 2 (luteolin 5-glucoside) was hydrolysed during methylation with methyl sulphate, the position of the sugar in the diglucoside (5·2) was confirmed by methylation with diazomethane. In this case the product obtained gave a brown colour with ferric chloride solution before hydrolysis, showing that the 5-hydroxyl group was free. Band 5·2 is therefore luteolin 7-diglucoside (II; R = H, R′ = glucosylglucosyl). No compound of this structure has been so far reported in Nature. The second component (5·1) gave glucose and a mixture of two aglycones on hydrolysis, neither of which has been so far identified. Band (5·1) was in too low a concentration for further work to be carried out but from its position it was presumably a mixture of diglucosides.

The anthocyanin band (6) was separated into two compounds with *iso*propanol–2N-hydrochloric acid (Table 1). The major component on hydrolysis gave cyanidin (identified by its R_F value in butanol–2N-hydrochloric acid and distribution tests), arabinose, and glucose (Table 3). It is probably a 3 : 5-diglycoside but owing to fading of the aglycone at low concentrations a quantitative determination was not possible. An arabinoglucoside of cyanidin has been reported in the beans of *Forastero cacao* (Forsyth, *Biochem. J.*, 1952, **51**, 511). The second anthocyanin was present in too low concentration for complete identification, but on hydrolysis gave an aglycone indistinguishable from pelargonidin.

The reference compounds required for this investigation were prepared by oxidative cyclisation from the corresponding chalkones.

Experimental

M. p.s are corrected.

Separation of the Flavonoid Compounds from "Dandy."—Petals (88·5 g.) of "Dandy" (a blue garden variety of *Dahlia variabilis*) were crushed under 0·01N-ethanolic hydrochloric acid (400 ml.), kept at 0° for 1 week, and filtered (total solids, 14·5 mg./ml.; total sugar, 6 mg./ml.). The extract (10 ml.) was applied as a streak from a teat pipette in 0·5—1-ml. portions to Whatman's No. 3 paper 22 × 18", which had been previously washed with water for 24 hr. in a chromatographic cabinet and dried at room temperature. The chromatogram was developed overnight with butanol–acetic acid–water (6 : 1 : 2) (this approximates to the organic phase of the conventional 4 : 1 : 5 solvent) and carefully dried in a current of air in a fume cupboard. The positions of the bands were marked under an ultra-violet lamp. The separated components were eluted from the paper in a conventional chromatographic tank with either cold aqueous ethanol (40—70%, more aqueous solvents being used for components having low R_F values) for the flavones, or cold aqueous dilute hydrochloric acid for the anthocyanins. This process took 1—3 days. Each of the resulting solutions was concentrated to small volume *in vacuo* and purified by re-running in the same solvent system and elution as before. The purified bands were then tested for homogeneity on Whatman's No. 1 paper in other solvents and, when mixtures, separated in a similar way to that used for the original extract. Compounds having R_F values less than 0·4 in butanol–acetic acid–water (6 : 1 : 2), except the anthocyanins, were separated from free sugars on Whatman's No. 3 paper by acetone–water (1 : 3) in which the flavones have much lower R_F values (0—0·1) than those of the sugars (0·8—0·9). The anthocyanin band was freed from the faster-running sugars (R_F 0·7—0·8) during separation by *iso*propanol–2N-hydrochloric acid. The results of the primary separation are given in Table 1.

Examination of the Separated Flavone Glycosides.—The R_F values in several solvents and

TABLE 2. *R_F values and colour reactions of the glycosides and corresponding aglycones.*

