531. *The Water-soluble Polysaccharides of* Cladophora rupestris *and of* Chaetomorpha *spp. Part II.*[1] *The Site of Ester Sulphate Groups and the Linkage between the Galactose Residues*

By Sir Edmund Hirst, W. Mackie, and Elizabeth Percival

Partial hydrolysis of the starch-free water-soluble sulphated polysaccharide from *Cladophora rupestris* led to the isolation and characterisation of L-arabinose 3-sulphate, D-galactose 6-sulphate, 1,3- and 1,6-linked D-galactobioses, and a 1,4- or 1,5-linked L-arabinobiose 3-sulphate. Confirmation of the existence of arabinose 3-sulphate and of a 1,4- or 1,5-arabinose linkage in the *Cl. rupestris* polysaccharide was obtained by the isolation of crystalline 2-*O*-methyl-L-xylose after treatment with sodium methoxide.

Evidence is presented of the essential similarity of the *Cl. rupestris* polysaccharide with the sulphated polysaccharides from *Chaetomorpha linum* and *Ch. capillaris*.

Earlier studies [1] on the water-soluble polysaccharides of *Cladophora rupestris* established the presence of D-galactose, L-arabinose, D-xylose, L-rhamnose, and D-glucose in the molar proportions of 2·8 : 3·7 : 1·0 : 0·4 : 0·2, together with ester sulphate (16% as SO_3^{2-}). Methylation and periodate oxidation provided evidence for the presence of 1,3-linked galactose, 1,3-linked arabinose, and 1,4-linked and end-group xylose. The cleavage of 66% of the galactose units by periodate indicated that galactose linkages other than 1,3- were present in the polysaccharide. No definite evidence of the distribution of sulphate groupings was obtained, although from their apparent stability to alkali it was suggested that some of these might be situated on C-4 of galactose units.

[1] I. S. Fisher and Elizabeth Percival, *J.*, 1957, 2666 is considered to be Part I.

Evidence that part of the glucose was present as a separate polysaccharide of the laminarin type was also presented, and more recent work [2] resulted in the separation and characterisation of a starch-type polysaccharide. Application of numerous fractionation methods [1] suggested that the rest of the material comprised a single heteropolysaccharide but unequivocal proof of this was not obtained.

The present work describes further studies on the starch-free water-soluble polysaccharide from *Cl. rupestris* and a similar extract from the botanically closely related genus *Chaetomorpha*. Owing to the difficulty of harvesting large quantities of *Chaetomorpha* spp. large scale experiments were carried out on *Cl. rupestris* extract (A_1), and, where it was considered appropriate, analogous studies of *Ch. capillaris* (A_2) and *Ch. linum* (A_3) polysaccharides were also made.

Before extraction of the weeds with hot water all the samples of dried material were decolourised with butan-1-ol and acetone. The quantity of polysaccharide lost by this treatment was negligible.

The three polysaccharides, after removal of starch,[2] had very similar properties (Table 1) apart from the slightly lower protein contents of the extracts from *Chaetomorpha*. But

TABLE 1

Polysaccharide	Sugar * (%)	$[\alpha]_D$	Sulphate (%) [3]	Ash (%)	Protein (%) [4]
A_1	43·3	+53°	12·3	8·1	25·6
A_2	43·5	66	15·2	9·2	19·4
A_3	43·5	75	15·5	9·3	18·8

* Percentage sugar contents (calc. as monosaccharides) were obtained by treating aliquots of standard polysaccharide solutions with the phenol–concentrated sulphuric acid reagent.[5] The optical densities of the solutions were compared with aliquots of a standard solution treated in the same way. The standard solution contained galactose (38%), arabinose (42%), xylose (12%), rhamnose (5%), and glucose (3%). The figures obtained cannot be regarded as absolute values, but are useful for comparison of different polysaccharide fractions.

this was probably due to treatment with trichloroacetic acid during extraction.[1] Polysaccharide (A_1) used for purification and fractionation experiments was not treated with trichloroacetic acid.

The three polysaccharide extracts gave rise to the same sugars on acid hydrolysis (Table 2) although polysaccharide (A_1) contained greater proportions of galactose than the *Chaetomorpha* extracts.

TABLE 2

Molar proportions of sugars *

Polysaccharide	Galactose	Arabinose	Xylose
A_1	3·1	3·2	1·0
A_2	1·4	3·7	1·0
A_3	1·8	3·7	1·0

* Cf. C. M. Wilson, *Analyt. Chem.*, 1959, **31**, 1199.

