433. *Structural Investigations on the Water-soluble Polysaccharides from the Green Seaweed* Acrosiphonia centralis (Spongomorpha arcta).

By J. J. O'Donnell and Elizabeth Percival.

Water-soluble sulphated polysaccharides containing D-glucose, D-xylose, L-rhamnose, and D-glucuronic acid, together with small amounts of D-galactose and D-mannose, have been extracted from *Acrosiphonia centralis*. Chloroform-extraction of the acetylated material separates *ca.* 9% of a glucose-rich fraction resembling starch. Further study of the main polysaccharide material revealed the presence of end-group xylose, 1 : 4-linked xylose and rhamnose. and a relatively large proportion of triply linked rhamnose. Sulphate groups are attached to rhamnose and/or xylose residues. All the uronic acid residues appear to be present as end-groups linked to rhamnose. 4-*O*-β-D-Glucopyruronosyl-L-rhamnose was separated and characterised. This polysaccharide is compared with the water-soluble polysaccharides of *Cladophora rupestris* and of *Ulva lactuca*.

In view of the close botanical relation between the green seaweeds, *Cladophora rupestris* and *Acrosiphonia centralis* (*Spongomorpha arcta*), and of the studies on the water-soluble polysaccharide of the former,[1] it seemed desirable to determine whether *A. centralis* synthesised similar material. Collection of pure species of the latter weed is difficult, and we are grateful to Dr. R. Lewin who sent us from Nova Scotia sufficient material for a preliminary investigation. It was at once apparent that the two water-soluble extracts were different and that large-scale investigations of *A. centralis* were warranted. Mr. H. Powell very kindly collected pure species of this weed from a single area at Millport, and the structural investigations now reported have been carried out on the water-soluble polysaccharides isolated from this material.

After removal of free sugars and most of the colouring matter by extraction with aqueous ethanol, water-soluble polysaccharide material was separated (mainly as the ammonium salt) from the residual weed by extraction with aqueous ammonium oxalate. Relatively little glucose remained in the weed after this extraction and it follows therefore that the cellulose content of *A. centralis* must be very small.

In Table 1 the properties of the water-soluble extract and the molar proportions of the sugars present in a partial hydrolysate are compared with those for similar extracts from *Cladophora rupestris* [1] and from *Ulva lactuca*.[2] The neutral sugars in the partial hydrolysate from *A. centralis* were separated and characterised as crystalline sugars or their derivatives. Hydrolysis for longer periods or with more concentrated acid led to extensive degradation.

[1] Fisher and Percival, *J.*, 1957, 2666.
[2] Brading, Georg-Plant, and Hardy, *J.*, 1954, 319.

The sulphate content (3·8%) calculated from the sulphated ash comprises half the total sulphate (7·8%), providing evidence of sulphate esters in the polysaccharide.

The water-soluble polysaccharide material from *C. rupestris* (see Table 1) has a positive

TABLE 1.

Molar proportions	*Cladophora rupestris* [1]	*Acrosiphonia centralis*	*Ulva lactuca* [2]
Arabinose	3·7	—	—
Galactose	2·8	0·1	—
Xylose	1·0	1·6	1·3
Rhamnose	0·4	1·4	4·4
Glucose	0·2	1·0	1·0
Mannose	—	0·2	—
Uronic acid (%)	3·0	19·3	20·8
SO_4^{2-} (%)	16·1	7·8	17·5
Ash (%)	13·7	10·0	19·0
$[\alpha]_D$ (in H_2O)	+69°	−31°	−47°

rotation and consists mainly of arabinose, galactose, and xylose units: the polysaccharides from the two other green seaweeds have negative rotations and comprise mainly glucose, xylose, rhamnose, and uronic acid residues. Although all three extracts contain ester sulphate groups it is plain that the extract from *A. centralis* more nearly resembles that from *U. lactuca*. The difference in the relative proportions of the sugars in the two extracts containing much uronic acid may be due, at least in part, to the resistance of polyuronides to hydrolysis; the *A. centralis* hydrolysate comprises *ca.* 45% of oligouronic acids containing uronic acid and sugar residues.

The sulphate (7·8%) and uronic anhydride (20%) in *A. centralis* extract correspond to about one residue to every eight and four sugar units respectively. A hydrolysate with 20% of uronic acid, present as unhydrolysed barium uronosylaldose, would contain *ca.* 16% of aldose and 6% of barium; *i.e.*, 40—45% of the neutralised hydrolysate would consist of barium uronates, which is in agreement with the experimental findings.

The free-acid polysaccharide has an equivalent weight of 459. In the absence of sulphate and ash, a 20% uronic acid content corresponds to an equivalent weight of 870. A sulphate content of 7·8% represents 67 g. (0·70 equivalent) of sulphate in 870 g. Hence 870 g. correspond, not to 1, but to 1·70 equivalents, and the calculated equivalent weight of a polysaccharide containing 20% of uronic acid and 7·8% of sulphate is 870/1·70, *i.e.*, 512. The uronic acid and sulphate content were determined on neutral polysaccharide which contained 10% of ash; it is permissible therefore to deduct 10% in the above calculation of the theoretical equivalent weight. The final calculated value then becomes 461, in excellent agreement with the experimental value.

The periodate consumption by the polysaccharide corresponds to *ca.* 1 mole, and the formic acid release to *ca.* 0·6 mole, for every anhydro-sugar residue. These results indicate that many of the units have free contiguous hydroxyl groups. An oxopolysaccharide was isolated, after dialysis, in 68% yield, and hydrolysis showed that this contained intact xylose and rhamnose units. There is, therefore, an indication of the presence of either 1 : 3-linked units or of branch points in the molecule.

