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The Preparation and Properties of Aucubin, Asperuloside and Some Related Glycosides

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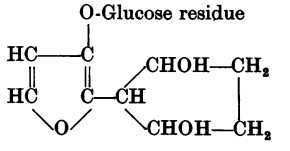
Some of the many types of glycosides which have been described have been thought to be specific products of narrow taxonomic groups of plants. However, more and more of them are proving to occur widely in the plant kingdom. The work described in this paper shows that aucubin, asperuloside and related glycosides, which are probably derived from substituted furan aglycones, constitute one such widely occurring type.

Aucubin was first isolated by Bourquelot & Hérissey (1905) from the leaves of *Aucuba japonica* (family Cornaceae). It is a monohydrate of a monoglucoside of an unstable aglucone, which these workers were unable to isolate or identify. Asperuloside was first isolated by Hérissey (1925*a*, *b*, 1926*a*, *b*, 1927, 1933) from several members of the family Rubiaceae. He showed that it, too, is a monoglucoside of an unstable aglucone, which, like aucubin, loses 1 mol. of water when heated to 105–110°.

Karrer & Schmid (1946) suggested for aucubin the substituted furan structure (I) which explains the properties described by Bourquelot & Hérissey (1905) and Bergmann & Michalis (1927). This



(I)

glucoside has now been isolated from plants belonging to the following families: Cornaceae (Bourquelot & Hérissey, 1905), Garryaceae (Hérissey & Lebas, 1910), Plantaginaceae (Bourdier, 1907), Orobanchaceae (Bridel, 1929), Globulariaceae (Zellner, 1934), Scrophulariaceae (Braecke, 1923*a*), and Eucommiaceae (Plouvier, 1944).

In the present work an improved method for the isolation of glycosides (Hill & Van Heyningen, 1951) has been applied to the preparation of aucubin and asperuloside, and both glycosides can now be prepared in quantity and in a pure form. Aucubin has been obtained from the leaves of *Aucuba japonica* and *Melampyrum arvense* and, for the first time, from *Buddleia globosa* (family Loganiaceae). Asperuloside has been isolated from *Galium aparine*, varieties of *Rubia tinctorum* and, for the first time, from *Daphniphyllum macropodum* (family Euphorbiaceae). New chemical properties of the two glucosides are described, some of which support the suggestion of Hérissey (1925*a*) that aucubin and asperuloside have a similar structure, while others sharply distinguish the two glycosides. There seems to be little evidence that these two glycosides are derived from coumarin, as stated by Armstrong & Armstrong (1931). It has also been shown that other, less well known, glycosides could be conveniently placed in a group with aucubin and asperuloside.

EXPERIMENTAL

All melting points quoted from the authors' observations are corrected.

The preparation of the glycosides and their derivatives

Glycosides and other substances are adsorbed from aqueous solutions by charcoal. Sugars, salts and some other substances are removed from the adsorbed mixture by washing with water. The glycosides remain on the charcoal and are eluted with aqueous ethanol, from which some of them may be easily obtained crystalline.

The preparation of aucubin from Aucuba japonica, Melampyrum arvense *and* Buddleia globosa. Fresh leaves of *A. japonica* (1200 g.) were chopped into small pieces and added to 2·5 l. of boiling water containing 3 g. $CaCO_3$. Boiling was continued for 3 hr. The cooled extract was poured off, the leaves squeezed in a coarse cloth to remove as much fluid as possible and the combined extract filtered through kieselguhr. Animal charcoal (20 g.), previously boiled for about 10 min. with distilled water to remove air, was stirred with the filtrate for 15 min. and then filtered off with kieselguhr. Charcoal (500 g.), boiled as before, was stirred with the filtrate for 15 min. adsorbing most of the aucubin. The charcoal was then filtered off with a large quantity of kieselguhr and the pad washed with 700 ml. distilled water. The adsorbed glucoside was eluted by washing with 7 l. 50% (v/v) ethanol, the eluate reduced to 200 ml. *in vacuo* in the presence of a little $CaCO_3$, filtered and then reduced *in vacuo* to 100 ml. The aucubin crystallized on standing at room temperature: it was filtered off, redissolved in the minimum volume of hot 90% (v/v) ethanol, boiled for a few minutes with animal charcoal, filtered hot and recrystallized twice from 90% ethanol giving 20 g. aucubin, m.p. 176–177°. This material (14 g.) recrystallized three times from 90% ethanol yielded 11·6 g. of aucubin, m.p. 180–181°, $[\alpha]_D^{18°}$ – 164·7° (*l*, 2; *c*, 1·5 in water).

The same method was used to prepare aucubin from *Melampyrum arvense* and *Buddleia globosa*. Yields and constants are recorded in Table 1.

Samples of aucubin hexa-acetate and the two monobromo-hexa-acetyl aucubins *A* and *B* were prepared, the former by the method of Karrer & Schmid (1946) and the two latter by the method of Bergmann & Michalis (1927).

The preparation of asperuloside from Rubia tinctorum *and* Daphniphyllum macropodum. The fresh tips (1730 g.) of the young shoots of *Rubia tinctorum* were extracted with 1400 ml. of 0·33 N-HCl in four equal batches. The shoots were gently pounded in a pestle and mortar until reduced to a pulp, stood at room temperature for 30 min. and then squeezed through coarse calico. The extract was filtered with kieselguhr.