None of the polysaccharides contained detectable amounts of 3,6-anhydrogalactose,[6] uronic acid,[7] or amino-sugar.[8]

Anion exchangers such as DEAE(diethylaminoethyl)-[9] and ECTEOLA(epichlorohydrin-triethanolamino)-cellulose [10] and DEAE-Sephadex [11] have been used to separate mixtures

[2] J. Love, W. Mackie, J. P. McKinnell, and Elizabeth Percival, *J.*, 1963, 4177.
[3] A. S. Jones and D. S. Letham, *Chem. and Ind.*, 1954, 662.
[4] R. Belcher and A. L. Godbert, "Semimicro Quantitative Organic Analysis," Longmans, Green, London, 1945.
[5] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Analyt. Chem.*, 1956, **28**, 350.
[6] W. Yaphe, *Analyt. Chem.*, 1960, **32**, 1327.
[7] E. A. McComb and R. M. McCready, *Analyt. Chem.*, 1952, **24**, 1630.
[8] C. Cessi and F. Piliego, *Biochem. J.*, 1960, **77**, 508.
[9] H. Neukom, H. Deuel, W. J. Heri, and W. Kundig, *Helv. Chim. Acta*, 1960, **43**, 64.
[10] N. R. Ringertz and P. Reichard, *Acta Chem. Scand.*, 1960, **14**, 303.
[11] M. Schmidt, *Biochem. Biophys. Acta*, 1962, **63**, 346.

of acidic polysaccharides as well as mixtures of neutral and acidic polysaccharides. Experiments using DEAE-cellulose columns in the chloride and phosphate forms and DEAE-sephadex in the chloride form under the conditions recommended by earlier workers [9,11] produced essentially the same separation on each column (Table 3).

TABLE 3

Fractionation of polysaccharide A_1 on DEAE-cellulose

Tube No.	Wt. (mg.)	Galactose	Arabinose	Glucose	Xylose	Rhamnose
9—15	8 (B)	Trace	Trace	S	S	—
16—58	31 (C)	S (2·1)	S (2·6)	W—M	M (1·0)	W
59—115	232 (D)	S (2·4)	S (2·7)	Trace	M (1·0)	W
116—140	38 (E)	S (2·5)	S (2·8)	Trace	M (1·0)	W
	(D_1)	(2·6)	(2·9)		(1·0)	

The proportions of sugars in an acid hydrolysate were determined by visual examination of a paper chromatogram and are denoted by letters: S, major component; M, medium component; W, minor component. The figures represent the molar proportions determined by the method of Wilson (footnote, Table 2).

TABLE 4

Investigation of polysaccharide fractions

Polysaccharide	$[\alpha]_D$	Sugar (%)	SO_3^{2-} (%)	Protein (%)	Ash (%)
(C)	+59°	52·1	11·3	—	—
(D)	66	49·1	13·3	16·9	9·4
(E)	50 *	—	14·0	—	—
(D_1)	80	52·2	19·1	8·1	10·0

* Cloudy solution.

The small quantity of fraction (B) precluded further structural studies on this material. Apart from a slight variation in sulphate content (Table 4) and the higher glucose content (2·5%) of (C), the three fractions (C), (D), and (E) were very similar and can, for the purposes of structural investigation, be regarded as essentially the same polysaccharide. None of them gave a positive reaction for 3,6-anhydrogalactose. It was unexpected that these three fractions each contained a greater proportion of xylose than the original polysaccharide (A_1) (Tables 2 and 3), since similar treatment of *Codium fragile* polysaccharide provided a purified arabino-galactan by removal of xylose residues.[12]

Polysaccharide (D_1) (Tables 3 and 4) was obtained in 44% yield by treatment of fraction (D) with charcoal. The use of columns of sulphoethylsephadex and carboxymethylcellulose in protein removal was also studied but both failed to lower the protein content of the carbohydrate material.

Polysaccharide (C) was treated separately with salivary α-amylase and sodium metaheriodate and yielded residual polymers containing 1·7 and 0·5% of glucose, respectively. Thus, of the total glucose (2·5%) in polysaccharide (C) 32% was degraded by α-amylase and was presumably residual starch which had escaped precipitation. Of the rest of the glucose, 20% was not cleaved by periodate, as for example β-1,3-linked laminarin (in keeping with earlier findings), and the remaining 48% was unattacked by α-amylase and was cleaved by periodate. It is surprising, however, that these neutral glucans were not separated from the strongly acidic polysaccharide on the anion exchangers. Clearly, the use of DEAE-cellulose as a test of polysaccharide homogeneity must be made with caution.

Various methods for the elimination of sulphate without hydrolysis of the polysaccharide were investigated. The formation of the methylsulphate esters of polysaccharide (A_1) [13] was attempted. However, complete esterification was not achieved and both reductive and acidic desulphation of the partially esterified material caused extensive degradation. Treatment of polysaccharide (A_1) with methanolic hydrogen chloride also

[12] J. Love and Elizabeth Percival, *J.*, 1964, 3338.
[13] G. Coleman, M. Higgs, A. Holt, and M. Malvin, *Chem. and Ind.*, 1963, 376.

gave a low recovery of polysaccharide (K) (SO_3^{2-}, 2·3%) and an examination of the supernatant solution showed that reaction for 24 hr. caused the liberation of major quantities of galactose and xylose and only traces of arabinose (Table 5).

