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PLANT POLYPHENOLS—XIV.
CHARACTERIZATION OF FLAVONOID GLYCOSIDES BY ACIDIC AND ENZYMIC HYDROLYSES*

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Abstract—The rates of hydrolysis of more than a hundred flavonoid glycosides have been measured, using N HCl, β-glucosidase, β-glucuronidase or anthocyanase. The results show that such measurements are useful (*a*) in distinguishing *O*- from *C*-glycosides, (*b*) in characterizing flavonoids having sugars attached to more than one hydroxyl group, and (*c*) for determining suitable conditions for the isolation of intermediate glycosides. Flavonols with glucuronic acid or glucose attached to the 7-hydroxyl are readily distinguished from those having rhamnose attached by their resistance to acid hydrolysis; times for complete hydrolysis are 180, 25 and 5 min respectively. β-Glucosidase attacks the 3-, 7- and 4′-*O*-glucosides of quercetin at the same rate, but preferentially removes glucose from the 7-hydroxyl group of quercetin 7-glucoside-3-sophoroside. Anthocyanase hydrolyses anthocyanidin 3-galactosides, 3-glucosides and 3-(diglycosides) much more rapidly than 3-rhamnosides, 3-arabinosides and acylated glycosides.

These procedures have been applied, in combination with other methods, to the identification of flavonol glycosides present in species of *Allium*, *Helleborus*, *Lathyrus*, *Matthiola*, *Potentilla* and *Tulipa*. New glycosides now characterized are: the 7-glucuronide-3-rutinosides of kaempferol and quercetin, the 7-rhamnoside-3-lathyroside and 7-rhamnoside-3-(rhamnosylarabinoside) of kaempferol, and the 7-glucoside-3-(xylosyl-glucoside), the 3,4′-diglucoside, the 3,7-diglucuronide and the 7-glucoside-3-(caffeoylsophoroside) of quercetin.

INTRODUCTION

ALTHOUGH it is accepted that flavonoid aglycones such as quercetin and cyanidin are almost universally distributed among higher plants,[1] it has only recently become apparent that some of their glycosidic forms are very restricted in distribution and thus of potential phytochemical interest.[2,3] This is particularly true of flavonoids having more than two monosaccharide units; of the eighteen known classes of anthocyanidin glycoside, no fewer than seven have three sugar units.[2,4] Similarly, of the forty flavonol glycosidic types, fourteen have three sugars;[3] and a flavonol with three glucoses and a rhamnose is known in *Solanum* seed [5] and one with five sugars has been partly characterized from *Equisetum* leaf.[6] While these are all *O*-glycosides, a series of flavonoid *C*-glycosides have also been found to occur widely in plants.[7,8] The best-characterized *C*-glycosides are all flavone derivatives (i.e. are glycoflavones) but flavanone and isoflavone *C*-glycosides have also been described.[9,10] These

* Part XIII. J. B. HARBORNE and E. HALL, *Phytochem.* **3**, 453 (1964).

[1] T. SWAIN and E. C. BATE-SMITH, in *Comparative Biochemistry* (Edited by M. FLORKIN and H. S. MASON), Vol. IIIA, p. 755, Academic Press, New York (1962).
[2] J. B. HARBORNE, in *Chemical Plant Taxonomy* (Edited by T. SWAIN), p. 359, Academic Press, London (1963).
[3] J. B. HARBORNE, *Biochemistry of Phenolic Compounds*, p. 129, Academic Press, London (1964).
[4] J. B. HARBORNE, *Phytochem.* **2**, 85 (1963).
[5] J. B. HARBORNE, *Biochem. J.* **84**, 100 (1962).
[6] S. BECKMANN and H. GEIGER, *Phytochem.* **2**, 281 (1963).
[7] L. HÖRHAMMER and H. WAGNER, in *Chemistry of Natural Phenolic Compounds* (Edited by W. D. OLLIS), p. 185, Pergamon Press (1961).
[8] L. J. HAYNES, *Advances in Carbohydrate Chem.* **18**, 227 (1963).
[9] W. E. HILLIS and A. CARLE, *Aust. J. Chem.* **16**, 147 (1963).
[10] S. SHIBATA, T. MURAKAMI and Y. NISHIKAWA, *J. Pharm. Soc., Japan* **79**, 757 (1959).

C-glycosides either occur as such or, more frequently, in *O*-glycosidic combination. While no flavonol *C*-glycoside (or glycoflavonol) has yet been fully characterized, such a structure was recently proposed to explain the resistance to acid hydrolysis of some flavonols occurring in certain plants of the families Leguminosae, Liliaceae, Ranunculaceae and Rosaceae.[11,12]

The problem, therefore, of characterizing flavonoid glycosides has become much more complex, depending as it does on distinguishing not only between *O*- and *C*-glycosides, but also between a range of different *O*-glycosidic combinations. Whilst a variety of methods (e.g. those based on spectral measurements,[13] H_2O_2 oxidation,[14] etc.) exist for doing this, there is little information about the response of the various glycosidic bonds to hydrolytic attack by acid or enzyme. Thus, on the one hand, it has been assumed that all *O*-glycosidic bonds are split by acid treatment (2 N HCl) at 100° for 30 min;[15] on the other, there are reports of *O*-glycosides requiring several hours' heating with acid for complete hydrolysis.[16] Similarly, while most workers report that *C*-glycosides are unaffected by heating with 2 N acid for 24 hr, there is some evidence that hydrolysis does occur after shorter periods of heating.[8] Furthermore, while the acid-catalysed hydrolyses of many simple glycosides have been studied rather carefully (e.g.[17]), there is no precise information about the rate of hydrolysis of the more complex flavonol glycosides such as rutin.

This lack of information became particularly apparent when re-examination of some of the "glycoflavonols" in the plants mentioned above showed them to be flavonol *O*-glycosides which were readily susceptible to enzymic hydrolysis.[18] Again, although β-glucosidase (β-D-glucoside glucohydrolase) and β-glucuronidase (β-D-glucuronide glucuronohydrolase) are used occasionally for purposes of flavonoid characterization, they are not used routinely and there is little information about their effect on glycosides other than simple β-glucosides or β-glucuronides. A third enzyme, anthocyanase, is available for the hydrolysis of anthocyanins,[19] but, apart from a brief study by Forsyth and Quesnel[20] of the properties of cacao anthocyanase and our own preliminary observations,[21] little is known about its substrate specificity.