The filtrate was stirred for 10 min. with 30 g. of previously boiled charcoal. The charcoal was removed by filtration with kieselguhr and the filtrate stirred with a further 100 g. charcoal for 10 min. A large bulk of kieselguhr was added and the suspension filtered on a Büchner funnel, washed with 300 ml. distilled water and the glycoside eluted with 3 l. 50% ethanol. The eluate was reduced to 150 ml. *in vacuo* in the presence of 2 g. $CaCO_3$. Ethanol (150 ml. of 95%) was added and the light gelatinous precipitate removed by filtration through kieselguhr. The filtrate was reduced to a syrup by distillation *in vacuo* and dried in a desiccator over $CaCl_2$. The glucoside was extracted from the residue with hot ethanol, filtered and reduced to a small volume *in vacuo*. The fine white crystals of asperuloside obtained on standing at 0° were filtered off, washed with a little ice-cold ethanol, recrystallized twice from 95% ethanol and dried in air; 10 g. of asperuloside with the following constants were obtained; m.p. 125–127°, $[\alpha]_D^{18°}$ – 204° (*l*, 2; *c*, 1·7 in water) for the anhydrous glucoside, solubility in water 4% at 18°, 0·2% at 0°; C, 50·6; H, 5·5%; N, S and halogens were absent. Calc. for $C_{17}H_{24}O_{11}$: C, 50·6, H, 5·7% and a molecular weight of 404, Hérissey (1925*a*) found a molecular weight of 409 by the cryoscopic method.

Table 1. *Yields and constants of aucubin*

Source	Method of preparation	Amount of material (g. fresh wt.)	Amount of purified aucubin (g.)	M.p.	$[\alpha]_D$
Aucuba japonica seeds	Bourquelot & Hérissey (1905)	500	15	181°	– 164·9° at 18°
Aucuba japonica leaves	Charcoal adsorption	1200	16·5	181°	– 164·5° at 18°
Melampyrum arvense foliage	Charcoal adsorption	330	2·9	181°	– 165° at 16°
Buddleia globosa leaves	Charcoal adsorption	200	0·7	182°	– 166° at 17°
Plantago lanceolata unripe fruit	Karrer & Schmid (1946)	2000	20	182·3°	– 162° at 15°

Table 2. *Yields of asperuloside*

Source	Method of preparation	Amount of material: Fresh wt. (g.)	Amount of material: Dry wt. (g.)	Amount of asperuloside (g.)	M.p.
Galium aparine seedlings	Hérissey (1926*c*)	—	8980	4·5	126–127°
Galium aparine young shoot tips	Charcoal adsorption	600	Approx. 90	6·5	125–127°
Rubia tinctorum young shoot tips	Charcoal adsorption	1730	Approx. 260	10·0	125–127°
Daphniphyllum macropodum mature leaves	Charcoal adsorption	315	—	2·0	126–129°

This method was used to prepare asperuloside from *Galium aparine* and *Daphniphyllum macropodum*, for leaves of which we are indebted to Mr J. Gilmour, Director of the Royal Horticultural Society's Garden. The purified product from *Daphniphyllum* had the following constants: m.p. 126–129°, mixed m.p. with the sample from *Rubia tinctorum* 126–129°; $[\alpha]_D^{18°}$ – 196 (*l*, 2; *c*, 1·64 in water), for anhydrous material – 200°, C, 50·7; H, 5·6%.

The samples for elementary analysis were recrystallized from wet ethyl acetate, dried in the air for 2 days and then to constant weight over $CaCl_2$ in an atmospheric desiccator. A sample from *Daphniphyllum* lost the equivalent of 0·5 mol. of water of crystallization, one from *Rubia tinctorum* lost the equivalent of 1 mol. These materials were used for analysis. However, if dried further by heating at 110° to constant weight both samples appear to lose a further 0·5 mol. of water. Yields are recorded in Table 2.

Acetyl asperuloside. A crystalline acetyl derivative of asperuloside was obtained by the procedure described by Karrer & Schmid (1946) for the preparation of aucubin hexa-acetate. Crystalline asperuloside (2·1 g.) was heated to 37° for 24 hr. with 12·8 ml. anhydrous pyridine and 10·7 ml. acetic anhydride. Then, following the original procedure in detail, 2·7 g. of the acetyl derivative, m.p. 153°, were obtained. The melting point could not be raised by further recrystallizations. The compound is soluble in ethanol, methanol, methanol-water mixtures and ethyl acetate and is readily crystallized from these solvents. (Found: C, 53·7; H, 5·4 mol.wt. by the camphor method 757; $C_{33}H_{40}O_{19}$ requires C, 53·5; H, 5·4%; and mol.wt. 740.) Assuming that the molecular weight of anhydrous asperuloside is 404, from the empirical formula, these constants correspond to an octa-acetate, calculated mol.wt. 740. This could not be checked by the determination of acetyl groups by the standard methods which employ acid hydrolysis. It was also impossible to resort to saponification because the glucoside itself reacts with dilute alkali to produce an indeterminate amount of acid.

Some properties and reactions of aucubin and asperuloside

Ultraviolet absorption spectra. The ultraviolet absorption spectrum of asperuloside was determined in both neutral and alkaline solutions. The curves are shown in Fig. 1, together with that for aucubin.

General reactions. Neither aucubin nor asperuloside give colour reactions with $FeCl_3$ and they do not reduce Fehling's solution. Both substances reduce Tollens's $AgNO_3$ reagent slowly in the cold and in a few seconds on heating to 100°. Asperuloside reacts more readily than aucubin. The rate of reaction at 100° is comparable with that of the phenolic glucoside salicin. The chromogenic structures of both substances are stable to alkali and their characteristic colour reactions still occur after boiling with N-NaOH. Karrer & Schmid (1946) showed that aucubin could be recovered after boiling with saturated $Ba(OH)_2$ for 1 hr. Although strong alkali does not affect the capacity of asperuloside to give its colour reactions with acids, dilute alkali causes a rapid, irreversible change in its structure and acid is produced. The