TABLE 5

The action of methanolic hydrogen chloride on polysaccharide A_1

Time (hr.)	Sample	Wt. (mg.)	SO_3^{2-} (%)	Galactose	Arabinose	Xylose	Glucose	Rhamnose
24	Supernat.	—	—	S	W	M	W	—
	Residue	120	12·1	M	S	W	W	W
48	Supernat.	—	—	W	S	W	W	W
	Residue	80	3·6	S	S	M	W	W
96	Supernat.	—	—	S	S	M	W	W
	Residue (K)	40	2·3	S (2·9) *	S (3·8) *	M (1·0) *	W	W

* The figures in parentheses are the molar proportions of the sugars in the final residue.

Comparison of the molar proportions of galactose : arabinose : xylose in polysaccharide (K) with those of the earlier trichloroacetic acid-treated polymer [1] and with untreated polysaccharide (A_1) (Table 2) confirms that acidic reagents bring about a preferential cleavage of galactose and xylose units from the polysaccharide. It is interesting, too, that the trichloracetic acid-treated *Chaetomorpha* polysaccharides (A_2) and (A_3) (Table 2) contain the same proportions of arabinose and xylose as the acid-treated *Cladophora* samples, but have lower proportions of galactose.

Examination of partial acid hydrolysates of polysaccharides (A_1), (A_2), and (A_3) revealed the same chromatographic and ionophoretic pattern from each polysaccharide. In addition to the expected neutral sugars a number of charged components Table 7 and neutral oligosaccharides were present. Large-scale hydrolysis of polysaccharide (A_1) was followed by separation of the acidic fraction (S) from the neutral fraction (R). Fraction (S), after further purification on charcoal and on filter sheets, yielded three compounds (S_A), (S_B), and (S_C) isolated as their ammonium salts. These were characterised respectively as galactose 6-sulphate, arabinose 3-sulphate, and 4-*O*-β-L-arabinopyranosyl-L-arabinopyranose and 5-*O*-β-L-arabinopyranosyl-L-arabinofuranose with an ester sulphate linked to C-3 of the non-reducing moiety. Characterisation was by chromatographic mobility,[14] the products formed on hydrolysis and on methylation, and, in the case of the two monosaccharides, the reduction of periodate and the rate of hydrolysis of the ester sulphate. The reduction of *ca.* 3·5 moles and 3·3 moles of periodate by galactose 6-sulphate and arabinose 3-sulphate, respectively, is in keeping with the results of earlier workers on galactose 6- and glucose 3-sulphates.[15] From a study of the rate of hydrolysis of the ester sulphates[16] the galactose 6-sulphate was shown to have a half-life of 1·61 hr. comparable with the recorded figures (1·55 hr.) for galactose 6-sulphate. In contrast the arabinose 3-sulphate had a half-life of 0·80, a figure which lies between equatorial (0·42) and axial (1·45) hexose sulphates.[16] However, since this is the first reported occurrence of a sulphated arabinose derivative no data on pentose sulphates are available for comparison and, as will be suggested later, it is possible that more than one conformation of arabinose 3-sulphate may exist in its equilibrium mixture. This would produce an overall hydrolysis rate made up of contributions from each conformation.

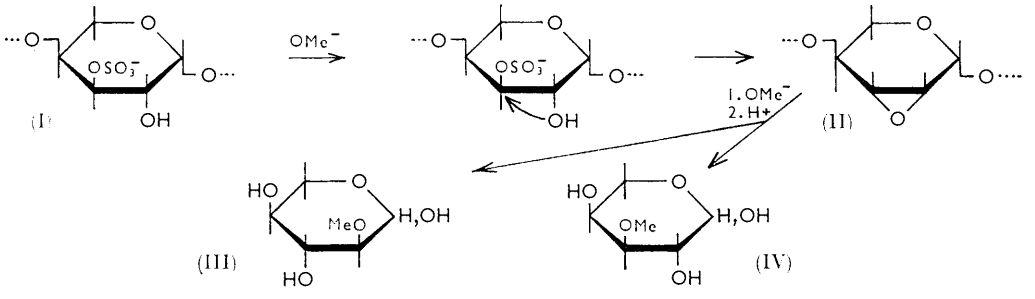
The action of alkali and of sodium methoxide on the polysaccharides[17] confirmed the presence of ester sulphate groups on C-3 of arabinose and revealed that the galactose 6-sulphate residues must be linked or sulphated at C-3 since they did not yield any appreciable quantity of 3,6-anhydrogalactose on alkali treatment.[18] 3-Sulphated arabinose units