Compound [a]	R_F in: BuOH–AcOH–H_2O (6 : 1 : 2)	BuOH–H_2O Plain paper	BuOH–H_2O Borate paper [b]	BuOH–H_2O Phosphate paper [c]	EtOAc–H_2O Plain paper	EtOAc–H_2O Borate paper [b]	EtOAc–H_2O Phosphate paper [c]	$COMe_2$–H_2O (1 : 1)	Colour reactions: u.-v. light	Colour reactions: u.-v. light + NH_3 vapour
Aglycone 1 from hydrolysed extract	0·89	0·88	0·46	0·84	0·88	—	—	0·59	Dark ochre	Bright green
Aglycone 2 from hydrolysed extract	0·82	0·73	0·02	0·69	0·86	—	—	0·31	,,	Green-yellow
Band 1	0·89	0·88	0·46	0·84	0·88	0·75	0·87	0·59	,,	Bright green
,, 2	0·75	0·60	0·02	0·26	0	—	—	0·42	,,	Green-yellow
,, 3·1	0·62	0·42	0·15	0·32	0·12	—	—	0·59	,,	Bright green
,, 3·2	0·62	0·19	0·04	0·04	0·12	—	—	0·59	,,	,,
,, 4	0·40	0·27	0·04	0·17	0·01	—	—	0·69	,,	,,
,, 5·1	0·23	0·09	0·06	0·07	0	—	—	0·53	Light ochre	,,
,, 5·2	0·23	0·05	0·02	0·04	0	—	—	0·38	Dark ochre	Green-yellow
Partially hydrolysed band 4	0·59	0·42	—	—	—	—	—	0·49	,,	Bright green
Partially hydrolysed band 5·2	0·39	0·21	—	—	—	—	—	0·45	,,	Green-yellow
Aglycone from band 2	0·82	0·73	0·02	0·69	0·86	0	0·80	0·31	,,	,,
,, ,, 3·1	0·89	0·86	0·46	0·84	0·91	0·75	0·87	0·59	,,	Bright green
,, ,, 3·2	0·89	0·89	0·46	0·84	0·91	0·75	0·87	0·59	,,	,,
,, ,, 3·2	0·95	0·89	0·88	—	—	—	—	0·79	Light ochre	Green
,, ,, 4	0·89	0·87	0·46	0·84	0·90	0·75	0·87	0·61	Dark ochre	Bright green
,, ,, 5·1	0·92	0·91	0·94	0·87	0·92	0·96	0·90	0·71	Green	Green
,, ,, 5·1	0·79	0·74	0·44	—	—	—	—	0·89	Light green	,,
,, ,, 5·2	0·82	0·73	0·02	0·69	0·86	0	0·80	0·59	Dark ochre	Green-yellow
Rhoifolin	0·40	0·23	0·04	0·17	—	—	—	0·64	,,	Bright green
Apigenin 7-glucoside [d]	0·59	0·42	0·15	0·32	—	—	—	0·49	,,	,,
Luteolin 7-glucoside [d]	0·39	0·21	—	—	—	—	—	0·42	,,	Green-yellow
Apigenin	0·89	0·88	0·46	0·84	0·90	0·75	0·87	0·59	,,	Bright green
Luteolin	0·82	0·73	0·02	0·69	0·86	0	0·80	0·31	,,	Green-yellow
Naringenin	0·93	0·89	0·88	0·87	0·93	0·91	0·91	0·79	Light ochre	Green

[a] See Table 1. [b] R_F dependent to some extent on amount of material applied. [c] Papers must be run as soon as possible after preparation. [d] Prepared from partially hydrolysed "crude apiin" (Nordström *et al., loc. cit.*).

colour reactions of the glycosides were determined on Whatman's No. 1 paper. The results are given in Table 2.

The ultra-violet absorption spectra of the glycosides in ethyl alcohol and 0·002M-sodium ethoxide solution were determined as described by Mansfield *et al.* (*loc. cit.*). The reference solvent was prepared separately for each glycoside by eluting a strip of the same R_F value from a blank chromatogram and treating it as in the purification of the glycoside itself. The results are shown in Table 4.

Identification of the Flavone Glycosides.—(a) *Qualitative hydrolysis.* The glycoside (*ca.* 100 μg.) in alcohol (1 ml.) and 2N-sulphuric acid (1 ml.) was heated at 100° for 1 hr., then cooled,

TABLE 3. *Identification of the sugars produced by hydrolysis of " Dandy " glycosides.*