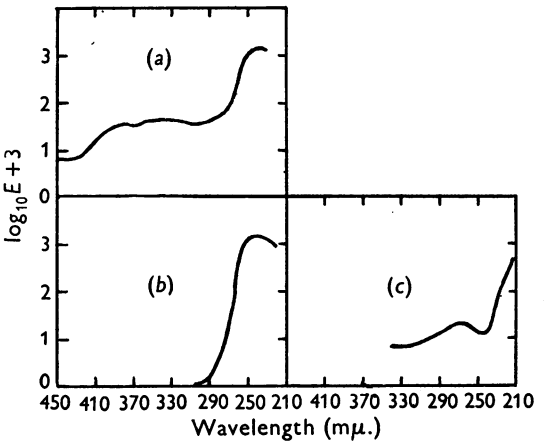


Fig. 1. Absorption spectra of (*a*) solution of 10·95 mg. asperuloside in 100 ml. 0·1 N-NaOH, allowed to stand at room temperature for 2 hr. before measurement. (*b*) Solution of 12·85 mg. asperuloside, recrystallized from water, in 100 ml. water. (*c*) Solution of 27·1 mg. aucubin in 100 ml. water.

accompanying change in the ultraviolet absorption spectrum is shown in Fig. 1. 6·05 and 7·55 mg. of asperuloside were allowed to stand for 5 min. with 5 ml. N/70-NaOH. The remaining NaOH was titrated with N/70-HCl, 3·45 and 3·25 ml. being required respectively, using phenolphthalein as an indicator. Solutions of the pure glucoside are neutral, so that this corresponds to the release of 1·3–1·4 equiv. of acid per g.mol. of glucoside. More acid is produced on standing. When alkali is first added the solution becomes yellow but later this colour fades. A crystalline product could not be obtained from asperuloside after treatment with NaOH.

The action of bromine. Aqueous and alcoholic solutions of aucubin and asperuloside rapidly decolorize Br_2 solutions.

Bergmann & Michalis (1927) have shown that three bromine substitution products of aucubin hexa-acetate are obtained by the action of Br_2 in the cold. Asperuloside acetate does not take up Br_2 in the cold and does so only very slowly on heating.

The action of acids. Aucubin and asperuloside are acid labile and produce coloured acid-degradation products. If the reaction takes place in glacial acetic acid containing a small amount of Cu^{++}, clear blue solutions are obtained. A convenient reagent (reagent *A*) may be prepared by mixing 10 vol. of glacial acetic acid, 1 vol. of 0·2% solution of $CuSO_4.5H_2O$ and 0·5 vol. of conc. HCl.

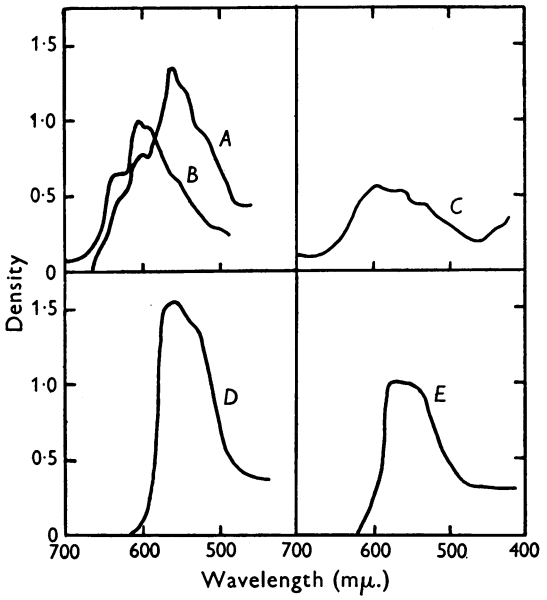


Fig. 2. Absorption spectra of pigments obtained by heating the following mixtures of substances, contained in 1 ml. of water, with 11 ml. of reagent *A* for 5 min. on a boiling-water bath: *A*, 0·488 mg. asperuloside and 4·94 mg. fructose: *B*, 0·488 mg. asperuloside; *C*, 0·432 mg. aucubin; *D*, 0·55 mg. asperuloside brominated in ethanol and 4·94 mg. fructose; *E*, 0·848 mg. bromo-hexa-acetyl aucubin and 4·94 mg. fructose.

If asperuloside (0·5 mg.) is heated to boiling with 2–3 ml. of reagent *A* for 0·5–1 min. a stable, bright-blue colour develops. The same colour develops when the glucoside is heated with the reagent in a boiling-water bath for 3–5 min. with occasional shaking. The cooled solution has two well defined absorption bands, a narrow band I with a centre and maximum at 645 mμ. and a broader band II with a centre and maximum at 600 mμ. This spectrum, measured in a Hilger-Nutting Visual Spectrophotometer, is shown in Fig. 2.

Bands I and II of the blue derivative obtained from asperuloside appear to be characteristic of two forms of the glucoside. If the glucoside is obtained by recrystallization from 90% ethanol, band I is weak or absent and band II is proportionately stronger. If the glucoside is crystallized from water, band I is strong and band II is proportionately weaker. In the latter case the relative strengths of the two bands may be reversed by the addition of a little ethanol to the glucoside before heating with reagent *A*. After the action of alkali on asperuloside, band I is strong and band II is very weak.

Aucubin boiled for 0·5–1 min. with reagent *A* gives a blue of a different tint. There is a single spectral absorption band at 590–600 mμ. (see Fig. 2). Aqueous solutions of aucubin heated with reagent *A* in a boiling-water bath frequently give a green colour, but solid aucubin always gives the blue colour just described.

The effect of other substances on the development of colour from asperuloside, aucubin and their derivatives by the action of reagent A. The colour produced by the action of reagent *A* upon the two glucosides and their derivatives is affected by other substances, particularly by ketohexoses, pyrroles, indoles and phenols. In the examples discussed below colour tests were made by heating both the added substance alone, and the added substance plus glucose, with reagent *A*. In some cases colour changes were observed, but these were relatively weak and distinctly different from the colours obtained with the two glucosides and their derivatives.