[14] D. A. Rees, *J.*, 1963, 1821.
[15] J. R. Turvey, N. J. Clancy, and T. P. Williams, *J.*, 1961, 1692; J. R. Turvey and D. A. Rees, *Nature*, 1961, **189**, 831.
[16] D. A. Rees, *Biochem. J.*, 1963, **88**, 343.
[17] F. H. Newth, *Quart. Rev.*, 1959, **13**, 30; R. E. Parker and N. S. Isaacs, *Chem. Rev.*, 1959, **59**, 737.
[18] E. G. V. Percival, *Quart. Rev.*, 1949, **3**, 369.

should yield some xylose units on treatment with alkali and this was revealed in the higher proportion of xylose units in the alkali-treated polysaccharide (H) (1 mole in 5·3 moles) compared with the starting material (D_1) (1 mole in 6·5 moles).

Confirmation was obtained by treatment of polysaccharide (A_1) with sodium methoxide.[19] Hydrolysis of the resultant polysaccharide and separation of the sugars in the derived syrup gave a crystalline material which was characterised as 2-*O*-methyl-L-xylose. Traces of 3-*O*-methylarabinose and 3-*O*-methylxylose were also tentatively identified. The same sugars were recognised qualitatively from similar treatment of polysaccharides (A_2) and (A_3). These observations together with the partial hydrolysis studies confirm that the sulphate groups are similarly linked in the *Cladophora* and *Chaetomorpha* polysaccharides and provides another point of resemblance between the two genera.

The 2-*O*-methyl-L-xylose (III) can only have arisen from 3-sulphated L-arabinose (I) in the polysaccharide through the intermediate formation of the 2,3-epoxide (II).[17] Attack by the methoxide ion (OMe^-) on the epoxide ring (II) can then occur either at C-2 or C-3 yielding 2-*O*-methyl-L-xylose (III) or 3-*O*-methyl-L-arabinose (IV), respectively. Since



the major derivative was 2-*O*-methyl-L-xylose attack must have been mainly at C-2. (2-Sulphated L-arabinose on similar treatment should yield 3-*O*-methyl-L-xylose and 2-*O*-methyl-L-arabinose.) The trace quantities of 3-*O*-methylarabinose and 3-*O*-methylxylose detected, precluded their characterisation as D- or L-sugars, and so it is not clear whether they are derived from sulphated D-xylose or L-arabinose units in the polysaccharide. 2-Sulphated D-xylose in *Ulva lactuca* polysaccharide gave rise to 2-*O*-methyl-D-xylose on sodium methoxide treatment.[19]

TABLE 6

Infrared absorption peaks ($cm.^{-1}$) of sugar sulphates *

	Primary OH	Equatorial OH	Axial OH
Sulphated glucose–galactose	820	830	850
Galactose 6-S (S_A)	820		
Arabinose 3-S (S_B)	770	818—830	852—855
Polysaccharide A_1	810—840		
Partly desulphated A_1	819 (sharp peak)		
Polysaccharides A_2 and A_3		825—835	

* Cf. A. G. Lloyd and K. S. Dodgson, *Biochim. Biophys. Acta*, 1961, **46**, 116; A. G. Lloyd, K. S. Dodgson, R. G. Price, and F. A. Rose, *ibid.*, p. 108.

It is known that sulphate esters of D-galactose and D-glucose exhibit a general infrared absorption band about 1240 $cm.^{-1}$. They also show specific bands which are considered to be characteristic of the type of ester sulphate as shown in Table 6, in which are also given the infrared spectra of the substances under investigation.

Whilst galactose 6-sulphate had the characteristic infrared spectrum of a primary hydroxyl sulphate it can be seen that arabinose 3-sulphate revealed three bands. The second corresponds to an equatorially disposed sulphate grouping and agrees with the

[19] Elizabeth Percival and J. K. Wold, *J.*, 1963, 5459.

suggestion [20] that the most stable conformation of the L-arabinopyranose unit is C1, in which the C-3 hydroxyl has an equatorial disposition. The significance of the small peak at 770 cm.$^{-1}$ is not known but the third peak is considered to arise from axially disposed sulphate groupings. The presence of more than one sulphate absorption band in this sugar can be explained if different conformations of the sugar (including the furanose-ring forms) are present in its equilibrium mixture.

The absorption band shown by polysaccharide (A_1) supports the presence of both equatorial and primary sulphated hydroxyl groups. In contrast the absorption of *Chaetomorpha* polysaccharides (A_2) and (A_3) suggests that these polymers contain a smaller proportion of primary sulphated hydroxyl corresponding with the lower galactose content of these polysaccharides. The spectrum of the partially desulphated polysaccharide (A_1) shows absorption only for primary sulphated hydroxyl. Such a sulphate would be more resistant to acidic hydrolysis than secondary equatorial sulphated hydroxyls.[16] In the polysaccharide, the arabinose 3-sulphate units are probably held in a single conformation and consequently the infrared absorption of the monosaccharide equilibrium mixture might be expected to differ from the sulphated monomer units within the polysaccharide.