	R_F values in :			
Sugar from band [a]	BuOH–AcOH–H_2O (6 : 1 : 2)	PhOH–H_2O	EtOAc–pyridine–H_2O [b] (2 : 1 : 2)	Colour with *p*-anisidine hydrochloride [c]
2	0·15	0·38	0·28	Brown
3·1	0·15	0·38	0·31	Brown
3·2	0·15	0·38	0·31	Brown
4	0·15	0·38	0·28	Brown
	0·34	0·64	0·54	Light brown
5·1	0·15	0·37	0·28	Brown
5·2	0·15	0·37	0·28	Brown
6·2	0·15	0·38	0·28	Brown
	0·19	0·54	0·32	Red
Partially hydrolysed band 4	0·15	0·38	0·28	Brown
Glucose	0·15	0·38	0·28	Light brown
Rhamnose	0·34	0·64	0·54	Brown
Arabinose	0·19	0·54	0·32	Red

[a] See Table 1. [b] Jermyn and Isherwood, *Biochem. J.*, 1949, **44**, 402. [c] Hough, Jones, and Wadman, *J.*, 1950, 1702.

TABLE 4. $\lambda_{max.}$, $\Delta\lambda$, and $\Delta D(\%)$ *of the ultra-violet absorption spectra in ethyl alcohol and* 0·002M-*sodium ethoxide of the glycosides and corresponding aglycones.*

	Band I			Band II		
Compound [a]	$\lambda_{max.}$ (EtOH)	$\Delta\lambda$ [b]	ΔD (%) [c]	$\lambda_{max.}$ (EtOH)	$\Delta\lambda$ [b]	ΔD (%) [c]
Band 1	335	63	44	269	10	17
,, 2	349	51	−26	265	5	45
,, 3·1	334	57	18	270	5	24
,, 3·2	340	56	1	270	6	6
,, 4	335	63	98	268	0	−21
,, 5·1	335	65	43	268	4	24
,, 5·2	350	52	12	259	8	18
Aglycone from band 2	350	54	19	258	12	29
,, ,, 3·1	335	63	51	270	7	29
,, ,, 3·2	335	63	43	270	7	17
,, ,, 3·2	—	—	—	290	40	12
,, ,, 4	335	64	83	269	10	18
,, ,, 5·1	325	65	−32	275	55	− 1
,, ,, 5·2	350	54	34	258	10	20
Rhoifolin	335	60	72	270	0	− 6
Apigenin 7-glucoside	335	63	66	268	1	−20
Apigenin	335	63	67	269	10	32
Luteolin	351	52	39	256	13	14
Naringenin	—	—	—	286	43	35

[a] See Table 1. [b] $\Delta\lambda$ is the difference in $\lambda_{max.}$ of the band in ethyl alcohol and 0·002M-sodium ethoxide. [c] ΔD (%) is the difference (%) in the optical density at the peak in alcohol and 0·002M-sodium ethoxide.

and the aglycone was extracted with ethyl acetate. The extract was washed with water and after removal of the solvent the flavone dissolved in alcohol (*ca.* 0·5 ml.). Each aglycone was identified by comparing its R_F values in various solvents, colour reactions, and ultra-violet absorption spectra with synthetic standards. The reference solvent for the spectra of the

aglycones was prepared by treating the solutions used for the glycosides under the same conditions of hydrolysis. The results are shown in Tables 2 and 4.

The aqueous phase and the washings from the hydrolysis were combined, and the acid was removed with the bicarbonate form of the basic ion-exchange resin " Dowex 2 " (prepared according to Piez *et al.*, *loc. cit.*). The resin was removed by centrifuging and washed with water, and the aqueous liquors were concentrated to *ca.* 0·5 ml. for identification of the sugars. The results are shown in Table 3.

(b) *Quantitative hydrolysis.* (i) Determination of aglycone concentration. The glycoside (*ca.* 100—300 μg.) in alcohol (2 ml.) and 2N-sulphuric acid (2 ml.) was heated for 2 hr. at 100° under reflux, cooled, and diluted to a known volume (depending on concentration) with alcohol. The concentration of the aglycone was determined spectrophotometrically, standards being previously determined in the same acid medium.

(ii) Determination of sugar concentration. A second portion of the glycoside solution used for the above determination (volume depending on sugar concentration) was taken to dryness in a $\frac{3}{4}$" standard colorimeter tube (Unicam D.G. spectrophotometer), and anthrone solution (10 ml.; 0·2% in 95% sulphuric acid) followed by water (5 ml.) added. The contents were mixed and the absorbance measured at 620 mμ after 15 min. A control experiment with the corresponding sugar was carried out simultaneously, the sugar solution being in this case added directly to the reagent. This method of addition (water or sugar solution to reagent) gave more reproducible results than the conventional reverse procedure (Morris, *loc. cit.*). The results are shown in Table 5.