Attempted fractionation of the free polysaccharide was unsuccessful. Acetylation, followed by chloroform-extraction, gave, however, a glucose-rich acetate (A) (9%), $[\alpha]_D$ +71° (devoid of sulphate). The deacetylated material resembled starch in its reactions with iodine. Methylation of this acetate (A) and hydrolysis of the product, followed by separation and characterisation of the methylated sugars, confirmed this structure, and also indicated some branching at position 6 of the sugar units. 2 : 3 : 4 : 6-Tetra- (1 part), 2 : 3 : 6-tri- (4 parts), and 2 : 3-di-*O*-methylglucose (1 part) were separated and identified. 2 : 3 : 6-Tri-*O*-methylmannose (*ca.* 10%) was also separated from this hydrolysate, proving the presence of 1 : 4-linked mannose residues, and suggesting the presence of a small quantity of a mannan or glucomannan. The presence of small amounts of 2 : 3 : 4-tri- and

2 : 3-di-*O*-methylxylose and of methylated acids is considered to be due to incomplete separation of the main polysaccharide material.

After removal of the glucose-rich fraction from the acetylated *A. centralis* extract the residual material (B), $[\alpha]_D$ −38°, was methylated and the product (OMe, 35·6%) hydrolysed. 2 : 3 : 4-Tri-*O*-methylxylose (1 part), 2 : 3-di-*O*-methylxylose (3 parts), 2 : 3-di-*O*-methylrhamnose (3 parts), and 2-*O*-methylrhamnose (5 parts), together with small quantities of 2 : 3 : 6-tri- and 2 : 3-di-*O*-methylglucose, were separated and characterised. Since both the methylated glucoses separated from this fraction were also present in the hydrolysate of the methylated fraction (A), their presence in both fractions is probably due to incomplete separation of the glucan from the polysaccharide(s) acetate.

These observations reveal structural similarities between the polysaccharides of the two weeds, *Ulva* and *Acrosiphonia*. Both polysaccharides carry xylose end groups and have 1 : 4-linked xylose and rhamnose. The isolation of a monomethylrhamnose, in relatively large quantity, from the methylated *Acrosiphonia* extract, is evidence that rhamnose occurs at branch points in the molecule.

In spite of the use of a wide variety of hydrolytic conditions no-one, so far as the authors are aware, has yet succeeded in isolating glucuronic acid or its lactone from any of the green seaweeds, the conditions required for complete hydrolysis invariably leading to degradation. Fractionation of the acidic material from the partial hydrolysate of the extract from *A. centralis* led to the separation of di- (10%), tri- (4%), and tetra-oligouronic acids (3%). A large polymeric fraction (65%) was also isolated, prolonged acid hydrolysis of which afforded the original mixture of oligouronic acids. Glycosidation, reduction, and hydrolysis of the first three fractions gave syrups consisting of varying proportions of glucose and rhamnose. The syrup from the first fraction had $[\alpha]_D$ −6° and was shown by quantitative chromatography [3] to contain equimolar proportions of D-glucose and L-rhamnose, the former being characterised by oxidation by the specific enzyme,[4] glucose oxidase, and the latter by formation of the authentic crystalline benzoylhydrazone. It is considered therefore that the glucose arose from the reduction of glucuronic acid residues. The ester glycoside of this fraction consumed 2·8 moles of periodate per mole, indicating that linkage occurred through position 2 or 4 of the rhamnose residue; methyl 2- or 4-glucuronosylrhamnoside requires 3 mols. of periodate for complete oxidation, whereas methyl 3-glucuronosylrhamnoside reduces only 2 mols. Methylation of the reduced ester glucoside confirmed these findings. Crystalline 2 : 3 : 4 : 6-tetra-*O*-methylglucose and syrupy 2 : 3-di-*O*-methylrhamnose were separated from the hydrolysate of the methylated disaccharide. The tetra-*O*-methylglucose could only have arisen from glycosidically linked glucuronic acid, and the biuronic acid has therefore the constitution 4-*O*-β-D-glucopyruronosyl-L-rhamnose, the β-configuration being inferred from the negative specific rotation. The chromatographic mobility ($R_{glucose}$ *ca.* 1·0) of this fraction is high for an aldobiuronic acid (cf. 2-*O*-D-glucuronosyl-L-rhamnose, $R_{glucose}$ 0·24) and it seemed likely that, in the free state, it existed as the lactone. Chromatographic support for this conclusion was obtained, inasmuch as it behaved in the same way as glucurone on similar treatment. Both migrated back from the starting line on ionophoresis at pH 5·5, indicating the absence of charged groups at this pH, whereas at pH 10 their respective M_G values are 0·75 and 0·96; in addition they each gave two spots if treated with ammonia before development. In this connection it is worth noting that the equivalent weight (328) of this fraction is closer to the calculated value (322) for the lactone of glucuronosylrhamnose than to the calculated value for the free biuronic acid (340).

The trisaccharide had equivalent weight 249 and molecular weight 508: a diglucuronosyl-*O*-rhamnose would require 257 and 514 respectively. In agreement with this structure, glycosidation, reduction, and hydrolysis afforded glucose and rhamnose in the molar proportions of *ca.* 2 : 1.

[3] Pridham, *Analyt. Chem.*, 1956, **28**, 1967.
[4] Keilin and Hartree, *Biochem. J.*, 1948, **42**, 230.

The tetrasaccharide fraction was difficult to purify and, although equimolar proportions of glucose and rhamnose were obtained on hydrolysis of the reduced material, the values for the equivalent and molecular weights cannot be explained on this basis and further evidence must be awaited.

Reduction and hydrolysis of the glycoside ester of the acidic polymeric fraction afforded equimolar proportions of glucose and rhamnose, together with a very small amount of xylose. The low yield (24%) from this reaction is attributable to the low solubility of the material in the organic solvents employed for reduction.