The neutral syrup (R) from partial acid hydrolysis was examined primarily for hetero-oligosaccharide fragments, since these would provide positive proof that polysaccharide (A_1) is a heteropolymer. It was only possible to separate two pure compounds, (R_1) and (R_2), and these were identified respectively as 6-*O*-β-D-galactopyranosyl-D-galactose and 3-*O*-β-D-galactopyranosyl-D-galactose on the basis of chromatographic mobility, specific rotation, and the products formed on acid hydrolysis and on methylation. No evidence for the presence in syrup (R) of hetero-oligosaccharides was obtained.

The release of oligosaccharides during partial acetolysis [21] of polysaccharide (A_1) was therefore studied. This procedure generally results in liberation of increased quantities of oligosaccharides.[22] However, in the present experiments very complex mixtures were obtained and it was impossible to separate a single pure hetero-oligosaccharide although the two galactobioses isolated from the acid hydrolysate were again separated and characterised.

Fractionation studies on the *Cladophora* polysaccharide allowed the separation of a purified galactose–arabinose–xylose polymer but it is still not certain that this main fraction is truly a single polysaccharide. Although the balance of evidence suggests that this is so all attempts to obtain unequivocal proof by the isolation of hetero-oligosaccharides from fragmentation studies were unsuccessful. It appears that the sulphated polysaccharides of *Cladophora* and *Chaetomorpha* are best regarded as a family of related, polydisperse molecules, in the same way as the uronic acid-containing sulphated polymers found in other genera of green algæ [19] and the sulphated galactans of red algæ.[23]

The arabino-galactans of the genera *Cladophora* and *Codium* [12] have some features in common, such as 6-sulphated and 1,3-linked galactose, but in spite of this they are apparently quite dissimilar substances. For, although each polymer contains alkali-labile ester sulphate, this is mainly linked to arabinose units in *Cladophora* and to galactose units in the *Codium* polysaccharide. This fundamental structural difference is revealed when the polysaccharides are treated with sodium methoxide. Major proportions of monomethylpentose are incorporated into the *Cladophora* polysaccharide, whereas the *Codium* polymer appears to be virtually unaffected.

Experimental

The analytical methods used have been described by McKinnell and Percival.[24] In addition, the following chromatographic solvent (9) was used: ethylacetate–pyridine–water (8 : 2 : 1)

[20] R. E. Reeves, *Adv. Carbohydrate Chem.*, 1951, **6**, 107.
[21] J. Love and Elizabeth Percival, *J.*, 1964, 3345.
[22] K. Morgan and A. N. O'Neill, *Canad. J. Chem.*, 1959, **37**, 1201.
[23] Elizabeth Percival, Proc. 4th Internat. Seaweed Symposium, 1963, p. 27.
[24] J. P. McKinnell and Elizabeth Percival, *J.*, 1962, 3141.

and the proportions of solvent (3) were changed to 18 : 3 : 1 : 4. Ionophoresis of sulphated sugars was carried out on Whatman No. 1 or 3MM paper in pyridine–acetic acid buffer (0·05N, pH 6) for 1·5—3 hr. at 300V. M_{GS} is the rate of travel relative to that of galactose 6-sulphate after allowance for endosmosis migration. Gas–liquid chromatography [25] was carried out on a Pye Argon Chromatograph in which argon was employed as the mobile gas phase. The stationary liquid phase was supported on Celite and was either butane-1,4-diol succinate polyester 175° or polyphenol at 200°. Retention times are expressed relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside.

Extraction of the Polysaccharide.—Dried *Cl. rupestris* harvested in October from the shore near Dunbar, Scotland, was frozen in liquid nitrogen, crushed to a powder, and treated successively with aqueous n-butanol (half-saturated with water) and cold acetone until no more colouring matter was removed. The residual weed was isolated as a grey powder. The organic solutions after concentration to small volume were extracted with ethyl acetate–water (1 : 1; 500 ml.). Addition of ethanol (6 vol.) to a portion of the aqueous solution did not yield a precipitate. Concentration of the aqueous layers to dryness and hydrolysis of the residue with *N*-sulphuric acid at 100° gave trace quantities of galactose, glucose, and arabinose (paper chromatography).

Portions of the decolourised weed were treated separately with water (*a*) at room temperature, (*b*) at 60—70° and (*c*) at 95—100°; (*b*) and (*c*) were under nitrogen. At intervals, aliquots were removed, clarified on the centrifuge and the supernatants tested with dilute iodine solution. No change of colour was observed with (*a*) or (*b*) after several days and hours, respectively. After extraction for 20 min. the solution from (*c*) gave an intense blue coloration.