TABLE 5. *Aglycone : sugar ratio for the glycosides.*

Band [a]	Concn. (mg./ml.) Aglycone [b]	Glucose	Aglycone : sugar [b]	Band [a]	Concn. (mg./ml.) Aglycone [b]	Glucose	Aglycone : sugar [b]
2	0·080	0·058	1 : 1·14	4	0·455	0·334 [d]	1 : 1·10 : 1·10
	0·095	0·066	1 : 1·12		0·455	0·340 [e]	1 : 1·12 : 1·12
3·1	0·340	0·268	1 : 1·18	5·2	0·140	0·198	1 : 2·24
	0·360	0·276	1 : 1·14		0·130	0·182	1 : 2·22
3·2	0·077 A, 0·022 N	0·063	1 : 0·96 A [c]				
	0·106 A, 0·013 N	0·077	1 : 0·98 A [c]				

[a] See Table 1. [b] A = Apigenin; N = naringenin. [c] Based on naringenin : glucose = 1 : 1. [d] Also rhamnose 0·306 mg./ml. [e] Also rhamnose 0·310 mg./ml.

Determination of the Position of Sugar Substituent.—(*a*) The glycoside (*ca.* 500 μg.), anhydrous potassium carbonate (0·1 g.), and freshly distilled methyl sulphate (0·1 ml.) were refluxed for 6 hr. in dry acetone (2 ml.). The solvent was removed under reduced pressure and the product heated with 2N-sulphuric acid (3 ml.) for 2 hr. at 100°. On cooling, the partially methylated aglycone was extracted with chloroform, the extract washed with water, and after removal of the solvent the compound was dissolved in alcohol. The partial methyl ethers were then identified by comparing their R_F values, colour reactions, and ultra-violet absorption spectra with those of synthetic standards. As in the previous case, the reference solutions were prepared by treating the eluates from the blank chromatograms as described above. The results are shown in Tables 6 and 7.

(*b*) Methylation of bands 2 and 5·2 with diazomethane was carried out in the normal manner.

Identification of the Anthocyanidin Glycosides.—Qualitative hydrolysis of the main anthocyanidin component (band 6·2, Table 1) was carried out in a manner similar to that described for the flavones. The aglycone was extracted with amyl alcohol. By distribution tests (Robinson and Robinson, *Biochem. J.*, 1931, **25**, 1693) and its R_F value (0·70 in butanol–2N-hydrochloric acid; Bate-Smith, *loc. cit.*), it was shown to be cyanidin (R_F 0·70). The aqueous solution, after removal of acid, showed the presence of arabinose and glucose (Table 3).

Attempts to estimate the cyanidin resulting from hydrolysis were abandoned since the colour faded at the concentration used. It was not possible therefore to determine the sugar : aglycone ratio.

The second anthocyanin band (6·1; Table 1) on hydrolysis and extraction with amyl alcohol as above gave an aglycone having the same R_F value (0·81 in butanol–2N-hydrochloric acid) and colour reactions as pelargonidin. The sugars were in too low concentration to be identified.

Isolation of Apigenin from " Dandy."—(*a*) The original extract (100 ml.) was heated with an equal volume of 2N-hydrochloric acid for 2 hr. at 100°. The cooled solution was extracted

with ethyl acetate (3 × 200 ml.), and the extract washed with water, taken to small volume, and separated on Whatman's No. 3 paper in butanol–acetic acid–water (6 : 1 : 2) as described above. The major component (R_F 0·89) was eluted with 70% alcohol, and most of the alcohol

TABLE 6. *R_F values and colour reactions of the partial methyl ethers obtained from the glycosides.*