Methylation studies of the acidic material confirmed these results. After removal of the neutral methylated sugars the methylated oligouronic acids were separated on thick paper. After glycosidation, reduction, and hydrolysis, the first fraction gave equimolar proportions of 2 : 3 : 4-tri-*O*-methyl-D-glucose and 2-*O*-methyl-L-rhamnose. This fraction therefore consisted of 2 : 3 : 4-tri-*O*-methyl-4-*O*-D-glucopyruronosyl-2-*O*-methyl-L-rhamnose and almost certainly originated from the same portion of the molecule as the 4-*O*-D-glucopyruronosyl-L-rhamnose, isolated from the hydrolysate of the unmethylated extract. Furthermore it follows that these units must be linked to the rest of the molecule through the hydroxyl group on position 3 of the rhamnose. Each of the remaining acidic fractions was similarly treated and each gave rise to approximately equimolar proportions of 2 : 3 : 4-tri-*O*-methylglucose and a non-reducing syrup with a high chromatographic mobility (R_G 1·15). The latter material did not reduce periodate and gave a positive test for methoxyl. These properties are in agreement with the structure, 1 : 4-anhydro-2-*O*-methylrhamnitol. Although it has been shown that rhamnitol, on treatment with acid, gives the 1 : 4-anhydro-derivative [5] it is difficult to see how this product could have arisen from the methylated acid fraction. Only if cleavage of the glycosidic links occurred before or during the reduction would rhamnitol be produced. Although comparison with reduced 2-*O*-methylrhamnose suggests that this non-reducing syrup derived from the acid fraction probably arose from rhamnose residues in the polysaccharide its characterisation as 1 : 4-anhydro-2-*O*-methylrhamnitol can only be regarded as tentative.

Methylation failed to reduce the proportion of sulphate groups in the polysaccharide (B). The allocation of these groups to individual sugar residues is a difficult problem; their attachment to glucose residues is very unlikely in view of the separation of a sulphate-free glucan and of the small amount of glucose in polysaccharide (B). The reduced glucuronic acid residues were isolated solely as 2 : 3 : 4-tri-*O*-methylglucose and must therefore occur as glycosidically linked end-groups with all the hydroxyl groups free for methylation. This leaves only xylose and/or rhamnose for union with the sulphate groups. 2 : 3-Di-*O*-methylxylose and 2 : 3-di-*O*-methylrhamnose were isolated in approximately equal quantities together with about twice as much mono-*O*-methylrhamnose. There are barely enough xylose residues to accommodate all the sulphate groups. Furthermore, if sulphate is linked to position 4 of those xylose units isolated as 2 : 3-di-*O*-methylxylose then practically all the xylose residues in the molecule are present as end-groups, many of which are sulphated. A likely site, therefore, for some at least of the sulphate groups, is on the rhamnose residues.

The polysaccharide(s) (B) must have a highly complicated structure. At least part of the molecule consists of 1 : 3-linked rhamnose residues carrying glucuronic acid units linked at position 4. The results of methylation studies have revealed the presence of the following residues:

GA...	...4 R 1...	...4 R 1... 3 ⋮	...4 X 1...	X 1...	...4 G 1...	...4 G 1... 6 ⋮
(25%)	(11%)	(21%)	(11%)	(4·2%)	(1·7%)	(0·6%)

GA = glucuronic acid, G = glucose, R = rhamnose, X = xylose.

[5] G. Buchanan, personal communication.

The rest of the material (*ca.* 24%) comprises the non-reducing material isolated from the acid fraction.

Experimental

Analytical Methods.—All solutions were evaporated under reduced pressure below 60°. Paper partition chromatography was carried out on Whatman No. 1 paper with upper layers of the following solvent systems (v/v) (1) butan-1-ol–benzene–pyridine–water (5 : 1 : 3 : 3); (2) ethyl acetate–pyridine–water (10 : 4 : 3); (3) ethyl acete–acetic acid–formic acid–water (18 : 4 : 1 : 5); (4) ethyl acetate–acetic acid–water (3 : 1 : 3); (5) butan-1-ol–acetic acid–water (4 : 1 : 5); (6) butan-1-ol–ethanol–water (4 : 1 : 5); (7) ethyl methyl ketone half saturated with water plus ammonia (99 : 1); (8) ethyl acetate–acetic acid–water (9 : 2 : 2). Papers were sprayed with a saturated aqueous solution of aniline oxalate. R_G, R_{GI}, G_{GA}, and R_F are the rates of travel relative to tetramethylglucose, glucose, glucuronic acid (glucurone co-spotted with ammonia), and the solvent front respectively. Ionophoresis [6] was carried out (750 v; 12·5 mA) in borate buffer at pH 10, and the ionophoretograms were sprayed with saturated aqueous aniline oxalate containing 20% of acetic acid. Specific rotations were measured for solutions in water at 18°.

Acrosiphonia centralis (100 g. dry), collected from Millport in April and June 1957, was thoroughly washed with sea-water, dried in a current of air at room temperature, and extracted under reflux with 85% aqueous ethanol until the alcoholic extracts were colourless. The residual weed was then exhaustively extracted at 95° with 1% ammonium oxalate solution and with water, and at room temperature with 0·01N-hydrochloric acid, 2% aqueous sodium carbonate, and 5% aqueous potassium hydroxide. The extracts were each dialysed until free from inorganic material, and the polysaccharides isolated by freeze-drying. Chromatography [3] of the hydrolysate of each extract revealed that they all contained the same sugar residues, and in approximately the same proportions. The weed remaining after extraction with ammonium oxalate was kept for 10 days in 72% sulphuric acid solution.[7] The filtered solution was diluted with water to a strength of N, then heated at 100° for 7 hr. The resulting syrup contained glucose : xylose : rhamnose (1 : 1 : 1) together with minute quantities of oligouronic acids (paper chromatography, visual examination).

Extraction with ammoniun oxalate gave the highest yield (12% of the dry weight of weed) of water-soluble polysaccharide, and a comparison of the properties of this extract with those of the hot water extract (4·5% yield) demonstrated the essential similarity of the two materials:

Extract	Ash (%)	Sulphate (%)	Nitrogen (%)
Hot water	13·9	7·8	1·0
Ammonium oxalate	10·0	7·8	4·2

In view of these findings all the subsequent investigations were carried out on an ammonium oxalate extract.

The polysaccharide (12 g.) was isolated, as an off-white powder, by freeze-drying. It had $[\alpha]_D$ −31° (*c* 1·2) [Found: N (as ammonium salt by direct distillation with alkali) 3·6, (Kjeldahl) 4·2; ash (direct) 10·0; (as sulphate) 11·8; SO_4^{2-} in ash 32; total SO_4^{2-}, 7·8; uronic anhydride,[8] 20·3%].