In order, therefore, to extend the use of controlled acid and enzymic hydrolysis in the study of these glycosides, the rates of hydrolysis of series of known flavonoids have been examined and the results are reported here. Using these and other methods, some of the acid-resistant flavonol glycosides in the Leguminosae, Liliaceae, Ranunculaceae and Rosaceae have been identified.

RESULTS

The Acid Hydrolysis of Flavonoids

Twenty flavonoid monoglycosides were hydrolysed by heating at 100° in 2 N HCl-ethanol (1:1) in stoppered tubes. An acid–alcohol mixture was chosen in order (*a*) to bring some

[11] K. EGGER, *Z. Naturforsch.* **14b**, 401 (1959).
[12] E. C. BATE-SMITH and T. SWAIN, *Chem. & Ind. (London)* 1132 (1960).
[13] L. JURD, in *Chemistry of Flavonoid Compounds* (Edited by T. A. GEISSMAN), p. 107, Pergamon Press (1962).
[14] B. V. CHANDLER and K. A. HARPER, *Aust. J. Chem.* **14**, 586 (1961).
[15] E. C. BATE-SMITH, *Sci. Proc. Roy. Dublin Soc.* **27**, 165 (1956); *J. Linn. Soc., London (Botany)* **58**, 95 (1962).
[16] S. HATTORI, in *Chemistry of Flavonoid Compounds* (Edited by T. A. GEISSMAN), p. 317, Pergamon Press (1962).
[17] W. G. OVEREND, C. W. REES and J. S. SEQUEIRA, *J. Chem. Soc.* 3429 (1962).
[18] J. B. HARBORNE, *Chem. & Ind. (London)* 222 (1962).
[19] H. T. HUANG, *J. Agr. Food Chem.* **3**, 141 (1954); *Nature* **177**, 39 (1956).
[20] W. G. C. FORSYTH and V. C. QUESNEL, *Biochem. J.* **65**, 177 (1957).
[21] J. B. HARBORNE and H. S. A. SHERRATT, *Biochem. J.* **65**, 24P (1957).

aqueous-insoluble glycosides into solution, (*b*) to prevent the aglycone from crystallizing out during hydrolysis and (*c*) to prevent loss of aglycone by auto-oxidation (cf. Nordstrom *et al.*).[22]

Samples were taken at suitable time intervals and the aglycone and glycoside present were separated by paper chromatography in 15% aq. acetic acid. The aglycone and glycoside spots were cut out, eluted with 70% ethanol and their proportions estimated spectrophotometrically; the rates of disappearance of glycoside and of appearance of aglycone were in broad agreement with each other. The results, based on disappearance of the glycosides and expressed in terms of the time required for 50 per cent hydrolysis, are given in Table 1. The

TABLE 1. RATES OF HYDROLYSIS OF FLAVONOID GLYCOSIDES

Glycoside*	Time required for 50% hydrolysis (min)	Estimated time for complete hydrolysis (min)
Rapidly hydrolysed:		
Kaempferol 3-rhamnoside	0·72	2–3
Quercetin 3-rhamnoside	0·72	
Quercetin 3-galactoside	1·5	4–6
Kaempferol 3-glucoside	1·8	
Kaempferol 7-rhamnoside	2·0	
Quercetin 3-glucoside	2·3	
Slowly hydrolysed:		
Quercetin 4′-glucoside	5·0	8–10
Apigenin 7-glucoside	5·2	15
Kaempferol 7-glucoside†	7·8	20–25
Quercetin 7-glucoside	12	
Peonidin 3-galactoside	15	30–45
Cyanidin 3-glucoside	15	
Luteolin 7-glucoside	16	
Kaempferol 3-glucuronide	19	45–60
Apigeninidin-5-glucoside	28	
Quercetin 3-glucuronide	35	
Acid resistant:		
Quercetin 7-glucuronide	88	180
Apigenin 4′-glucuronide‡	98	
Kaempferol 7-glucuronide	101	
Apigenin 7-glucuronide	134	250

* All have β-linked sugars, except the rhamnosides, which are shown by enzymic experiments (see p. 112) to be α-rhamnosides.

† Dihydrokaempferol 7-glucoside and naringenin 7-glucoside were hydrolysed at approximately the same rate as this glycoside.

‡ Measured by hydrolysing apigenin 7,4′-diglucuronide and following the rate of disappearance of the 4′-glucuronide intermediate.

times for complete hydrolysis could not be measured with the same precision since rates of hydrolysis fell off towards the end of the reaction. The rates for seven quercetin glycosides are shown in Fig. 1.

The results show that the most important factor in determining the rate of acid hydrolysis is the nature of the sugar moiety. The monosaccharides can be placed in the following order, based on ease of hydrolysis: L-rhamnose = L-arabinose > D-glucose≏D-galactose >

[22] C. G. NORDSTRÖM, R. HAKALAX and L. J. KARUMAA, *Suomen Kemistilehti* **36B**, 102 (1963).