Partial methyl ether from band [a]	R_F in : C_6H_6–$MeNO_2$–H_2O [b] (3 : 2 : 5)	R_F in : BuOH–H_2O Borate paper	R_F in : BuOH–H_2O Phosphate paper	Colour u.-v. light	Colour u.-v. light + NH_3 vapour
2 [c]	0·95 (0·91)	0·92 (0·80)	0·77 (0·55)	Brown (blue)	Brown (blue)
3·1	0·46	0·80	0·70	Blue	Green-blue
	0·46	0·51	0·62	V. pale blue	Yellow
3·2	0·46	0·80	0·70	Blue	Green-blue
	0·46	0·51	0·62	V. pale blue	Yellow
4	0·46	0·51	0·62	V. pale blue	Yellow
5·2	0·35	0·26	0·34	Pale lilac	Orange-yellow
4′ : 5-Di-*O*-methylapigenin	0·46	0·51	0·62	V. pale blue	Yellow
4′ : 7- ,, [c]	0·97	0·93	0·84	Brown	Brown
5 : 7- ,, ...	0·46	0·80	0·70	Blue	Green-blue
Tri-*O*-methylapigenin	0·91	0·87	0·79	Blue	Blue
3′ : 4′ : 5-Tri-*O*-methyl-luteolin	0·35	0·26	0·34	Pale lilac	Orange-yellow
3′ : 4′ : 7- ,, [c]	0·95	0·92	0·77	Brown	Brown
3′ : 5 : 7- ,, ...	0·72	0·67	0·55	V. pale blue	Yellow-green
4′ : 5 : 7- ,, ...	0·73	0·77	0·55	Dark blue	Red-purple
Tetra-*O*-methyl-luteolin	0·91	0·80	0·55	Blue	Blue

[a] See Table 1. [b] Simpson and Garden, *J.*, 1952, 4638. [c] Gave a positive ferric chloride reaction.

TABLE 7. *$\lambda_{max.}$, $\Delta\lambda$, and $\Delta D(\%)$ of the ultra-violet absorption spectra in ethyl alcohol and 0·002M-sodium ethoxide of the partial methyl ethers from the glycosides.*

Partial methyl ether from band [a]	Band I $\lambda_{max.}$ (EtOH)	Band I $\Delta\lambda$ [b]	Band I ΔD (%) [c]	Band II $\lambda_{max.}$ (EtOH)	Band II $\Delta\lambda$ [b]	Band II ΔD (%) [c]
2	335	—	—	270	18	1
3·1	327	53	5	262	7	29
3·2	325	50	4	260	8	40
4	325	38	−32	264	10	63
5·2	334	26	−43	242	33	15
4′ : 5-Di-*O*-methylapigenin	325	35	−32	260	12	70
4′ : 7- ,, ...	325	—	—	271	21	28
5 : 7- ,, ...	327	53	48	259	3	−10
Tri-*O*-methylapigenin	322	0	0	265	0	0
3′ : 4′ : 5-Tri-*O*-methyl-luteolin	333	27	−35	240	35	46
3′ : 4′ : 7- ,,	338	—	—	272	16	1
3′ : 5 : 7- ,,	335	65	35	245	10	−4
4′ : 5 : 7- ,,	334	46	−48	245	14	37
Tetra-*O*-methyl-luteolin	330	0	0	240	0	0

[a] See Table 1. [b, c] As for Table 4.

removed, yielding a dark brown solid. Recrystallisation of this from aqueous alcohol gave pale yellow crystals (27·5 mg.), m. p. 340°, which did not depress the melting point of synthetic apigenin (Found : C, 65·9, 66·4; H, 5·9, 6·2. Calc. for $C_{15}H_{10}O_5$: C, 66·7; H, 3·7%). The R_F values and colour reactions of the product from " Dandy " were identical to those of synthetic apigenin (Table 2).

(*b*) Removal of the alcohol from the eluate of band 1 (Table 1) from 40 ml. of original extract gave a precipitate which after recrystallisation from aqueous alcohol gave pale yellow crystals (2·4 mg.), m. p. 340° alone or admixed with synthetic apigenin.

Isolation of Rhoifolin from " Dandy ".—The material is band 4 (Table 1) from 30 ml. of original extract was purified as described above, yielding a brown amorphous solid, m. p. 263—265° (decomp.; softens at 200—205°) alone or mixed with rhoifolin supplied by Professor Hattori (Found : C, 51·9, 51·6; H, 5·7, 5·7. Calc. for $C_{27}H_{30}O_{14}.3H_2O$: C, 51·3; H, 5·7%)

(Hattori and Matsuda, *loc. cit.*, state that rhoifolin forms a trihydrate which softens at 200—203° and melts at 265° with decomp.).