Attempted Fractionation of the Aqueous Extract.—10% Aqueous cetyltrimethylammonium bromide (5 c.c.) was added dropwise, with stirring, to an aqueous solution of the extract (189 mg. in 20 c.c.). Regeneration [9] of the polysaccharide from the complex and from the supernatant liquid gave 132 mg. (SO_4^{2-}, 8·1%) and 37 mg. (SO_4^{2-}, 9·5%) respectively. Four successive fractionations in all were carried out; in each case hydrolysates of material both from the complexes and the supernatant layers revealed the presence of glucose, xylose, and rhamnose with no significant difference in their relative proportions (paper chromatography).

Treatment of the extract with aqueous solutions of copper sulphate and copper acetate failed to achieve fractionation.[10]

Equivalent-weight Determination.—An aqueous solution of the polysaccharide (133 mg. in

[6] Foster, *Chem. and Ind.*, 1952, 1050.
[7] Monier-Williams, *J.*, 1921, **119**, 803.
[8] Swenson, McCready, and Maclay, *Ind. Eng. Chem. Anal.*, 1946, **18**, 290.
[9] Bera, Foster, and Stacey, *J.*, 1955, 3788.
[10] Hough, Jones, and Wadman, *J.*, 1952, 3393.

20 c.c.) was recycled four times through a column of Amberlite IR-120H resin (10 g.), and the eluant freeze-dried to a white powder (Found: ash, nil; SO_4^{2-}, 7·5%). The equivalent weight of this material, determined by titration with 0·01N-sodium hydroxide, was 459 (mean of several determinations).

Hydrolysis of the Polysaccharide.—(*a*) The polysaccharide (0·335 g.) was hydrolysed with N-sulphuric acid (30 c.c.) at 100°. The course of the hydrolysis was followed by chromatography and measurement of specific rotation and reducing power (hypoiodite reduction [10]) on aliquot parts:

Time (hr.)	0	0·5	1	2	4	6	8
Iodine (0·01N; c.c.)	1·9	5·4	7·1	8·6	9·7	—	—
$[\alpha]_D$	−29°	−12°	−4°	+2°	+6°	+4°	+6°

Chromatography (solvent 1) revealed that glucose, xylose, and rhamnose were released within the first hour, accompanied by traces of neutral oligosaccharides; the oligosaccharides had entirely disappeared in 8 hr. The presence of uronic acids was indicated by a series of pink spots near the starting line. Traces of mannose and galactose were also visible.

(*b*) The polysaccharide (1 g.) was heated under reflux with 3% methanolic hydrogen chloride (25 c.c.) for 6 hr.; 15% methanolic hydrogen chloride (15 c.c.) was then added at 16-hourly intervals, four times in all (total hydrolysis time 70 hr.). Water (30 c.c.) was added and the mixture heated at 100° for 6 hr. Chromatography in solvent (3) failed to reveal the presence of glucuronic acid or its lactone.

(*c*) The polysaccharide (1 g.) was heated with 98% formic acid (50 c.c.) under reflux in nitrogen for 7 hr. The neutral sugars were separated by elution through a column of IR-45B resin (acetate form), and the column was washed with water until the eluant gave a negative Molisch reaction. The acid fraction was recovered by elution with N-formic acid. After repeated extraction with ether the aqueous-acid fraction was evaporated to dryness and exhaustively extracted with ethanol. Chromatography (solvent 3) of the concentrated ethanolic extracts revealed spots of R_{Gl} 1·05, M_G 0·75, and R_{Gl} 0·88 and 0·63 respectively (cf. glucurone R_{Gl} 2·4).

Partial Hydrolysis of the Polysaccharide and Separation of the Sugars.—The polysaccharide (3·25 g.) was hydrolysed with N-sulphuric acid for 7 hr. at 100°. After neutralisation of the cooled solution with barium carbonate, the hydrolysate (2·2 g.) was obtained as an amorphous solid. Quantitative analysis of the neutral sugars in this hydrolysate by a colorimetric method [3] gave the molar proportions of glucose (1·0), xylose (2·1), rhamnose (1·3); and by sodium periodate oxidation [11] glucose (1·0), galactose (0·12), mannose (0·21), xylose (2·2), and rhamnose (1·6). A portion of the hydrolysate (1·75 g.) was fractionated on a cellulose column (2·8 × 60 cm.) with butan-1-ol half saturated with water as eluant. R_F values are recorded for solvent (1).

Fraction 1. A yellow syrup (28·8 mg.), R_F 0·09, $[\alpha]_D$ −4° (*c* 2·29) (Found: OMe, nil), gave a positive Selivanoff test.

Fraction 2. Crystalline L-rhamnose hydrate (139 mg.), m. p. and mixed m. p. 68°, R_F 0·55; the derived benzoylhydrazone had m. p. and mixed m. p. 186°.

Fraction 3. A syrup (18·5 mg.), R_F 0·46, was chromatographically distinct from ribose and gave a positive Selivanoff test.

Fraction 4. Crystalline D-xylose (264 mg.), m. p. and mixed m. p. 145°, R_F 0·44, $[\alpha]_D$ +19° (*c* 1·0). The derived dibenzylidene dimethyl acetal had m. p. 186°.

Fraction 5. A syrup (39·0 mg.), R_F 0·38. Although contaminated with xylose it was mainly mannose (solvents 1 and 3); its identity was confirmed by isolation of mannose phenylhydrazone m. p. and mixed m. p. 188°.

Fraction 6. A syrup (56·6 mg.), R_F 0·33, $[\alpha]_D$ +53° (*c* 0·25); its identity as D-glucose was established by formation of the dichlorophenylhydrazone, m. p. and mixed m. p. 153°.

Fraction 7. A syrup (21 mg.), R_F 0·28, chromatographically identical with galactose (solvents 1 and 3). The derived diethyl mercaptal had m. p. and mixed m. p. with the diethyl mercaptal of D-galactose, 140—142°.