D-glucuronic acid. The resistance of *O*-glucuronides (or *O*-glucosiduronic acids) to hydrolysis is a well-known phenomenon; polysaccharides containing uronic acid residues[23] and saponins containing glucuronic acid[24] are also particularly resistant to acid hydrolysis. This resistance is apparently due to the fact that the conformation of glucuronides is different from that of other glycosides and not because of the polar nature of the carboxylic acid grouping in the 6-position.[25] The position of substitution is also important: 3-glycosides are hydrolysed more rapidly than 4'-glycosides which are, in turn, hydrolysed more quickly than 7-glycosides (Table 1 and Fig. 1). These results agree with those of Simpson and Beton,[26] who have observed similar variations in the ease of demethylation of 3-, 7- and 4'-*O*-methylated

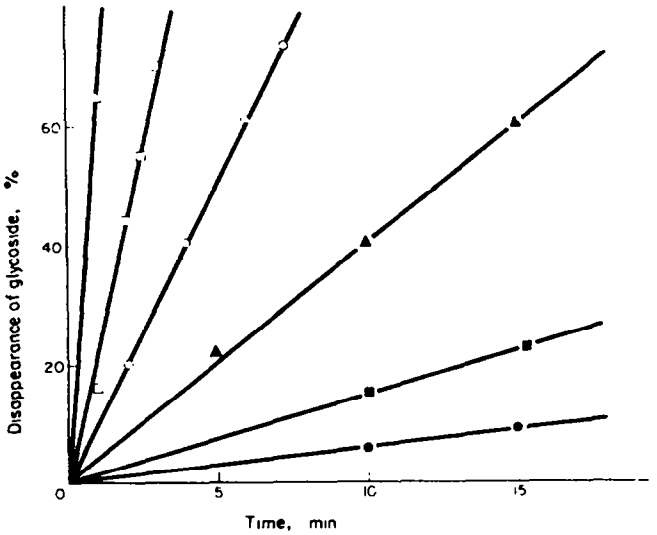

FIG. 1. RATES OF ACID HYDROLYSIS OF QUERCETIN MONOGLYCOSIDES.
△—3-Rhamnoside, □—3-glucoside and 3-galactoside, ○—4'-glucoside, ▲—7-glucoside, ■—3-glucuronide, ●—7-glucuronide.

flavones. The remarkable resistance of apigenin 7-*O*-glucuronide, which is only completely hydrolysed after 4 hr heating with acid, is thus due to the combination of these two factors, presence of an acidic sugar and position of substitution.

By contrast, the nature of the aglycone has less effect on the rate of hydrolysis of the glycoside. Thus, analogous glycosides of kaempferol and quercetin behave similarly and the rates of hydrolysis of the 7-*O*-glucosides of flavonols, flavones, flavanones and dihydroflavonols (Table 1) are all of the same order. Anthocyanins, however, differ from the other flavonoids in carrying a net positive charge and are, as expected, considerably more resistant to acid hydrolysis than flavonol glycosides. For example quercetin 3-glucoside is hydrolysed within 5 min but cyanidin 3-glucoside requires 30 min for complete hydrolysis. Aromatic acyl groups attached to the sugars of flavonol glycosides and anthocyanins also increase

[23] R. L. WHISTLER and W. M. CORBETT, in *The Carbohyrates* (Edited by W. PIGMAN), p. 641, Academic Press (1957).
[24] O. JEGER, *Fortschr. Chem. Org. Naturstoffe* 7, 1 (1950).
[25] T. E. TIMELL, *Chem. & Ind. (London)* 503 (1964).
[26] T. H. SIMPSON and J. L. BETON, *J. Chem. Soc.* 4065 (1954).

resistance to acid hydrolysis. For example, tiliroside (kaempferol 3-*O*-(*p*-coumaroylglucoside))[27] is not completely hydrolysed by 30 min heating with acid.

Several flavone *C*-glycosides (including vitexin and lutexin) and the xanthone *C*-glucoside, mangiferin, were heated for 24 hr at 100° with the alcohol–acid mixture used in the above experiments. No appreciable hydrolysis had occurred at the end of the treatment and it is clear that these *C*-glycosides are much more resistant to hydrolysis than the most recalcitrant *O*-glycoside, i.e. apigenin 7-glucuronide.

Enzymic Hydrolysis of Flavonol Glycosides

The effects of β-glucosidase and anthocyanase* on a series of kaempferol and quercetin glycosides were studied and the results are shown in Table 2. The β-glucosidase used was from a commercial source and had β-galactoside (β-D-galactoside galactohydrolase) activity,

TABLE 2. ENZYMIC HYDROLYSIS OF FLAVONOL GLYCOSIDES*
(A, WITH GLUCOSIDASE; B, WITH ANTHOCYANASE)

Rapid (< 1 hr)	Slow (1–24 hr)	None (after 24 hr)
	A	
3-Glucoside	3-Gentiobioside	3-Sophoroside†
7-Glucoside‡		3-Rhamnoside
4′-Glucoside		3-Rutinoside
3,7-Diglucoside		7-Rhamnoside-3-sophoroside
3,4′-Diglucoside		7-Rhamnoside-3-glucosylsophoroside
7-Glucoside-3-sophoroside§		7-Rhamnoside-3-rhamnosylgalactoside
3-Galactoside		3-(*p*-Coumaroylglucoside)
	B	
3-Rhamnoside	3-Gentiobioside	
3-Rutinoside	7-Rhamnoside-3-sophoroside	
3-Glucoside	7-Rhamnoside-3-glucosylsophoroside	
3,7-Diglucoside		

* In most cases, both kaempferol and quercetin glycosides were studied; in some instances, only one or other of the two glycosides was available.

† The 3-sophoroside only shows slight hydrolysis after 24 hr, and is not hydrolysed completely, even after 21 days' incubation (cf. L. BIRKOFER and C. KAISER, *Z. Naturforsch* **17b**, 359 (1962)).

‡ 7-*O*-Glucosides of dihydrokaempferol, of naringenin and of luteolin were also rapidly hydrolysed.

§ Gives the 3-sophoroside (see text).

but no appreciable α-glucosidase activity. The anthocyanase, a crude enzyme mixture from *Aspergillus niger* and also commercially available, was used because preliminary studies indicated that, besides having β-glucosidase, it contained appreciable amounts of α-rhamnosidase (α-L-rhamnoside rhamnohydrolase). Thus, it hydrolysed methyl α-D-glucose, methyl α-D-mannose, maltose, rutinose (6-*O*-α-L-rhamnosyl-D-glucose) and rutin.[28] Although α-rhamnosidase has been found in a number of higher plants,[29] the fungal preparation is a most convenient source of this enzyme.

Results with β-glucosidase were as expected: all monoglucosides were rapidly hydrolysed,

* Strictly speaking, this name should be reserved for the enzymes which specifically hydrolyse anthocyanins (see later). It is used here to indicate a crude enzyme preparation, which contains a number of hydrolases.