Decolourised weed (30—40 g. batches) in water (500 ml.) thoroughly saturated with nitrogen was heated to 90—95° with continuous stirring.[2] In this way 100 g. of decolourised weed were extracted and the combined extracts were concentrated to small volume. Starch (1·0 g.) [2] was precipitated as the starch–iodine complex from the derived solution by the method of Steiner and Guthrie [26] and the sulphated polysaccharide (A_1) (13·8 g.) was recovered from the supernatant after dialysis, concentration, and freeze-drying.

Ch. capillaris (harvested at North Berwick, Nov., 1958) and *Ch. linum* (harvested in Holland, 1961) were similarly extracted, except that they were treated with trichloroacetic acid before dialysis in order to remove contaminating protein,[1] and yielded polysaccharides (A_2) (*ca.* 11 g. from 50 g.) and (A_3) (4·7 g. from 50 g.), respectively.

Properties of the Extracted Polysaccharides.—The three polysaccharide extracts had the properties given in Table 1. Each extract gave, after hydrolysis with N-sulphuric acid for 3 hr. at 100°, arabinose, galactose, and xylose in the molar proportions given in Table 2 and trace amounts of rhamnose and glucose, together with a number of amino-acids including glycine and leucine (paper chromatography, spray *a* and ninhydrin spray). All the extracts were devoid of 3,6-anhydrogalactose [6] and amino-sugars [8] and contained little, if any, uronic acid.[7]

(*a*) Polysaccharide (A_1) (512 mg.) in water (50 ml.) was applied to the top of a DEAE-cellulose column (42·0 × 3·6 cm.) in the chloride form.[19] After allowing the polysaccharide to drain in, gradient elution with sodium chloride (water–0·5M-sodium chloride, 1 l. each; 0·5M-sodium chloride–2M-sodium chloride, 1 l. each). The eluate was collected in 25 ml. fractions, aliquots of which were analysed for their carbohydrate content [5] and an elution graph was drawn. (The recovery after allowance for ash and protein was 67%.) Tubes were combined and analysed as shown in Tables 3 and 4.

(*b*) Gradient elution through a DEAE-cellulose column in the phosphate form [19] buffered at pH 5·9 (NaH_2PO_4–Na_2HPO_4, 0·1M–0·3M; NaH_2PO_4–Na_2HPO_4–0·3M-NaOH) gave the same fractionation pattern as (*a*) above.

(*c*) Gradient elution of polysaccharide from DEAE-Sephadex A50 grade (Pharmacia) in the chloride form as under (*a*) above gave no obvious difference in the fractionation.

Treatment with Salivary α-Amylase.—Polysaccharide (C) (14·2 mg.), in water (5 ml.) containing sodium chloride (1 mg.) and salivary α-amylase (20 mg.), was incubated at 37° for 24 hr. The residual polymer (9·6 mg.) isolated by freeze-drying after dialysis still contained 1·7% of glucose.[27]

Action of Alkali on Fraction (D_1).—Polysaccharide (D_1) (see p. 2960) (30·0 mg.) in water

[25] G. O. Aspinall, *J.*, 1963, 1676.
[26] E. T. Steiner and J. D. Guthrie, *Ind. Eng. Chem., Analyt.*, 1944, **16**, 736.
[27] J. W. White and M. H. Subers, *Analyt. Biochem.*, 1961, **2**, 380.

(5 ml.) containing potassium borohydride (30 mg.) was left overnight at room temperature, to prevent end-group degradation by the alkali. To the resulting mixture, potassium borohydride (30 mg.) and sodium hydroxide (2N; 5 ml.) were added, and the solution was heated at 80° for 7 hr. Further additions of potassium borohydride (1—2 mg.) were made every hour. After dialysis against frequently changed distilled water the derived solution was concentrated and freeze-dried to a white solid (H) (24·2 mg., 81%). It had $[\alpha]_D$ +65° (*c* 0·5) and contained 60% of sugar comprising galactose, arabinose, and xylose in the molar proportions 2·3 : 2·0 : 1·0 (Found: SO_3^{2-}, 13·8; 3,6-anhydrogalactose [6] *ca.* 0·2%).

Desulphation Studies.—(*a*) *Treatment with methanolic hydrogen chloride.* Polysaccharide (A_1) (203 mg.) was shaken with 1% dry methanolic hydrogen chloride (40 ml.) at room temperature. The insoluble polysaccharide was removed after 24 hr. and 48 hr. and shaken with fresh 1% methanolic hydrogen chloride. A portion (20 mg.) was removed at each stage for estimation of sulphate, and acid hydrolysis. The supernatant was also hydrolysed. The results are given in Table 5.

After 96 hr. treatment with 0·1% methanolic hydrogen chloride, the residual polysaccharide (*ca.* 25%) still contained 7·1% of ester sulphate.