The effect of ketoses. The presence of large quantities of aldoses has no effect on the colour developed by heating aucubin, asperuloside and their derivatives with reagent *A*. Ketohexoses cause a shift of absorption towards the blue and the solution assumes a red colour proportionate to the amount of sugar added. With asperuloside and its derivatives this effect is marked. With aucubin it is not so strong. The effect with hexa-acetyl aucubin is exceptional in that the colour becomes olive-green, but there is a marked reddening with the two monobromo-acetyl aucubins. The absorption spectra of some of the pigments produced by fructose are shown in Fig. 2. In each case the molecular concentration of glucoside was approximately the same. The following ketohexoses were tested with asperuloside, brominated asperuloside and monobromo-hexa-acetyl aucubin A and gave a well defined red reaction: fructose, L-sorbose, D- and L-psicose, as the diacetone derivatives, which are hydrolysed to the free sugar during the reaction, and D-tagatose. Sucrose, raffinose, stachyose and irisin, which contain combined fructose, also gave the reaction.

The ketopentoses D- and L-adonose (erythro-2-ketopentose), regenerated from the *o*-nitrophenylhydrazones, did not produce red colours. The ketotetrose, L-erythrulose, regenerated from the *o*-nitrophenylhydrazone, suppressed the development of colour from asperuloside. It gave a brick-red colour with asperuloside, previously treated with Br_2 in ethanolic solution, and a bright-red colour with bromo-hexa-acetyl aucubin A. Aldoses, dihydroxyacetone, glyceraldehyde and pyruvate had no effect.

This reaction shows the similarity between aucubin and asperuloside. It provides a useful specific test for ketohexoses in the presence of aldoses and other substances and offers the basis of a method for their estimation. Under the above conditions, about 20 mol. of ketose per mol. of glucoside must be added to convert the whole of it to the red pigment. With a fixed amount of asperuloside, 0·488 mg., there is a linear relation between the extinction at 565 mμ. and the amount of added fructose over the range 2–5 mg. fructose. The volume of the solution was 12 ml., 1 ml. aqueous solution of

the glucoside and fructose and 11 ml. reagent *A*. The value of the extinction at 565 mμ., which is the peak of the characteristic band of the red pigment, measured in a Hilger-Nutting Visual Spectrophotometer, are recorded in Table 3.

Table 3. *Relation between fructose concentration and extinction at* 565 mμ.

Fructose (mg./12 ml. solution)	Extinction at 565 mμ.
1·97	1·12
2·97	1·22
4·94	1·45

Fructose (2 mg.) in a final volume of 12 ml. gives a strong colour. Brominated asperuloside gives hardly any colour with reagent *A* unless fructose is added. So that if this compound is used and the volumes are proportionately reduced to 1–2 ml. total the reaction may prove to be of use for quantitative work down to 50 μg. ketohexose.

The effect of tryptophan and other heterocyclic nitrogen compounds. Tryptophan behaves like a ketohexose in changing the colour from blue towards red, in the case of asperuloside, aucubin, hexa-acetyl aucubin and bromohexa-acetyl aucubin, and in producing a red-purple colour with brominated asperuloside. No other amino-acid has a marked effect, although cysteine intensifies band I of the asperuloside colour. Combined tryptophan reacts like free tryptophan. With asperuloside and tryptophan the purple colour develops rapidly at 70°, whereas with ketose the temperature must be raised to 100° before the reaction will occur readily.

Pyrrole compounds produce a similar effect. With the ethyl ester of 3:5-dimethylpyrrole-2-carboxylic acid asperuloside gives a bright magenta pigment with strong absorption between 500 and 550 mμ. Aucubin and its derivatives give intense purple colours. Carbazole, which gives colour reactions with the acid degradation products of sugars, gives a purple colour with asperuloside and a slight reddening of the colour with aucubin.

The effect of some phenolic substances. Some phenolic substances give a reddening of the colour with the two glucosides and their derivatives. Phenol, cresol, orcinol, resorcinol, pyrogallol, thymol, β-naphthol, syringin and phlorrhizin react in this way. There is no effect with quinol, salicylic acid and catechol. Phloroglucinol gives a red-brown colour with asperuloside and green with aucubin. Brilliant red-purple colours are obtained with orcinol, resorcinol and thymol.

Other colour reactions of aucubin and asperuloside. Since a furan structure has been proposed for aucubin, the reactions of the two glucosides with known colour reagents for furan derivatives were studied. These reactions are the $SbCl_3$ reaction (Wettstein & Miescher, 1943), the reaction with Ehrlich's aldehyde reagent (Reichstein, 1932), the reaction with vanillin and HCl (Asahina, 1924–6) and Shear's reaction with aniline hydrochloride (Levine, Seaman & Shaughnessy, 1933).

Antimony trichloride reaction. Karrer & Schmid (1946) found that $SbCl_3$ reacts with aucubin to give a strong blue-violet colour in the cold. To a few mg. of solid glucoside 0·1 ml. acetic anhydride and 0·4 ml. of a saturated solution of $SbCl_3$ in pure $CHCl_3$ containing 1% (v/v) ethanol were added. Controls without $SbCl_3$ remained colourless for 24 hr. The development of colour from aucubin and asperuloside in the cold is described in Table 4. If the solutions are warmed the colours develop rapidly.

Table 4. *The rate of development of colour from asperuloside and aucubin in the antimony trichloride reaction*

Time (min.)	Colour	
	Aucubin	Asperuloside
0	Pale yellow	—
2	Pale green	—
7	Pale blue	—
10	Pale blue-violet	Pale green
30	Strong blue-violet	Blue-green

Action of Ehrlich's reagent and vanillin in conc. HCl. The glucosides were tested with Ehrlich's aldehyde reagent under the strongly acid conditions described by Karrer & Schmid (1946). Asperuloside produces the same blue colour as aucubin but more slowly (Table 5). Both colours are stable.