[27] J. B. HARBORNE, *Phytochem.* **3**, 151 (1964).
[28] P. A. J. GORIN and A. S. PERLIN, *Can. J. Chem.* **37**, 1930 (1959).
[29] H. SUZUKI, *Arch. Biochem. Biophys.* **99**, 476 (1962).

whereas other glycosides, if attacked, were only slowly hydrolysed. The 3-, 7- and 4′-*O*-glucosides of quercetin were all rapidly hydrolysed by β-glucosidase, although Hörhammer *et al.*[30] have suggested that flavonol 4′-*O*-glucosides are rather resistant to hydrolytic enzymes. The difference in the rate of hydrolysis of flavonol 3-sophorosides and 3-gentiobiosides is noteworthy, but the disaccharides themselves are known to vary considerably in their susceptibility to hydrolysis.[31] The slow hydrolysis of the 3-sophoroside (β1→2 link) as compared with the 3-gentiobioside (β1→6 link) may be due to steric factors. The slow hydrolysis of these flavonol diglucosides means that monosaccharides may be removed preferentially from flavonols having two glucoses attached in one position and one in another. Indeed, quercetin 7-glucoside-3-sophoroside, on treatment with β-glucosidase for 1 hr, gave the 3-sophoroside in good yield.

The results with anthocyanase were largely complementary to those obtained with β-glucosidase. It hydrolysed all those flavonol glycosides (e.g. rhamnosides) which were not attacked by β-glucosidase or β-glucuronidase. It even attacked, albeit slowly, kaempferol 3-triglucoside-7-rhamnoside. Anthocyanase is also useful for confirming the presence of α-linkages in flavonol glycosides. For example, the linkage of the three common flavonol 3-rhamnosides (kaempferitrin, quercitrin and myricitrin) must be the expected α-linkage since none is hydrolysed by β-glucosidase but all are rapidly hydrolysed by anthocyanase.

Enzymic Hydrolysis of Anthocyanins

Before use, the commercial anthocyanase preparation was purified 15-fold by $(NH_4)_2SO_4$ precipitation; further purification with $(NH_4)_2SO_4$ gave no improvement in activity. The purified enzyme still retained considerable α-rhamnosidase and β-glucosidase activity towards

TABLE 3. ENZYMIC HYDROLYSIS OF ANTHOCYANINS*

Hydrolysis with anthocyanase†		
Rapid (< 4 hr)	Slow (4–48 hr)	None (after 72 hr)
3-Glucoside	3-Arabinoside	3-(*p*-Coumarylglucoside)-5-glucoside
3-Galactoside	3-Rhamnoside	3-(Caffeoylglucoside)-5-glucoside
5-Glucoside	3-Rutinoside	7-Glucoside-3-sophoroside‡
3,5-Diglucoside	5-Glucoside-3-rutinoside	
3-Gentiobioside	5-Glucoside-3-rhamnoside	
3-Sophoroside	5-Glucoside-3-sophoroside	
3-Sambubioside		

* Glycosides of more than one of the six common anthocyanidins (pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin) were tested in each class and there was no evidence that the structure of the aglycone moiety appreciably affected the rate of hydrolysis.

† Huang's conclusion[19] that cyanidin 3-glucoside is not hydrolysed by β-glucosidase was confirmed and extended to a range of other anthocyanins.

‡ The failure to hydrolyse this glycoside may have been due to the presence of an impurity inhibiting enzyme activity. Repeated attempts to purify this glycoside failed.

flavonols (see above). A range of different anthocyanins was incubated with the enzyme and the rates of hydrolysis were followed by taking aliquots at regular time intervals. The rate of disappearance of anthocyanin was noted; the anthocyanidin produced was irreversibly lost, due to pseudo-base formation at pH 4 and breakdown,[19] and did not interfere with spectral measurements on the anthocyanin in the visible region.

[30] L. HÖRHAMMER, R. HANSEL and W. ENDRES, *Arch. Pharm.* **289**, 133 (1956).

[31] H. BAUMANN and W. PIGMAN, in *The Carbohydrates* (Edited by W. PIGMAN), p. 562, Academic Press (1957).

The results (Table 3) show that all simple 3-*O*-β-glycosides were rapidly hydrolysed, α-glycosides were slowly hydrolysed and acylated pigments were not affected. The resistance to hydrolysis of the α-glycosides has preparative value, since good yields of 3-α-L-rhamnosides may be obtained from the corresponding 5-glucoside-3-rhamnosides.[4] The resistance of rhamnosides, arabinosides and rutinosides to anthocyanase indicates that this enzyme has very little α-anthocyanase activity. It differs in substrate specificity from the cacao anthocyanase of Forsyth and Quesnel,[20] which hydrolyses β-D-galactoside and α-L-arabinoside, but not β-D-glucoside or β-D-xyloside. Indeed, the fungal enzyme hydrolyses 3-β-D-glucoside and 3-β-D-galactoside at very similar rates (see Fig. 2).

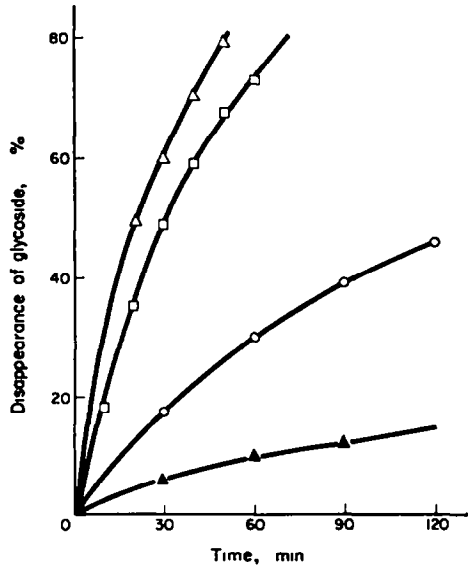

FIG. 2. RATES OF ANTHOCYANASE HYDROLYSIS OF CYANIDIN GLYCOSIDES.
△—3-Glucoside, □—3-galactoside, ○—3-sophoroside, ▲—3-rutinoside

Identification of Flavonol 3,7-Diglycosides

The use of acidic and enzymic hydrolyses as an aid to identification may be illustrated by reference to the acid-resistant flavonols found by Egger[11] and by Bate-Smith[12] in plants of the Leguminosae, Liliaceae, Ranunculaceae and Rosaceae. It was earlier shown[18] that these pigments are not glycoflavonols but are 7-*O*-glucosides or 7-*O*-glucuronides. It is now confirmed, from studying flavonol glycosides of representative species of these families, that only *O*-glycosides are present in these plants and that the 7-*O*-glycosides arise by acid hydrolysis of 3,7-diglycosides originally present in the plant extracts. Flavonol glycosides present in each of the four families mentioned will be considered in turn.