Fraction 8. Eluted with water, and isolated as an amorphous solid (820 mg.), R_F 0—0·07.

Examination of the acidic fraction. Fraction 8 (2·14 g., the combined products from two separations) was converted into the free acid (1·57 g.) by treatment with IR-120(H^+) resin and

[11] Hirst and Jones, *J.*, 1949, 1659.

an aqueous solution (10 c.c.), mixed to a slurry with cellulose powder, and freeze-dried. The product was applied to the top of a cellulose column (74 × 3·6 cm.) and eluted with solvent (8); R_{Gl} values recorded below are for solvent (3). Seven fractions were separated. Each of the fractions, after evaporation to small volume (10 c.c.), was treated with barium hydroxide (to remove traces of sulphuric acid) and filtered. The filtrate was exhaustively treated with ether in a continuous extractor to remove final traces of acetic acid. Deionisation with IR-120(H^+) resin afforded clear acidic syrups.

(*a*) Fraction 1 (0—2·5 l.) (144 mg.). Chromatography showed glucose, xylose, and rhamnose together with considerable streaking.

(*b*) Fraction 2 (2·5—3·6 l.). An aldobiuronic acid (227 mg.), $[\alpha]_D$ −6° (*c* 0·5), R_{Gl} 1·05, R_{GA} 0·85 (solvent 4); co-spotting with ammonium hydroxide before elution gave a second spot, R_{Gl} 0·57 (cf. D-glucurone R_{Gl} 2·4 and 1·0 under these conditions), M_G (in borate buffer pH 10) 0·75, (in acetate buffer pH 5·5) 0·0 (cf. glucose and glucurone which behaved similarly). Co-spotting with ammonia before ionophoresis in acetate buffer gave a second spot *ca.* 15 cm. from the starting line. Galacturonic acid in acetate buffer moves *ca.* 14 cm. in 5 hr. (Found: equiv., 328, by titration). The reducing power, compared with that of rhamnose monohydrate as standard, was measured by treatment with iodine in excess of sodium hydroxide solution. The mixture was set aside for 2 hr., then acidified with sulphuric acid and titrated with sodium thiosulphate. The aldobiouronic acid (12·4 mg.) had the reducing power of 6·55 mg. of rhamnose monohydrate. This corresponds to a molecular weight of 344. Hydrolysis of a portion with 2N-sulphuric acid gave only degradation products of the furfuraldehyde type (paper chromatography). A portion (204 mg. and 50 mg. from a previous fractionation) was heated under reflux with 2% methanolic hydrogen chloride for 6 hr. The resulting ester glycoside (266 mg.) was suspended in tetrahydrofuran (30 c.c.) which had been dried by distillation from sodium and from lithium aluminium hydride. A saturated suspension of the hydride in tetrahydrofuran (6 c.c.) was then added dropwise, and the solution refluxed for 1 hr. Dropwise addition of water (18 c.c.) was followed by filtration of precipitated aluminium hydroxide and treatment with IR-120(H^+) resin. After evaporation of the organic liquid the remaining aqueous solution (R) was diluted with distilled water to 50 c.c. To a portion of this (10 c.c.) an equal volume of 2N-sulphuric acid was added, and the mixture heated at 100° for 6 hr. Deionisation with IR-4B(OH^-) resin, evaporation to dryness, and methanol-extraction of the residue afforded a syrup (33·7 mg.). Chromatography (solvents 1 and 3), together with colorimetric estimation, revealed the presence of glucose and rhamnose in the molar proportion of 1 : 0·95. The hydrolysed material (30 mg.) was treated with glucose oxidase,[4] and the enzyme was later removed by addition of equivalent solutions of cadmium sulphate and barium hydroxide and filtration of the resulting precipitate. Evaporation followed by methanol-extraction yielded a syrup which contained only rhamnose (paper chromatography). The derived benzoylhydrazone had m. p. and mixed m. p. 185°.

A portion of the diluted solution (R) (4 c.c.) was deionised with IR-120(H^+) and IR-4B(OH^-) resin and evaporated to a clear glass (14·2 mg.) which was oxidised with 0·015M-sodium periodate (10 c.c.). The reduction of periodate after 44 hr. was found [12] to be equivalent to 2·8 moles of periodate per mole of disaccharide glycoside and proceeded only very slowly beyond this figure.

The remainder of solution (R) (containing 128 mg.) was methylated with 25% aqueous sodium hydroxide in nitrogen at 5°, and dimethyl sulphate (17 c.c.) was added dropwise with cooling and stirring during 6 hr. Next morning the mixture was remethylated twice more and then extracted with chloroform. Evaporation of the chloroform extracts afforded the reduced methylated disaccharide glycoside (98 mg.), $[\alpha]_D$ +7° (*c* 2·0 in methanol). Hydrolysis with aqueous-methanolic N-hydrogen chloride (1 : 1) gave a syrup (84 mg.) which was separated on 3MM paper (40 × 40 cm.) by elution with solvent (7). R_G values are recorded below for solvent (6).

Fraction (i). 2 : 3 : 4 : 6-Tetra-*O*-methylglucose (36 mg.), m. p. and mixed m. p. 94°, $[\alpha]_D$ 79° (*c* 0·9), R_G 1·0 (Found: OMe, 51·9. Calc. for $C_{10}H_{20}O_6$: OMe, 52·5%).

Fraction (ii). 2 : 3-Di-*O*-methylrhamnose (34 mg.), $[\alpha]_D$ +42° (*c* 2·9), R_G 0·86, M_G 0·02. Oxidation with periodate and chromatography of the product [13] revealed one spot, of R_F 0·81. Comparison with the oxidation product of authentic 2 : 4-di-*O*-methylrhamnose revealed spots of R_F 0·72, 0·78, and 0·85 (solvent 6). Cf. page 2177, fraction 2.

[12] Aspinall and Ferrier, *Chem. and Ind.*, 1957, 1216.
[13] Lemieux and Bauer, *Canad. J. Chem.*, 1953, **31**, 814.

(*c*) Fraction 3 (3·6—4·4 l.) (58 mg.). Chromatography showed this to be a mixture of fractions 2 and 4.