Partial Hydrolysis Material of Bands 4 and 5·2 from "Dandy."—The glycoside (*ca.* 200 μg.) in alcohol (1 ml.) was hydrolysed with 1% hydrochloric acid (1 ml.) at 100° for 10 min. The mixture was cooled, applied as a streak on Whatman's No. 3 paper, and separated in the normal manner. After elution, the intermediate monoside was identified in the usual way (Tables 2 and 3).

Determination of the Relative Concentration of the Different Components.—The original extract (2 ml.) was separated as described and the individual flavones were hydrolysed with 2N-hydrochloric acid (2 ml.) for 1 hr. and made up with alcohol to 50 ml. The aglycone concentration in each component was determined spectrophotometrically. The anthocyanin in band 6 was estimated after elution as cyanin.

Preparation of Reference Compounds.—All the flavones required for this investigation, except those described below, were prepared by recorded methods.

4′-Hydroxy-3′ : 5 : 7-trimethoxyflavone. Condensation of 2 : 4-di-*O*-methylphloracetophenone (2·9 g.) with 4-*O*-benzylvanillin (3·6 g.) in ethanol (40 ml.) in the presence of sodium hydroxide (5 g. in water, 5 ml.) overnight yielded *4-benzyloxy-2′-hydroxy-3 : 4′ : 6′-trimethoxychalkone* (2·9 g.). This recrystallised from alcohol in bright yellow plates, m. p. 134·5—136°, giving a brown colour with ferric chloride in alcohol (Found : C, 71·7; H, 5·7. $C_{25}H_{24}O_6$ requires C, 71·4; H, 5·8%). The chalkone (2·5 g.) was dehydrogenated with selenium dioxide (2·5 g.) in boiling amyl alcohol (40 ml.) during 18 hr. and after filtration the solvent was removed in steam. The resultant dark solid (1·1 g.) was recrystallised from aqueous acetic acid, yielding cream-coloured needles of *4′-benzyloxy-3′ : 5 : 7-trimethoxyflavone*, m. p. 208—209° (Found : C, 68·6, 68·6 H, 5·1, 5·5. $C_{25}H_{22}O_6,2H_2O$ requires C, 68·8; H, 5·5%). The benzyloxyflavone (0·9 g.) was hydrolysed with acetic acid (7 ml.) and concentrated hydrochloric acid (5 ml.) for 1 hr. at 100°. After steam-distillation and recrystallisation from alcohol, *4′-hydroxy-3′ : 5 : 7-trimethoxyflavone* was obtained as pale yellow needles, m. p. 223—224° (0·6 g.) (Found : C, 62·0;H, 5·4. $C_{18}H_{16}O_6,H_2O$ requires C, 61·9; H, 5·8%).

3′-Hydroxy-4′ : 5 : 7-trimethoxyflavone.—A solution of 2 : 4-di-*O*-methylphloracetophenone (2·2 g.), 3′-*O*-benzyl*iso*vanillin (2·7 g.) in alcohol (30 ml.) was treated with sodium hydroxide (4 g. in water, 4 ml.) overnight, yielding *3-benzyloxy-2′-hydroxy-4 : 4′ : 6-trimethoxychalkone* (2·6 g.), which gave lemon-yellow needles (from alcohol), m. p. 106—107° (Found : C, 70·9; H, 6·1. $C_{25}H_{24}O_6$ requires C, 71·4; H, 5·8%).

This compound (1·8 g.) was dehydrogenated as before, yielding *3′-benzyloxy-4′ : 5 : 7-trimethoxyflavone*, cream-coloured needles (from alcohol), m. p. 190—191° (Found : C, 71·2; H, 5·4. $C_{25}H_{22}O_6$ requires C, 71·8; H, 5·3%). This material (0·9 g.) was debenzylated with acetic acid and concentrated hydrochloric acid as before and the product on recrystallisation from alcohol gave *3′-hydroxy-4′ : 5 : 7-trimethoxyflavone* as buff-coloured needles, m. p. 230—231° (Found : C, 65·0; H, 5·1. $C_{18}H_{16}O_6$ requires C, 65·5; H, 5·5%).

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