(1) *Flavonols of the Liliaceae.* Leaves of the cultivated tulip were reported to give, after acid hydrolysis at 100° for 20 min, two flavonols designated GK and GQ;[12] in order to distinguish them from similar substances from other sources, they will be referred to here as GK1 and GQ1. After purification by paper chromatography, these two substances were hydrolysed with acid. They proved to be very resistant, but were completely hydrolysed after 3–4 hr treatment to kaempferol or quercetin and D-glucuronic acid. In rate of hydrolysis, they were very similar to apigenin 7-glucuronide (see Table 1). On treatment with

β-glucuronidase. GK1 and GQ1 were rapidly and completely hydrolysed, thus proving that they were simple β-glucuronides. Spectral data (no shift in the presence of sodium acetate, see Table 4) show that they are 7-*O*-glycosides; furthermore, they differ in R_f value and colour reactions from the corresponding 3-glucuronides, isolated from *Phaseolus vulgaris*.[32] GK1 and GQ1 are thus kaempferol and quercetin 7-*O*-glucuronides respectively.

TABLE 4. CHROMATOGRAPHIC AND SPECTRAL PROPERTIES OF FLAVONOL GLYCOSIDES

Pigment	Source	R_f values in			
		BAW	H_2O	BEW	PhOH
GK1	*Tulipa* leaf	0·53	0·04	0·34	0·35
GQ1		0·26	0·02	0·25	0·18
TG1		0·26	0·93	0·17	0·39
TG2		0·20	0·90	0·12	0·28
GK2	*Lathyrus vernus* leaf	0·52	0·02	0·42	0·66
V4		0·20	0·70	0·07	0·54
GQ2		0·32	0·00	0·32	0·40
H1	*Helleborus foetidus* petal	0·21	0·64	0·47	0·39
H2		0·16	0·65	0·35	0·38
H1.2		0·14	0·61	0·20	0·37
LF4	*Lathyrus odoratus* petal	0·53	0·71	0·48	0·73
Incanin	*Matthiola incana* petal	0·58	0·59	0·54	0·72
PR1	*Potentilla reptans* leaf	0·16	---	0·23	0·27
Kaempferol 3-glucuronide		0·53	0·67	0·51	0·25
Quercetin 3-glucuronide		0·40	0·69	0·44	0·16
Robinin		0·48	0·54	0·40	0·72
Qu2	*Allium cepa* leaf	0·35	0·29	0·32	0·42
Qu3		0·23	0·03	0·19	0·34

	λ_{max} (mμ) in					
	95% EtOH		EtOH–NaOEt	EtOH–NaOAc	EtOH–H_3BO_3	EtOH–$AlCl_3$
	Band I	Band II	Band II	Band I	Band II	Band II
Kaempferol glycosides:						
3-Glucuronide	264	352	394	274	352	349, 380
7-Glycosides (GK1 and GK2)	268	368	422	268	370	355, 425
3,7-Diglycosides (TG1, PR1, V4, LF4, incanin)	267	350	388	267	351	348, 384
Quercetin glycosides:						
3-Glucuronide	258	364	419	264	374	364, 390
7-Glycosides (GQ1, GQ2)	257	375	421	256	385	405, 425
3,7-Diglycosides (TG2, H2, H1.2)	258	360	438	258	382	370, 400
Caffeoyl deriv. (H1)	254			256		
	268(infl.)	335	412	268(infl.)	364	334, 406
Qu2	259(infl.)		350			
	267	350	380(infl.)	270	352	348, 397
Qu3	256	371	427	256	371	351, 423
	265(infl.)	420(infl.)		265(infl.)		
Methylation and Hydrolysis product of Qu2	275	363	426	276	362	325, 417

[32] C. A. MARSH, *Nature* **176**, 176 (1955) (see also Experimental).

Examination of unhydrolysed extracts of *Tulipa* leaf showed that two glycosides TG1 and TG2 were present. They gave, as expected, GK1 and GQ1 on acid hydrolysis and were hydrolysed by β-glucuronidase to give kaempferol and quercetin 3-rutinoside, respectively. These results, together with the spectral data (Table 4), show that TG1 and TG2 are the 7-*O*-glucuronide-3-*O*-rutinosides of kaempferol and quercetin. Glycosides similar to TG1 and TG2 also occur in the flowers of most cultivated tulips. They are presumably characteristic of the genus, since Bate-Smith[33] has noted that GK1 and GQ1 are present in acid hydrolysates of the leaf extracts of some twenty-three *Tulipa* species.