(*d*) Fraction 4 (4·4—5·2 l.) (45 mg.), $[\alpha]_D$ +4° (*c* 0·5), R_{Gl} 0·88, R_{GA} 0·68 (solvent 8) (Found: equiv. 514, by titration; *M*, 508. Calc. for diglucuronosyl-*O*-rhamnose $C_{18}H_{28}O_{17}$: *M*, 516). Conversion into the ester glycoside and reduction, as for fraction 2, gave on hydrolysis a syrup consisting of glucose : rhamnose [3] 1·76 : 1.

(*e*) Fraction 5 (5·2—5·6 l.) (27 mg.) was a mixture of fractions 4 and 6.

(*f*) Fraction 6 (5·6—6·4 l.). The syrup (51 mg.), R_{Gl} 0·63, R_{GA} 0·44 (solvent 8) (Found: equiv. 690, by titration; *M*, 1050), on glycosidation, reduction, and hydrolysis, yielded a syrup containing glucose : rhamnose = 1 : 0·83.

(*g*) Fraction 7 (aqueous eluant) (974 mg.), R_{Gl} 0—0·4. Further hydrolysis (16 hr., 2N-HCl at 100°) of a portion of this syrup and chromatography of the hydrolysate revealed spots corresponding to the acidic oligosaccharides described above. Esterification, reduction, and hydrolysis afforded a syrup containing glucose : rhamnose = 1 : 0·88, together with a trace of xylose.

Periodate Oxidation of the Extract.—The extract (0·153 g.) in water (30 c.c.) was treated with 0·095M-sodium periodate (30 c.c.). The reduction of periodate and release of formic acid were measured on samples (1 c.c.) withdrawn at intervals: [1]

Time (hr.)	1·25	3·5	8	25	32	48	96
Moles of periodate reduced/anhydro-unit	0·47	0·61	0·70	0·90	0·98	1·08	1·20
Moles of formic acid released/ ,,	0·138	0·177	0·215	0·250	0·256	0·397	0·586

Oxidation was stopped after 96 hr. by passing sulphur dioxide through the cooled mixture. After dialysis, freeze-drying afforded the oxopolysaccharide (204 mg.) (Found: ash, 6·7; SO_4^{2-}, 8·6%). Chromatography of the hydrolysate (7 hr., N-H_2SO_4 at 100°)revea led the presence of small amounts of xylose and rhamnose.

Acetylation of the Polysaccharide.—The polysaccharide (13·8 g.), dispersed in formamide (100 c.c.) and pyridine (700 c.c.), was shaken with acetic anhydride (200 c.c.) during 72 hr. The mixture was added slowly with cooling to an equal volume of water. After dialysis and evaporation to small volume the polysaccharide (15·6 g.) was isolated by freeze-drying (Found: Ac 13·6%). Reacetylation gave an off-white powder (14·5 g.) (Found: Ac, 22·4%). Further treatment with acetic anhydride failed to increase the acetyl content. The acetylated material (12·7 g.) was exhaustively extracted with chloroform. Addition of light petroleum to the concentrated chloroform extracts afforded a white precipitate (polysaccharide acetate A; 0·91 g.). The chloroform-insoluble material (11·5 g.) will henceforth be called polysaccharide acetate (B).

Polysaccharide (*A*).—Polysaccharide acetate (A) had $[\alpha]_D$ +71° (*c* 1·2 in $CHCl_3$) (Found: SO_4^{2-}, nil). Deacetylation of a portion with sodium methoxide afforded a polysaccharide which gave a purple colour with iodine. This property was destroyed by prior treatment with α-amylase.[14] Hydrolysis of a portion and determination [3] of the molar proportions of the sugars present gave glucose : mannose : xylose = 1·0 : 0·31 : 0·17.

Deacetylation and methylation. The acetate (A) (0·82 g.), dissolved in ice-cooled 30% aqueous sodium hydroxide (20 c.c.), was treated dropwise with dimethyl sulphate (9 c.c.) with stirring in nitrogen during 6 hr. After being kept overnight the mixture was treated with a further quantity of sodium hydroxide and dimethyl sulphate as before.[15] The mixture was neutralised (pH 7) with sulphuric acid and dialysed until free from inorganic ions. Thallium hydroxide (2 g.) was added and the mixture freeze-dried. The resulting white powder was refluxed with methyl iodide (10 c.c.) until the solution was no longer alkaline (16 hr.).[16] After evaporation of the methyl iodide the residue was extracted at room temperature with methanol (3 × 25 c.c.), and with 50% aqueous methanol (3 × 25 c.c.); under reflux with 50% aqueous methanol (3 × 25 c.c.) and with water (3 × 25 c.c.). The combined extracts were methylated as before. After a third methylation and removal of the methyl iodide the residue was extracted with chloroform. Removal of the chloroform afforded a syrup (0·34 g.). Methylation with Purdie reagents twice gave a methylated product (0·324 g.) with $[\alpha]_D$ +42° (*c* 1·03 in $CHCl_3$) (Found: OMe, 38·6%). Hydrolysis with 4% methanolic hydrogen chloride (20 c.c.) under

[14] Rundle, Foster, and Baldwin, *J. Amer. Chem. Soc.*, 1944, **66**, 2116.
[15] Haworth, *J.*, 1915, **107**, 13.
[16] Fear and Menzies, *J.*, 1926, 937.

reflux for 3·5 hr. was followed by addition of N-hydrochloric acid (20 c.c.) and heating during a further 3·5 hr. The resulting syrup (0·28 g.) was fractionated on a cellulose column (1·9 × 45 cm.) with water-saturated light petroleum–butan-1-ol (7 : 3). R_G values are recorded below for solvent (6). The products of demethylation were identified by paper chromatography.[1]

Fraction 1 (1—71 c.c.). Syrupy 2 : 3 : 4 : 6-tetra-*O*-methylglucose (21 mg.), R_G 1·0, $[\alpha]_D$ +73° (*c* 0·24) (Found: OMe, 51·0. Calc. for $C_{10}H_{20}O_6$: OMe, 52·5%). Demethylation gave only glucose. The derived aniline derivative had m. p. and mixed m. p. 135°.