Asahina (1924–6) showed that when substituted furan compounds such as furfurals are treated with a saturated solution of vanillin in cold conc. HCl, colours develop. Aucubin rapidly gives a red colour and asperuloside gives a magenta colour more slowly.

The reactions with Ehrlich's reagent and with vanillin show a similarity. By analogy with pyrrols (Fischer & Orth, 1934), it may be suggested that the aldehyde group of the reagent condenses with two molecules of the furan compound to produce a substance analogous to the triphenylmethane type which is oxidized to give coloured products.

Reaction with aniline hydrochloride. Furan compounds react with aniline salts to produce coloured derivatives such as the well known red colour formed by the action of aniline acetate on furfural. Asperuloside reacts in the cold with Shear's reagent (1 vol. of conc. HCl dissolved in 15 vol. of

Table 5. *Rate of development of colour from aucubin and asperuloside with Ehrlich's reagent*

Time of heating to 75° ...	Colour					
	0 sec.	30 sec.	60 sec.	90 sec.	4 min.	10 min.
Glucoside						
Aucubin	Yellow	Blue-violet	Blue-violet	Blue-violet	Blue-violet	Blue-violet
Control, no Ehrlich reagent	—	Faint green	Brown	Brown	Brown	Brown
Asperuloside	Yellow	Yellow	Yellow	Yellow	Faint green	Blue-violet
Control	—	—	—	—	Faint brown	Brown

aniline). It gives a yellow, brown and finally an intense green colour. Aucubin does not react in the cold; on heating to 100° for a short time it gives a brown colour indistinguishable from that obtained with glucose under the same conditions.

Reaction of asperuloside with amines. Reactions with amines under neutral or faintly acid conditions sharply distinguish asperuloside from aucubin, which gives no colour reactions with amines. Asperuloside reacts with primary amines to form strongly coloured products. A few mg. each of amine and asperuloside heated in an open dish on a boiling-water bath with a few drops of 10% (v/v) acetic acid, 2–3 ml. water and an excess of sodium acetate produce a strong colour, usually within 10 min. In most cases the pigment is soluble in water, insoluble in organic solvents, and blue or blue-violet in colour. With methylamine the reaction is very rapid, and a strong colour develops in 1 or 2 min. This pigment is blue-violet with a marked absorption band from 530 to 610 mμ. It will also develop on standing at room temperature. It is insoluble in amyl alcohol. Octadecylamine reacts much more slowly and heat is required. The blue pigment produced is soluble in amyl alcohol, which suggests that these blue substances are condensation products of the whole amine molecule with the whole or part of the asperuloside molecule.

All the naturally occurring amino-acids, except cysteine, and a number of di- and tri-peptides react with asperuloside, and in all cases the pigment is blue or blue-violet with a characteristic absorption band similar to that obtained with methylamine. The proteins wheat gluten and gelatin reacted to give blue-violet colours. Cysteine and pyrrolidonecarboxylic acid do not react to give coloured products, but cysteic acid gives the usual colour.

Some amino compounds are either non-chromogenic with asperuloside or do not give the typical reaction, which appears to require a free amino group. Secondary and tertiary amines do not react as a rule, and while L-leucine behaves like the other α-amino acids acetyl L-leucine gives no colour. Amides such as succinamide, acetamide and urea are not chromogenic but formamide produces the blue-violet colour. Hydroxylamine and Ehrlich's aldehyde reagent are not chromogenic with asperuloside, but aniline gives a strong green-blue colour. Heated under the mildly acid or neutral conditions of this test ammonia produces a soluble blue-violet colour and a blue-violet precipitate, but under other conditions a different reaction occurs. If asperuloside is allowed to stand with ammonium acetate in the cold a deep-brown colour gradually develops. Dimethylamine reacts rapidly in the cold to produce a brown pigment, *o*-phenylenediamine gives a black precipitate on heating, hydrazine a strong red-brown and phenylhydrazine an olive-green. Glucosamine gives first a violet colour and then a strong brick-red with two absorption bands in the visible spectrum, one at 540–560 mμ., maximum at 550 mμ. and a weaker but sharper band at 600 mμ.

The substances which give characteristic red pigments with asperuloside when heated with reagent *A* give no coloured products under these faintly acid or neutral conditions. Naturally tryptophan is an exception and gives a typical amine reaction.

These reactions with amines do not seem to be due to the presence of a furan nucleus in asperuloside, but rather to the presence of carbonyl groups or potential carbonyl groups, and may be compared with the purple colour reactions of ninhydrin with amino-acids. This interpretation is supported by the fact that, after standing with N/10-NaOH for 5 min., asperuloside no longer gives the amine colour reaction, whereas those reactions which can be attributed to a furan structure are unaffected. Further support is lent to this view by the fact that aucubin gives no colour reactions with primary aliphatic amines.

Action of carbonyl reagents. Since the above reactions suggest that asperuloside may contain or generate carbonyl groups, further tests were made. The Tollens silver nitrate test and the action of Schiff's reagent suggest that there is no aldehyde group. It has already been shown that asperuloside reacts with *o*-phenylenediamine and hydrazine. It also reacts with hydroxylamine hydrochloride in acid solution in the cold, as may be shown by Duke's (1944) test. The reagent is made by dissolving 0·5 g. hydroxylamine hydrochloride in 95% ethanol containing bromophenol blue and adjusting the pH to 3·7–3·9. If an aldehyde or ketone is added to this solution hydroxylamine combines with the carbonyl groups and an equivalent amount of HCl is released. Titration back to pH 3·7–3·9 gives an estimate of the carbonyl radical. Asperuloside (46·7 mg.) was dissolved in 3 ml. of the solution and allowed to stand at room temperature. At intervals the solution was titrated to pH 3·8 with N/70-NaOH. The figures in Table 6 suggest that carbonyl groups are slowly formed.