Bate-Smith[33] also found compounds similar to GK1 and GQ1 in acid-hydrolysed extracts of some other plants belonging to the Liliaceae, as well as of a few other related monocotyledons. The onion, *Allium cepa*, was chosen for study, partly because Herrmann[34] has already examined the flavonols present in the bulb. He found three quercetin glycosides, one of which he was able to identify as quercetin 4'-glucoside. One of the other glycosides present, Qu2, has now been identified as quercetin 3,4'-diglucoside (for details, see Table 4).* It is interesting that on acid hydrolysis Qu2 gives quercetin, the 4'-glucoside and glucose, whereas on β-glucosidase hydrolysis, the only intermediate that could be detected was the 3-glucoside. The third glycoside, Qu3, was not present in any quantity, but its spectral

TABLE 5. HYDROLYTIC PRODUCTS OF FLAVONOL GLYCOSIDES

Pigment	Reagent	Time of hydrolysis	Products (ratios in parentheses)
GK1	β-Glucuronidase	½ hr	Kaempferol and glucuronic acid
	Acid	3 hr	
GQ1	β-Glucuronidase	½ hr	Quercetin and glucuronic acid
	Acid	3 hr	
TG1	β-Glucuronidase	3 hr	Kaempferol 3-rutinoside and glucuronic acid
	Acid	½ hr	GK1
TG2	β-Glucuronidase	3 hr	Quercetin 3-rutinoside and glucuronic acid
	Acid	½ hr	GQ1
GK2	β-Glucosidase	10 min	Kaempferol and glucose
	Acid	25 min	
V4	β-Glucosidase	3 hr	Kaempferol 3-sophoroside
	Acid	15 min	GK2
GQ2	β-Glucosidase	10 min	Quercetin and glucose
	Acid	15 min	
H1	Alkali	2 hr	H1.2 and caffeic acid
	Acid	45 min	Caffeic acid, caffeoylglucose and GQ2
H2	Acid	45 min	GQ2, quercetin (0·8), glucose (2·0) and xylose (1·17)
	β-Glucosidase	2 hr	Quercetin 3-xylosylglucoside
H1.2	Acid	45 min	GQ2, quercetin (1·0), and glucose (2·89)
LF4	Acid	10 min	Kaempferol (0·78), xylose (1·04), rhamnose (1·0), galactose (1·13) and kaempferol 7-rhamnoside
Incanin	Acid	5 min	Kaempferol (0·87), arabinose (1·17), rhamnose (2·0) and kaempferol 7-rhamnoside
Qu2	β-Glucosidase	3 hr	Quercetin, glucose and quercetin 3-glucoside
	Acid	10 min	Quercetin, glucose and quercetin 4'-glucoside

* This glycoside has also been reported to occur in horse-chestnuts (H. WAGNER, *Naturwiss.* **48**, 54 (1961), but its presence in this source could not be confirmed in this laboratory or by A. H. WILLIAMS (private communication).

[33] E. C. BATE-SMITH, unpublished results.

[34] K. HERRMANN, *Arch. Pharm.* **291**, 238 (1958).

characteristics (Table 4), R_f values and colour reactions suggest that it is the 7,4'-diglucoside of quercetin. None of these three glycosides appears to be present in the leaf of the onion; instead, a substance behaving like a flavonol 3,7-diglycoside is present.

(2) *Flavonols of the Leguminosae.* The only plant of this family reported to contain a glycoflavonol is *Lathyrus vernus.* Re-examination of the flavonol (GK2) produced on acid hydrolysis of leaf extracts showed it to be kaempferol 7-*O*-glucoside. GK2 was identified by direct comparison of its properties with authentic material (Table 4) and by a study of its acid and enzymic hydrolysis. The unhydrolysed leaf extract of this plant contains at least five different flavonol glycosides. One of them, V4, gave kaempferol 3-sophoroside on β-glucosidase hydrolysis and, from its spectral characteristics and R_f, is provisionally identified as the 7-glucoside-3-sophoroside of kaempferol (Table 5). This kaempferol glycoside has been found in flowers of *Petunia*[35] and of *Galanthus nivalis.*[36]

Lathyrus vernus is distinct from all other *Lathyrus* species in producing kaempferol 7-glucoside on acid hydrolysis of a leaf extract. However, flavonol 3,7-diglycosides do occur in other species, notably in flowers of *L. odoratus.*[18] These are interesting since they represent a contrasting group of flavonol 3,7-diglycosides, the 7-sugars of which are rapidly lost on acid hydrolysis. The identification of kaempferol 3,7-dirhamnoside and kaempferol 3-lathyroside-7-rhamnoside (LF4) from this plant are therefore here included. The first of these glycosides has been found in several other legumes, but the second one is a new glycosidic type and is of interest because of its relationship to the anthocyanins with which it occurs.[4]

A flavonol 3,7-diglycoside similar to LF4 in its lability towards acid is incanin, a kaempferol derivative in white flowers of *Matthiola incana* (Cruciferae). The sugars are L-rhamnose (2) and L-arabinose (1) and the detection of kaempferol 7-rhamnoside as an intermediate of acid hydrolysis shows the position of one sugar. Attempts to determine the order of the other two sugars attached at the 3-position have so far failed.

(3) *Flavonols of the Ranunculaceae.* The presence in many plants of the Ranunculaceae (thirty-four out of fifty-one species examined) of one or other of two unusual flavonols related in structure to kaempferol and quercetin was reported by Egger in 1959.[11] Later, Bate-Smith and Swain[12] found that these two flavonols corresponded in R_f value to GK and GQ of *Tulipa.* Subsequently, Egger[37] isolated kaempferol 3,7-diglucoside from petals of *Paeonia albiflora* and concluded that the GK found in acid-hydrolysed extracts of this plant during his earlier survey arose from this pigment (i.e. was kaempferol 7-*O*-glucoside). *Helleborus foetidus*, a plant of the Ranunculaceae reported earlier to contain GQ, has now been examined and the flavonol formed after acid hydrolysis (GQ2) of a petal extract has been identified as quercetin 7-*O*-glucoside (see Tables 4 and 5). The petals contain two glycosides, H1 and H2, which have been identified by the usual procedures as quercetin 3-(caffeoylsophoroside)-7-glucoside and quercetin 7-glucoside-3-(xylosylglucoside). Deacylation of H1 gives quercetin 7-glucoside-3-sophoroside (H1.2), which has previously been identified in *Petunia* flowers[35] but the xylose-containing pigment, H2, is a new glycoside.