Fraction 2 (71—189 c.c.). A syrup (11·2 mg.) which revealed spots of R_G 1·0, 0·91, and 0·81, corresponding to 2 : 3 : 4 : 6-tetra- and 2 : 3 : 6-tri-*O*-methylglucose and 2 : 3 : 4-tri-*O*-methylxylose respectively, on chromatography. Demethylation gave glucose and xylose.

Fraction 3 (190—364 c.c.). A syrup (84 mg.), R_G 0·81, chromatographically identical with 2 : 3 : 6-tri-*O*-methylglucose in solvents (6) and (7), and having $[\alpha]_D$ +72° (*c* 0·78) (Found: OMe, 40·3. Calc. for $C_9H_{18}O_6$: OMe, 41·9%). It gave only glucose on demethylation. An aliquot part (*c* 1·12) was kept at room temperature in 0·1N-methanolic hydrogen chloride and the change in rotation observed:

Time (hr.)	0·2	1	4	5	21	29	40	45	
$[\alpha]_D$.....	+0·74°	+0·52°	+0·26°	+0·24°	−0·19°	−0·25°	−0·32°	−0·32°	Final $[\alpha]_D$ −0·29°

Hydrolysis with 0·5N-hydrochloric acid gave the parent sugar. The derived lactone had $[\alpha]_D$ +33°.

Fraction 4 (379—469 c.c.). A syrup (16·5 mg.), R_G 0·76 (cf. 2 : 3-di-*O*-methylxylose, R_G 0·76; 2 : 4-di-*O*-methylxylose R_G 0·69), M_G 0·02, $[\alpha]_D$ +9° (*c* 0·7); it gave only xylose on demethylation.

Fraction 5 (470—553 c.c.) (19 mg.), R_G 0·76. Demethylation gave mannose and xylose.

Fraction 6 (554—1036 c.c.). A syrup (16 mg.), $[\alpha]_D$ +16° (*c* 0·8 in MeOH), R_G 0·76 identical with 2 : 3 : 6-, and distinct from 2 : 4 : 6- and 3 : 4 : 6-tri-*O*-methylmannose run as controls. It gave mannose on demethylation.

Fraction 7 (1037—1365 c.c.). A syrup (27 mg.), $[\alpha]_D$ +52° (*c* 0·9 in acetone) R_G 0·56 (Found: OMe, 29·2. Calc. for $C_8H_{16}O_6$: OMe, 29·6%), gave only glucose on demethylation and was chromatographically identical with 2 : 3-di-*O*-methylglucose run as a control (solvents 6 and 7). The derived aniline derivative had m. p. 133°, undepressed on admixture with specimen kindly given by Professor M. Stacey, F.R.S.

Fraction 8 (aqueous eluant). An amorphous solid (12·3 mg.), $[\alpha]_D$ −9° (*c* 0·3). Deionisation [IR-120(H^+] resin) gave an acidic syrup which revealed spots of R_F 0·70, 0·45, 0·40, 0·34, and 0·15 respectively (paper chromatography, solvent 5).

Polysaccharide (B).—The chloroform-insoluble acetate (B) had $[\alpha]_D$ +38° (*c* 1·15 in C_5H_5N) (Found: Ac, 22·0; SO_4^{2-}, 5·7%). Determination of the molar proportions [3] of the sugars present in a hydrolysate gave galactose : glucose : xylose : rhamnose = 0·06 : 0·56 : 1·69 : 1·31. Deacetylation and methylation of the residual material (11·3 g.) as for polysaccharide (A) gave a methylated product (5·3 g.), $[\alpha]_D$ −28° (*c* 1·3 in $CHCl_3$) (Found: OMe, 35·1; SO_4^{2-}, 5·4%). Further Purdie methylations failed to raise the methoxyl content. Fractionation of the methylated material (5·12 g.) by treatment with light petroleum (b. p. 60—80°) and addition of increasing quantities of chloroform gave the following fractions. Complete dissolution occurred in a mixture containing 40% chloroform (v/v) :

Fraction	1	2	3	4	5	6	7	8
$CHCl_3$ in mixture (%)	0	5	10	15	20	25	30	40
Weight (mg.)	24	47	106	386	635	972	943	2060

A sample of each fraction was hydrolysed (N-H_2SO_4, 7 hr., 100°), and the hydrolysate examined by paper chromatography. Each of the hydrolysates contained methylated uronic acids (solvent 3) and exhibited no appreciable quantitative difference in the neutral methylated sugars present. The fractions were combined (5·1 g.) and hydrolysed as for the methylated polysaccharide (A) except that hydrolysis with aqueous acid was continued for 18 hr. The product (4·1 g.) was separated on a cellulose column (52 × 3 cm.) with water-saturated light petroleum–butan-1-ol (7 : 3); R_G values are recorded below for solvent (6).

Fraction 1. 2 : 3 : 4-Tri-*O*-methylxylose (87 mg.), R_G 0·95, $[\alpha]_D$ +22° (*c* 0·8) (Found: OMe, 48·0. Calc. for $C_8H_{16}O_5$: OMe, 48·1%). Demethylation afforded xylose. The aniline derivative had m. p. and mixed m. p. 97°.

Fraction 2. Crystallised slowly from methanol (230 mg.), this had m. p. 99°, R_G 0·88, M_G 0·0 (cf. 3 : 4-di-*O*-methylrhamnose M_G 0·36), $[\alpha]_D$ +42° (*c* 1·5) (Found: OMe, 32·4. Calc. for $C_8H_{16}O_5$: OMe, 32·3%). Demethylation gave rhamnose. The reduction of sodium periodate by this fraction and by 2 : 4-di-*O*-methylrhamnose was measured.[12] This was constant with both sugars after 20 hr. and corresponded to 1·6 and 0·9 mol. of periodate respectively. After addition of ethylene glycol the oxidation mixtures were examined by paper chromatography [13] (solvent 6). The product from fraction (2) gave one main spot, of R_G 0·81, whereas that from 2 : 4-di-*O*-methylrhamnose gave spots of R_G 0·72, 0·78, and 0·85 severally (aniline oxalate spray). *X*-Ray powder photographs of the crystalline sugar and of authentic 2 : 4-di-*O*-methylrhamnose were different.