(*b*) *Reductive fission.*[13] A 1% solution of polysaccharide (A_1) (1·0 g.) was converted into the free acid with Amberlite IR-120 H^+ resin, concentrated to 20 ml., and diluted with methanol to precipitation point. This was cooled to 0° and a chilled solution of diazomethane in ether was slowly added, with shaking, until the ethereal layer remained yellow. The mixture was shaken vigorously at 2° for 20 hr. and diluted with water. After removal of methanol and ether the residual solution was freeze-dried to a white solid (0·8 g.). An aqueous solution of this was still acid to litmus. A portion (315 mg.) was refluxed overnight in dry dioxan (80 ml.) containing lithium aluminium hydride (0·4 g.). The excess of hydride was removed with ethyl acetate, hydrochloric acid was added to pH 5, and the mixture was filtered. The filtrate and washings were dialysed and freeze-dried to a brown solid (13 mg.) (Found: SO_3^{2-}, 2·3%).

A second portion (208 mg.), after shaking overnight at room temperature with 1% methanolic hydrogen chloride (40 ml.), was neutralised with methanolic ammonia, concentrated, and freeze-dried (yield, 52 mg.; SO_3^{2-}, 2·3%).

(*c*) *Treatment with sodium methoxide.* Polysaccharide (A_1) (10 g.) after treatment with sodium borohydride and drying [19] was treated with sodium methoxide (6 g. sodium in 250 ml. dry methanol) under reflux for 24 hr. with further additions of sodium borohydride. Chromatographic examination of an acid hydrolysate of a portion of the recovered polysaccharide (6 g.) gave the same pattern of sugars together with two new substances R_{gal} 1·82 and 1·60 (solvent 2). Further treatment of portions of the recovered polysaccharide with fresh volumes of sodium methoxide for 24, 72, and 168 hr. failed to increase the proportions of the new substances.

Similar treatment of polysaccharides (A_2) and (A_3) (200 mg. each) followed by hydrolysis gave the same two new substances (paper chromatography).

Hydrolysis of the recovered polysaccharide from (A_1) (5·0 g.) with N-sulphuric acid (250 ml.) for 5 hr. at 100° gave, after neutralisation, a syrup (1·9 g.). This was applied to a column of cellulose (55 × 4 cm.) and the following fractions eluted with butan-1-ol, half-saturated with water:

Fraction 1 (75 mg.) had R_{gal} 1·82 (solvent 2); 3·92 (solvent 3), and was identical with 2-*O*-methylxylose in chromatographic mobility and colour (reddish black, spray *a*). Chromatography in solvent (7) revealed a trace of a second substance (R_{gal} 10·7 as compared with R_{gal} 9·8 for the major constituent) with a mobility identical with 3-*O*-methylxylose and which like the latter gave a bright red colour with spray (*a*), and a positive reaction with triphenyltetrazolium hydroxide.[28] Substance R_{gal} 9·8 gave no perceptible colour with triphenyltetrazolium hydroxide. Demethylation [29] of a portion (10 mg.) and chromatographic examination of the product in solvents (2, 3, and 9) showed only xylose. A second portion (1—2 mg.) was refluxed with 3% methanolic hydrogen chloride and, after neutralisation, examined by gas–liquid chromatography. Peaks with the retention times of the methyl glycosides of 2-*O*-methylxylose [25] together with small peaks, whose retention times were characteristic of 3-*O*-methylxylose, were observed. The remainder of the fraction deposited white needles from ethanol at room temperature. After washing with ethyl acetate–ethanol (2 : 1) and two recrystallisations

[28] D. J. Bell, "Modern Methods of Plant Analysis," ed. Paech and Tracey, Springer-Verlag, Berlin, 1955, Vol. II, p. 9.

[29] T. G. Bonner, E. J. Bourne, and S. McNally, *J.*, 1960, 2929.

from ethanol, they had m. p. 132—133°, depressed on admixture with authentic 2-*O*-methyl-D-xylose (m. p. 135°), but they gave an *X*-ray powder photograph identical with this latter substance. Crystalline fraction 1 had $[\alpha]_D$ −6·4 ⟶ −36·1° (constant 5 hr., *c* 0·5) (cf. 2-*O*-methyl-D-xylose, $[\alpha]_D$ +36°).

Fraction 2 (137 mg.) was a complex mixture of rhamnose, xylose, and substances with R_{gal} 6·1, 9·8, 10·7 (solvent 7). Successive separations on filter paper gave a small amount (1 mg.) of pure substance, R_{gal} 6·1 (solvent 7), 3·49 (solvent 3). It differed in mobility and colour [R_{gal} 1·6 (solvent 2), bright red, spray *a*] and its positive reaction with triphenyltetrazolium hydroxide [28] from authentic 2-*O*-methylarbinose. Gas–liquid chromatography of the derived methyl glycosides [25] showed two peaks whose retention values were characteristic of the methyl glycosides of 3-*O*-methyl-arabinose.