Table 6. *The reaction of asperuloside with hydroxylamine hydrochloride*

(Results expressed as ml. N/70-NaOH required to neutralize the HCl liberated. 7·8 ml. ≡ 1 carbonyl group per mol. At all times the control without NH_2OH showed no detectable free acid.)

Time (hr.)	N/70-NaOH (ml.)
15	2·05
17	2·65
18	3·17
39	5·52
63	6·77

Asperuloside acetate and aucubin do not react with hydroxylamine under these conditions. These results are in keeping with the presence of at least one potential carbonyl group in the asperuloside molecule, but the experiment is not conclusive for the glucoside undergoes chemical changes under mild conditions. The reactions with other carbonyl reagents are also inconclusive, and an adequate interpretation of these observations awaits a more rigorous chemical study.

Other glycosides similar to aucubin and asperuloside

Aucubin has been reported in many plants besides those from which it has been isolated. Extracts of some plants, particularly members of the family Scrophulariaceae, produce reducing sugar and a dark amorphous precipitate when treated with emulsin. This behaviour is characteristic of true aucubin-containing plants and is shared by those containing asperuloside. By means of the tests described in this paper it has been shown that while some of these glucosides are similar to aucubin they are not identical with it. In addition a very characteristic test with phloroglucinol and HCl, developed by one of us (R.H.) in collaboration with Mr J. L. Crosby, indicates this difference in a striking

manner and shows that similar glycosides occur in the family Labiatae.

The phloroglucinol blue reaction. If a few ml. of an extract of the tubers of *Stachys palustris* (Labiatae) in 50% ethanol is mixed with a few mg. of phloroglucinol and an equal volume of concentrated HCl in the cold, an intense, unstable, green-blue colour develops in a few seconds. These conditions are the same as for the well known phloroglucinol-HCl test for lignin. On dilution with water the colour becomes bright blue and may be extracted quantitatively with butanol or amyl alcohol. If the acid is removed by repeated washing with water the colour becomes rich purple. It is then more stable and may be extracted into alkaline aqueous solutions. With the exception of naphthoresorcinol no other phenolic compound has been found to give such a distinctive colour reaction with the glucoside. Extracts from some other Labiatae: *S. tuberifera, S. sylvatica, Teucrium scorodonia, Ajuga reptans* and *A. chamaepitys* give a similar reaction. These chromogenic substances appear to be glycosides, and unsuccessful attempts were made to isolate them from *Stachys palustris* and *Ajuga reptans*. They were readily adsorbed by charcoal and eluted by aqueous ethanol.

The phloroglucinol blue reaction is given by extracts of some members of the family Scrophulariaceae: *Antirrhinum major, Linaria vulgaris, Linaria cymbalaria, Verbascum thapsus* (and cultivated varieties) and *Scrophularia nodosa*. In these cases the reaction was slower and the first blue colour was of a greener hue, but on dilution with water it became bright blue. Unlike the substances from the Labiatae those from *Antirrhinum major* and *Linaria vulgaris* are stable and may be obtained in a crude amorphous form by the charcoal adsorption method. Filter-paper chromatography using butanol, acetic acid and water or butanol, pyridine and water systems showed that both *Linaria* and *Antirrhinum* contain two components giving identical phloroglucinol-HCl reactions.

Besides the very distinctive colour reactions with phloroglucinol or naphthoresorcinol and HCl all these substances give some of the colour reactions characteristic of aucubin and asperuloside. Some examples are given in Table 7.

Both aucubin and asperuloside react in the phloroglucinol-HCl test, but the colour produced is not blue; aucubin gives a purple colour rapidly, the control without phloroglucinol is brown; and asperuloside gives a purple colour, the control without phloroglucinol is grey-green.

Hitherto asperuloside has been thought to be a specific product of plants in the family Rubiaceae. However, as has been described earlier in this paper, it also occurs in *Daphniphyllum macropodum*, a Chinese plant of uncertain affinity, classified in the family Euphorbiaceae. Also, using the specific colour tests for asperuloside, which showed the presence of asperuloside in *D. macropodum*, two more closely allied glycosides have been detected. One of them, occurring in the uncommon saprophytic plant *Monotropa hypopitys* Walt. is the glucoside monotropéine isolated by Bridel (1923). *Monotropa* is classified in Ericales, family Pyrolaceae. The other glycoside occurs in *Genipa americana* (family Rubiaceae). There are large quantities of it in the pulp of the unripe fruit.

Both glycosides may be partially purified by the charcoal adsorption method. Their solutions give a blue colour on heating with reagent *A* and the spectrum has two characteristic absorption bands in the visible range, a strong band I at 630–640 mμ. and a weaker band II at 600 mμ. If heating is continued the colour becomes purple and a third band III appears at 550–570 mμ. It is unlikely that this is due to contamination with ketose or other substances known to give a purple reaction with asperuloside and reagent *A*, because these characteristic reddening reactions are all given on brief heating of the two glycosides with ketoses, heterocyclic N compounds or phenols and reagent *A*.

Both glycosides give the blue-violet reaction with primary amines which is characteristic for asperuloside. Bridel (1923) states that monotropéine is hydrolysed by emulsin to a blue product. We found that both monotropéine and asperuloside give a blue-violet, water-soluble pigment on incubation at 37° for about 1 hr. with crude emulsin and concluded that the colour is due to the reaction of the glycosides with primary amino groups in the enzyme preparation and does not necessarily depend upon hydrolysis of the glycosidic linkage, for the intensity of the colour varied very greatly with different samples of emulsin and, in the case of asperuloside, occurred after heat inactivation of the enzyme. In support of this it was found that the reaction between asperuloside and glycine to form the blue-violet pigment occurs at 37° in neutral solutions, and is appreciable after less than 30 min. incubation. Under these conditions monotropéine does not react unless emulsin is added; if glycine is also added the rate of colour development is greatly increased.