Fraction 3. 2 : 3 : 6-Tri-*O*-methylglucose (35 mg.), m. p. and mixed m. p. 122°, R_G 0·81, $[\alpha]_D$ +70° (*c* 0·95) (Found: OMe, 42·0. Calc. for $C_9H_{18}O_6$: OMe, 41·9%). Demethylation gave glucose.

Fraction 4. Syrupy 2 : 3-di-*O*-methylxylose (250 mg.), R_G 0·77 (cf. 2 : 4-di-*O*-methylxylose R_G 0·66), $[\alpha]_D$ +21° (*c* 3·0) (Found: OMe, 34·0. Calc. for $C_7H_{14}O_5$: OMe, 34·8%). Demethylation gave xylose. The aniline derivative had m. p. and mixed m. p. 145°.

Fraction 5. Syrupy 2-*O*-methylrhamnose (464 mg.), R_G 0·60, M_G 0·06 (cf. 3-*O*- and 4-*O*-methylrhamnose M_G 0·37 and 0·48 respectively), $[\alpha]_D$ +37° (*c* 3·5). The derived methylglycopyranoside reduced 1·1 moles of sodium metaperiodate per mole of glycoside. Paper chromatography of the oxidation products of the free sugar revealed a single spot of R_F 0·78. 4-*O*-Methylrhamnose on similar treatment gave a spot of R_F 0·95 (solvent 6). The aniline derivative had m. p. 146° undepressed on admixture with an authentic specimen of 2-*O*-methyl-*N*-phenylrhamnosylamine kindly supplied by Dr. L. Hough.

Fraction 6. Syrupy 2 : 3-di-*O*-methylglucose (14 mg.), R_G 0·52 (Found: OMe, 29·5. Calc. for $C_8H_{16}O_6$: OMe, 29·6%). Demethylation gave glucose. The aniline derivative had m. p. and mixed m. p. 132° [cf. polysaccharide (A), fraction 7].

Fraction 7. A syrup (26 mg.), R_G 0·30; in solvent (5), R_G 0·75.

Fraction 8, aqueous eluant. An amorphous solid (2·06 g.) which after treatment with IR-120(H^+) resin gave an acidic syrup (1·61 g.). Chromatography in solvent (5) gave spots of R_F 0·72, 0·53, 0·33, 0·25, 0·13, and 0·10; and R_G values of 0·87, 0·66, 0·41, 0·30, 0·16, and 0·12 respectively. Attempted separation on a cellulose column was unsuccessful, but was achieved on thick paper with this eluant: six fractions were collected.

The first (i), a syrup (161 mg.), R_G 0·87, $[\alpha]_D$ −12° (*c* 1·6), was converted into the ester glycoside and reduced with lithium aluminium hydride as for the aldobiuronic acid. This product was hydrolysed with N-hydrochloric acid at 100° for 4 hr. The resultant syrup (95 mg.) revealed two main spots, of R_G 0·84 and 0·61 respectively, together with a faint spot of R_G 1·15 (paper chromatography, solvent 6). Separation on thick paper (3MM) afforded: 2 : 3 : 4-tri-*O*-methylglucose (38 mg.) (characterised as below) and 2-*O*-methylrhamnose (36 mg.), R_G 0·61, $[\alpha]_D$ +34° (*c* 1·8), chromatographically and ionophoretically identical with fraction 5 separated from the hydrolysate of the neutral sugars from the methylated polysaccharide (B). After periodate oxidation this substance revealed a single spot, R_F 0·78 (solvent 6).

The other fractions (ii, 56 mg.; iii, 154 mg.; iv, 117 mg.; v, 360 mg.; and vi, 207 mg.) were each converted into their ester glycoside, reduced, and hydrolysed. Chromatography (solvent 6) of each hydrolysate revealed that fractions ii—v each gave two spots, of R_G 1·15 and 0·84. Fraction ii also gave a faint spot of R_G 0·61. Fraction vi gave the same spots as fractions ii—v and in addition spots of R_G 0·48, 0·33, 0·18, and 0·10 severally. Yields were as tabulated. Fractions ii—v were combined and separated into two further fractions (*a* and *b*) on thick paper.

Fraction	ii	iii	iv	v	vi
Weight (mg.)	56	154	117	360	207
Yield after reduction	31	92	68	204	82

(*a*) 2 : 3 : 4-Tri-*O*-methylglucose (142 mg. + 38 mg. from fraction i), R_G 0·84, $[\alpha]_D$ +53° (*c* 0·66) (Found: OMe, 41·2. Calc. for $C_9H_{18}O_6$: OMe, 41·9%), gave an infrared spectrum identical with that from 2 : 3 : 4-tri-*O*-methylglucose. The aniline derivative had m. p. 131°, not depressed on admixture with 2 : 3 : 4-tri-*O*-methyl-*N*-phenylglucosylamine, m. p. 139°.

(*b*) A yellow non-reducing syrup (113 mg.), R_G 1·15 (solvent 6), $[\alpha]_D$ +41° (*c* 2·0), was unattacked by periodate and gave a positive test for methoxyl. Comparison of the infrared spectra of this material with that of reduced (lithium aluminium hydride) 2-*O*-methylrhamnose

revealed that, while not identical in the finger-print region, the spectra indicated similarity in the molecular structure.

The authors are grateful to Professor E. L. Hirst for his interest and advice, and to Dr. J. G. Buchanan for help in the identification of the rhamnitol derivative. They are indebted to the Institute of Seaweed Research for a maintenance award (to J. J. O'D), and to Distillers Company and Imperial Chemical Industries Limited for grants.

CHEMISTRY DEPARTMENT, UNIVERSITY OF EDINBURGH. [*Received, January 27th,* 1959.]

[17] Hough, Powell, and Woods, *J.*, 1956, 4799.