(4) *Flavonols of the Rosaceae.* The distribution of GK and GQ in the Rosaceae is of systematic interest, since they are restricted to *Potentilla* and the two related genera, *Geum* and *Waldsteinia.*[38] In order to isolate material for re-investigation, attention was turned to the leaves of the common weed, *Potentilla reptans.* Difficulty was experienced in isolating

[35] L. BIRKOFER and C. KAISER, *Z. Naturforsch.* **17b**, 359 (1962).
[36] J. B. HARBORNE, unpublished results.
[37] K. EGGER, *Z. Naturforsch.* **16b**, 430 (1961).
[38] E. C. BATE-SMITH, *J. Linn. Soc., London (Botany)* **58**, 39 (1961).

flavonoids from this plant because of the large amounts of saponins and other interfering substances present. However, the spectral properties of the main glycoside present (PR1 Table 5) indicated that it was a quercetin 3,7-diglycoside. On hydrolysis with β-glucuronidase PR1 gave glucuronic acid and quercetin; PR1 was not hydrolysed by β-glucosidase and on acid hydrolysis, it gave a flavonol chromatographically identical with quercetin 7-*O*-glucuronide from *Tulipa*. These provisional results show that PR1 is quercetin 3,7-diglucuronide and it is thus unlikely that glycoflavonols are present in this plant.

DISCUSSION

In a recent review, Haynes[8] points out the difficulty of distinguishing unequivocally between flavonoid *O*- and *C*-glycosides from the results of hydrolytic studies. The present work shows that as long as acids as well as a range of enzymes are employed, there is rarely any difficulty in differentiating the two classes of glycoside.

Thus, all of some hundred *O*-glycosides studied were hydrolysed by acid or enzyme, without the use of "forcing" conditions.* The two types of reagent are often complementary in their action; for example, those glycosides (i.e. 7-*O*-glucuronides, 7-*O*-glucosides) which are the most resistant to acid hydrolysis are rapidly hydrolysed by the appropriate enzymes. Some *O*-glycosides have been reported to require prolonged heating (4–6 hr) but this is almost certainly because they are rather insoluble in aqueous acid. Hattori[16] states that luteolin 7-glucoside requires 6 hr heating with 30% H_2SO_4 for complete hydrolysis, but our results show that it is hydrolysed by N HCl and ethanol (1:1) within an hour. Diosmin, another glycoside said to be difficult to hydrolyse,[16] is a notoriously insoluble pigment. In the present experiments using an acid–alcohol mixture, the most refractory *O*-glycoside (i.e. apigenin 7-*O*-glucuronide) was hydrolysed completely after 4 hr heating at 100°. Although flavonol glycosides and anthocyanins having acylated sugars are resistant to enzymic attack, they are hydrolysed by acid only slightly less quickly than unacylated *O*- glycosides. In doubtful cases, these acyl groups should be removed by mild alkaline saponification before hydrolytic studies are attempted.

By contrast with all the *O*-glycosides mentioned above, *C*-glycosides are not appreciably hydrolysed when they are heated for 24 hr with the standard acid–alcohol reagent and no glycosidase is known which will hydrolyse carbon–carbon linked sugars. Thus, the difficulties described by Haynes are more apparent than real. It must be admitted, however, that this resistance to hydrolysis of *C*-glycosides is "negative" evidence of structure and more positive experiments using "forcing" conditions to produce the aglycone or its formyl derivative or its sugar are very desirable.

The present studies also show that measurements of the approximate rates of hydrolysis of *O*-glycosides by acid or enzyme often provide useful structural information. All the flavonoid monoglucosides examined were rapidly hydrolysed by excess β-glucosidase, whereas those flavonoids having *O*-D-glucosyl-D-glucose units (e.g. gentiobiose or sophorose) were only slowly hydrolysed. Thus, when the measurement of sugar–aglycone ratios is impracticable, enzymic evidence is of considerable value in establishing the presence or absence of simple *O*-mono-glucosidic links. The complementary use of acidic and enzymic hydrolyses in determining the structures of flavonoids having sugars attached at more than one hydroxyl group are illustrated here by studies of a number of new flavonol 3,7-diglycosides.

* That is heating with conc. acid at temperatures over 100° (e.g. with conc. H_2SO_4 or HI in phenol at the b.p.).

Anthocyanase has already been used to advantage for determining the structure of anthocyanins having rhamnose or rutinose substituents in the 3-position (see e.g.[3,4]).

The glycoflavonol structures, suggested by previous workers[11,12] for substances detected in the acid-hydrolysates of certain plant extracts, are no longer tenable because every pigment that has now been re-examined is readily hydrolysed by β-glucuronidase or β-glucosidase. Indeed, these substances have been shown to be 7-*O*-glucosides or 7-*O*-glucuronides, formed as intermediates in the acid hydrolysis of flavonol 3,7-diglycosides originally present in the leaf extracts. There is no definite proof that glycoflavonols occur in nature and it seems unlikely, if they do exist, that they are present at all frequently, as compared with glycoflavones such as vitexin.

TABLE 6. NATURAL DISTRIBUTION OF FLAVONOL 3,7-DIGLYCOSIDES*

Group and family	Genus	Glycosidic type (aglycone in parentheses†)
Monocotyledons		
Liliaceae‡	*Allium*	7,4′-Diglucoside (Qu)
		3,4′-Diglucoside (Qu)
	Tulipa	7-Glucuronide-3-rutinoside (Km, Qu)
Amaryllidaceae	*Galanthus*	7-Glucoside-3-sophoroside (Km)
Dicotyledons— Archichlamydeae		
Ranunculaceae§	*Helleborus*	7-Glucoside-3-sophoroside (Qu)
		7-Glucoside-3-xylosylglucoside (Qu)
	Paeonia	3,7-Diglucoside (Km)
Cruciferae	*Matthiola*	3-Arabinosylrhamnoside-7-rhamnoside (Km)
Leguminosae	*Celastrus*	3,7-Dirhamnoside (Km)
	Indigofera	3,7-Dirhamnoside (Km)
	Lathyrus	3,7-Dirhamnoside (Km), 3-lathyroside-7-rhamnoside (Km), 7-glucoside-3-sophoroside (Km)
	Lespedeza	3,7-Dirhamnoside (Km)
	Lotus	3,7-Dirhamnoside (Km)
	Phaseolus	7-Rhamnoside-3-robinibioside (Km)
	Pueraria	7-Rhamnoside-3-robinibioside (Km)
	Robinia	7-Rhamnoside-3-robinibioside (Km)
	Ulex	3,7-Diglucoside (Qu)
Rosaceae	*Potentilla*	3,7-Diglucuronide (Qu)
Dicotyledons— Sympetalae		
Apocynaceae	*Vinca*	7-Rhamnoside-3-robinibioside (Km)
Violaceae	*Viola*	7-Rhamnoside-3-rutinoside (Km, Qu)
Solanaceae	*Petunia*	7-Glucoside-3-sophoroside (Qu)
	Solanum	7-Rhamnoside-3-sophoroside (Km)
		7-Rhamnoside-3-triglucoside (Km)
Caprifoliaceae	*Viburnum*	3,7-Diglucoside (Km)