Our interest in *Genipa americana* was aroused by the description of the dyeing properties of the fruit given by Bancroft (1813). We suggest that the blue or purple colours observed to develop in the bruised tissues and extracts of *Genipa* fruit are due to a reaction between the asperuloside-like glycoside and some aliphatic amino compound also in the plant.

Filter-paper chromatography shows that both monotropéine and the glycoside from *Genipa* are more water-soluble than asperuloside and the ratio of the R_F values of

Table 7. *Colour reactions of glycosides from* Antirrhinum, Ajuga *and* Stachys

Source of glycoside	Colour developed		
Test	*Antirrhinum major*	*Ajuga reptans*	*Stachys palustris*
Phloroglucinol-HCl	Blue	Blue	Blue
Reagent *A*	Green-brown	Purple to blue	Violet to green
Reagent *A* and fructose	Red-brown	Brown to purple	Brown
Reagent *A* and fructose, glucoside previously treated with Br_2	—	Bright red	Brown-violet
Ehrlich's reagent	Green	Blue	Blue
Antimony trichloride	Violet	Brick red	Brown-violet
Shear's reagent in the cold	Red-brown	Brown	Bright red

these compounds and asperuloside run in butanol-acetic acid-water system (Partridge & Westall, 1948) is 1·6:1.

Bridel (1923) showed that monotropéine is a monoglucoside and contains an acidic group.

DISCUSSION

The glycosides aucubin and asperuloside are both derived from aglycones which cannot be isolated in the free state. They give dark resinous products when the glucose is removed, either by mild acid hydrolysis or by the action of hydrolytic enzymes derived from plant sources. It has now been found that if the acid hydrolysis is carried out in the presence of oxygen and a catalyst (reagent *A*) definite coloured products are obtained, which show characteristic absorption spectra.

A variety of reactions given by aucubin and asperuloside have been compared; and it has been concluded that asperuloside has similarities to aucubin which would be explained if the aglycone of asperuloside had a substituted furan structure like that ascribed to the aglycone of aucubin by Karrer & Schmid (1946). Asperuloside is a more reactive and, in certain respects, less stable glycoside than aucubin, as may be seen from the comparison in Table 8.

If the 'aucubin type' of glycosides can be accepted, it is clear that the group is of very wide distribution, and must therefore represent a fairly common feature of plant metabolism. Aucubin itself has already been isolated from members of seven families of plants, and its isolation from *Buddleia*, described here, has added an eighth. The occurrence of aucubin in *Eucommia ulmoides*, described by Plouvier (1944), is of both taxonomic and metabolic interest. This plant is difficult to classify and does not appear to be related to the families already known to produce aucubin.

The characterization of asperuloside as a probable representative of the 'aucubin type' of glycosides extends the taxonomic range of the class; and its isolation from *Daphniphyllum macropodum* (Euphorbiaceae) shows that this glycoside is not a specific product of the Rubiaceae. The detection of a glycoside akin to asperuloside in *Genipa americana* (Rubiaceae) is the first indication that other substances related to asperuloside may be produced by the Rubiaceae. Further, the characterization of 'monotropéine', a product of *Monotropa hypopitys* Walt., placed in the Ericales, family Pyrolaceae, now makes it reasonable to believe that asperuloside and very closely related glycosides may be of wide occurrence.

Table 8. *Summary of the chemical characteristics of aucubin and asperuloside*

	Aucubin	Asperuloside	Remarks
Empirical formula	$C_{16}H_{22}O_9$	$C_{17}H_{24}O_{11}$	Asperuloside has 2-O and 2-H atoms more than aucubin
Acetate	$C_{16}H_{16}O_9(COCH_3)_6$	$C_{17}H_{16}O_{11}(COCH_3)_8$	Asperuloside has 2-OH groups more than aucubin
Acetate + Br_2	Substitution	No substitution	No free —CH= in proposed furan nucleus of asperuloside
Reagent *A*	Blue	Blue	—
Furan reagents	+	+	Asperuloside reacts more slowly
Action of primary amino compounds	None	+ (negative after alkali)	Presence of potential carbonyl group or groups in asperuloside
Action of carbonyl reagents	None	+	
Stability to OH^-	Stable	Rapidly modified, acid liberated, no amine reaction; reaction with reagent *A* unaffected	Lactone group may be present in asperuloside

The colour reactions have been used to test glycoside preparations from a wide variety of plants, some of which have been said to contain aucubin. These reactions provide evidence for the definition of a class of glycosides of the 'aucubin type' showing a number of individual variations of structure (see Table 7). So far aucubin is the only member of this class on which sufficient data have been obtained to justify a constitutional formulation; and even some of the readily accessible plant sources, containing considerable amounts of glycosides of this class, have not, so far, yielded crystalline products.

The three glycosides discussed in the previous paragraph are sharply distinguished from the other glycosides of the 'aucubin type' by their ready reaction with primary amino compounds giving a purple colour. It is clear that this reaction, which is analogous to the reaction of ninhydrin with amino-acids, does not involve a furan nucleus. The capacity of the glycosides to give this reaction is destroyed by brief treatment with mild alkali, whereas the furan-like reactions with acid reagents are unaffected, even by much more drastic alkaline treatment.

The reaction with phloroglucinol and hydrochloric acid, together with some of the other reactions described in this paper, show that the 'aucubin type' of glycoside also occurs in the Labiatae. The aucubin-like glycosides which give the phloroglucinol-hydrochloric acid reaction are found particularly in the Scrophulariaceae, where several structural variants occur. Aucubin itself may be isolated from *Melampyrum* (Braecke, 1923*a*), *Veronica hederaefolia* (Charaux, 1922) and *Rhinanthus* (Braecke, 1923*b*), all Scrophulariaceae. Glycosides giving the phloroglucinol-hydrochloric acid reaction were found in the genera *Stachys* and *Ajuga* in the family Labiatae.