* Survey limited to angiosperms; two kaempferol 3,7-diglycosides have been found in the pteridophyte, *Equisetum*. Survey only includes fully characterized glycosides.

† Aglycone abbreviations: Km, kaempferol; Qu, quercetin.

‡ 3,7-Diglycosides also probably present in *Colchicum*, *Erythronium*, *Endymion*, *Galanthus*, *Hyacinthus*, *Veratrum* and *Zygadenus*.

§ 3,7-Diglycosides provisionally identified in *Actaea*, *Anemone*, *Aquilegia*, *Caltha*, *Clematis*, *Delphinium*, *Pulsatilla*, *Ranunculus* and *Thalictrum*.

The fact that the unusual substances found in the Liliaceae, Leguminosae, Ranunculaceae and Rosaceae are not, after all, glycoflavonols but are derived from flavonol 3,7-di-*O*-glycosides does not detract from their botanical interest: such glycosides do, in fact, appear

to be restricted in distribution to ten plant families (Table 6). But it should be remembered that surveys carried out on acid-hydrolysed plant extracts only detect flavonol 3,7-diglycosides in which the 7-sugar is glucose or glucuronic acid; rhamnose and other pentoses at the 7-position are rapidly lost under these conditions.

The occurrence of 3,7-diglycosides in such diverse families as those shown in Table 6 shows that these glycosides have no broad taxonomic significance and, in fact, are likely to be found in other unrelated families when sought. Studies of their occurrence at the generic and specific level are likely to be more rewarding. It already appears that the 7-glucuronide-3-rutinosides of *Tulipa* are characteristic of this genus, being present in most cultivated forms as well as twenty-three wild species.[33] Related flavonol 3,7-diglycosides occur in the quite distant genus *Potentilla*; here they are found in species of the subsection Gymnocarpae, but they do not appear in species of the Trichocarpae.[38] Finally, the distribution of 3,7-diglycosides in *Lathyrus* must be mentioned, since *L. vernus* is the only species to have a 3,7-diglycoside in which the 7-sugar is glucose rather than rhamnose, while *L. odoratus* is characterized by having a kaempferol 3-lathyroside-7-rhamnoside.

EXPERIMENTAL

Plant material. Most of the plants used were grown at this Institute. *Potentilla reptans* leaf and a tulip leaf extract were kindly provided by Drs. E. C. Bate-Smith and T. Swain, Cambridge.

Authentic flavonoids. The majority of these were available from earlier studies in this laboratory (for details see earlier papers in the "Plant Polyphenols" series). A few were kindly donated by Dr. H. W. Siegelman, Beltsville, and Professor L. Hörhammer, Munich. Kaempferol 7-rhamnoside was obtained by heating robinin at 100° with NHCl for 5 min and isolated by paper chromatography. Kaempferol and quercetin 3-glucuronide were isolated by paper chromatography from *Phaseolus vulgaris* leaf, in which they occur in conjunction with the corresponding 3-rutinosides. While the structure of quercetin 3-glucuronide has been established,[39] that of the kaempferol derivative has not. However, the spectral data given in Table 4 show that it is the 3-glucuronide and its sugar–aglycone ratio was measured and found to be 1:1.

Chromatography. This was carried out on Whatman No. 1 paper, using the following solvents: BAW, *n*-butanol:acetic acid:water (4:1:5); BEW, *n*-butanol:ethanol:water (4:1:2·2); PhOH, water-saturated phenol; and distilled water.

Spectroscopy. Measurements were made on a Unicam S.P. 500 spectrophotometer using well-described procedures.[4,5,13]

Enzyme sources. β-Glucosidase and β-glucuronidase were purchased from L. Light & Co. The anthocyanase preparation was provided in powder form by Rohm and Haas, Co., Pa., and was purified as follows. It was dissolved in water (1 g/5 ml) and the solution saturated with $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, redissolved in water, dialysed against water, and then freeze-dried. The resulting powder had 15 times the activity of the original material.

Acid hydrolysis. Solutions of flavonoids were prepared in 95% EtOH–2NHCl (1:1, by vol.) and 0·3 ml samples were heated in 10 ml securely stoppered test tubes at 100° for known time intervals, then quickly cooled. Aliquots (0·025 ml) were applied to Whatman No. 1 paper and the flavonoids were separated in 15% acetic acid. After the papers were

[39] I. A. PEARL and S. F. DARLING, *J. Org. Chem.* **28**, 1442 (1963).

thoroughly dried, the aglycone and glycoside spots were excised and eluted separately with 4 ml of 70% EtOH for 4 hr. The optical densities of the eluates were measured at the appropriate flavonoid long wavelength maxima.

Enzymic hydrolysis was carried out at 37° in an appropriate buffer solution, using excess enzyme and flavonoid (about 1 mg/ml); the rate of hydrolysis was followed chromatographically and, in the case of the anthocyanins, by spectral measurements in the visible region. For β-glucosidase and β-glucuronidase hydrolyses, acetate buffer, pH 5·0, was used; for anthocyanase hydrolysis, lactate buffer, pH 3·95, or acetate buffer, pH 4·0, was used. For anthocyanase and β-glucosidase, the hydrolysis of aesculin was included in every set of experiments as a control.

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