The 'aucubin type' of glycosides is thus characterized by the immediate formation of coloured, resinous secondary bodies on acid hydrolysis of the colourless glycoside and by reactions similar to substituted furan compounds. It cannot be suggested that the furan ring itself is necessarily present in all glycosides of this class, for other types of compounds give some of the reactions of substituted furans. Terpenes like geraniol give colour reactions with phloroglucinol and hydrochloric acid in the cold, and pyruvic acid gives one on heating. The antimony trichloride reaction (Levine & Richman, 1933) and the aniline hydrochloride reaction (Levine *et al.* 1933) are given by some terpenes. In certain cases the two reactions occur readily in the cold, the ease of reaction being increased by unsaturation and the presence of carbonyl groups.

The colour reactions given by the 'aucubin type' of glycosides provide a technique for the study of these substances in relation to the living plant. The only detailed chemical study of these glycosides is that of aucubin by Karrer & Schmid (1946).

SUMMARY

1. The method of Hill & Van Heyningen (1951) has been applied to the isolation of aucubin and asperuloside from plants.

2. Aucubin has been isolated for the first time from *Buddleia globosa* (family Loganiaceae), bringing the number of families known to produce aucubin to eight.

3. Asperuloside has been isolated for the first time from a non-rubiaceous plant, *Daphniphyllum macropodum* (Euphorbiaceae).

4. The empirical formula $C_{17}H_{24}O_{11}$ has been suggested for asperuloside.

5. A crystalline acetate of asperuloside has been obtained.

6. Aucubin, asperuloside and some of their derivatives are extremely acid-labile and under specified acidic conditions produce blue colours. In the presence of some phenols, ketoses and heterocyclic nitrogen compounds characteristic red colours are produced in this reaction.

7. These colour reactions and certain others are shared by other substances, which appear to be glycosides and which occur in the families Scrophulariaceae, Labiatae, Pyrolaceae and Rubiaceae.

8. It has been proposed on the basis of the work of Karrer & Schmid (1946) that all these glycosides could be regarded as substituted furan derivatives like aucubin.

9. The existence of a class of glycosides, typified by aucubin, is postulated. These glycosides are produced by a wide variety of plants.

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The Accumulation and Utilization of Asperuloside in the Rubiaceae

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The significance of glycosides in plant metabolism is obscure, and the present work has been undertaken as a contribution to its elucidation. Large amounts of glycosides are present in many plant cells and although these substances vary widely in their chemical constitution many of them exhibit a common pattern of distribution in the plant. They are accumulated in the vacuoles of young parts of plants during the period of cell expansion, and afterwards may be partly or completely removed as the part ages.

The plants investigated were: *Galium aparine* (cleavers, or goose-grass), *Asperula odorata* (woodruff) and *Rubia tinctorum* (dyer's madder), which belong to the tribe Stellatae of the family Rubiaceae. Most plants produce glycosides of a number of aglycone types, and the Stellatae are known to produce anthocyanins and related glycosides, which are universal among higher plants, anthraquinone glycosides, coumarin glycosides and asperuloside, which probably has a substituted furan structure (Trim & Hill, 1952).

Asperuloside was chosen for study because it occurs in very large quantities in the plants investigated, and because its chromogenic properties provide very simple means for its detection and estimation.

EXPERIMENTAL

The methods of growing and collecting the different plant materials will be described with the experiments in which they were used. The time of collecting was mid-morning. Extracts were made from fresh, weighed material unless otherwise stated. Dry weights were determined after drying equivalent batches of the material for several days at 37° and then over concentrated H_2SO_4 and solid NaOH *in vacuo*. The parts to be extracted were ground with an equal weight of 0·1 N-HCl and a little sand. A few drops of $CHCl_3$ were added and the mush allowed to stand for about 15 min. The ground tissues were then mixed with kieselguhr, filtered on a Büchner funnel and washed with water until the runnings were free from asperuloside. The extract was made up to an appropriate volume with water, neutralized with $CaCO_3$ and filtered.

Estimation of asperuloside. 1 ml. samples of the extracts were mixed with 11 ml. of reagent *A* (Trim & Hill, 1952) and heated on a boiling-water bath for 5 min. with occasional shaking. The characteristic blue derivative of asperuloside developed. The cooled solution was compared with a standard in a Duboscq-type visual colorimeter. Beer's Law applies over a wide range of concentrations as may be seen in Table 1. The effect of the addition of a plant extract is shown in Table 2. The colour developed under these conditions has an absorption spectrum with bands at 645 and 600 mμ. Early in the season, when most parts of the plants were young, very little extraneous colour was developed. Later, when the bulk of the tissues were older, the plants produced sufficient quantities of interfering substances to necessitate the use of a red filter when making the colorimetric measurements. The nature of the interference by substances such as phenols, ketoses and heterocyclic N compounds, which are likely to occur in plants, has been

Table 1. *Colorimetric estimation of known amounts of asperuloside*

Asperuloside (mg.)	Recovery, using a 1 mg. standard (%)
0·25	108
0·50	100
0·75	100
1·0	100

Table 2. *Colorimetric estimation of known amounts of asperuloside in the presence of an extract of* Galium aparine

Extract (ml.)	Asperuloside added as 0·1% solution (mg.)	Total found (mg.)	Recovered (mg.)
0·5	0·0	0·21	0·0
0·5	0·10	0·31	0·10
0·5	0·25	0·46	0·25
0·5	0·50	0·71	0·50
0·0	0·50	0·50	0·50