Partial Hydrolysis.—(*a*) Samples of polysaccharides (A_1, A_2, A_3) (*ca.* 0·1 g. each) were hydrolysed separately (N-sulphuric acid, 10 ml. at 100° for 1 hr.), neutralised, and the derived solutions deionised with Amberlite IR-120 H^+ form. Ionophoretic examination at pH 6 of the derived syrups showed the presence of several charged components, the pattern being identical for (A_1), (A_2), and (A_3) (Table 7). Major proportions of neutral sugars were also present.

TABLE 7

Charged substances in a partial hydrolysate

No.	1	2	3	4	5	6
Spray (*a*)	Brown	Pink	Brown	Pink	Yellow (trace)	Pink (trace)
M_{GS}	0·70	0·80	1·00	1·18	1·30	1·50

(*b*) Polysaccharide (A_1) (10 g.) in N-sulphuric acid (250 ml.) was heated at 100° for 1·5 hr. The resulting syrup was applied to a column (12 × 2·7 cm.) of Amberlite IR-400 A (acetate form) resin. From the aqueous eluate a syrupy mixture of neutral sugars (R) (2·7 g.) was obtained. From a 0·5N-sulphuric acid (2 l.) eluate an acidic fraction (S) free from neutral sugars was isolated as the ammonium salts.

Separation of the Acidic Fragments.—A solution of (S) in water was applied to a charcoal column (3 × 9 cm.) and elution with water gave three main fractions. It was found that the first two fractions, 582 and 180 mg., respectively, each contained the same two components in different proportions and that the third fraction (50 mg.) also contained two other substances of lower ionophoretic mobility. All three fractions were dissolved in water and eluted on 3MM filter-paper sheets with solvent (2) for 24 hr. Successive separations in this way gave samples of chromatographically (solvents 2 and 9) and ionophoretically homogeneous compounds (S_A) (95 mg.), (S_B) (56·5 mg.), and (S_C) (5 mg.) as their ammonium salts.

Fraction (S_A) had R_{gal} 0·55 (solvent 2; brown spot, spray *a*), M_{GS} 1·0, identical with galactose 6-sulphate, $[\alpha]_D$ +51° (*c* 1·3) calculated as the ammonium salt of a hexose monosulphate from the sugar concentration found by the method of Dubois *et al.*[5] It gave, after further hydrolysis (0·5N-HCl at 100° for 6 hr.), a qualitative test for sulphate and a single spot with the mobility of galactose on a paper chromatogram. It had DP [30] 1·03 and a molar ratio of galactose [5] to sulphate [3] of 1·0 : 1·15. A sample (1—2 mg.) was methylated [31] and the product treated with 3% methanolic hydrogen chloride for 12 hr. Gas–liquid chromatography [25] of the resulting glycosides gave peaks characteristic of methyl 2,3,4-tri-*O*-methylgalactosides.

Fraction (S_B), had R_{gal} 0·83 (solvent 2, pink spot, spray *a*) and M_{GS} 1·18, $[\alpha]_D$ +75° (*c* 0·6) (calc. as for fraction S_A) and DP [30] 1·02. It gave, after hydrolysis as for (S_A), a qualitative test for sulphate and a single spot with the mobility of arabinose on a paper chromatogram and a molar ratio of sulphate [3] to arabinose [5] of 1·09 : 1·00. After methylation [31] and methanolysis, gas–liquid chromatography [25] revealed peaks with the retention times of methyl 2,4- and 2,5-di-*O*-methylarabinosides.

Periodate Oxidation and Formaldehyde Release.—Solutions of (S_A) and (S_B) (0·500 ml.) containing 1·286 mg./ml. and 1·687 mg./ml., respectively, were each diluted with 0·03M-sodium metaperiodate solution (0·500 ml.) and set aside in the dark at room temperature. Aliquots (0·1 ml.) of each mixture were withdrawn at intervals and the reduction of periodate determined.[32]

[30] T. E. Timell, *Svensk Papperstidn.*, 1960, **63**, 668.
[31] O. Perila and C. T. Bishop, *Canad. J. Chem.*, 1961, **39**, 815.
[32] G. O. Aspinall and R. J. Ferrier, *Chem. and Ind.*, 1957, 1216.

TABLE 8

Reduction of periodate per mole of fractions (S_A) and (S_B)

Time (hr.)	0·75	2·5	4·0	24	96	192
Fraction (S_A)	3·08	3·36	3·56	3·67	3·79	
Fraction (S_B)	0·54	1·07	1·54	2·08	2·95	3·30

Aliquots of (S_A) and (S_B) 0·05—0·075 ml., respectively, and authentic glucose 3-ammonium sulphate (116—174 μg.) were oxidised with 0·015M-sodium metaperiodate in a total volume of 0·200 ml. (*a*) in unbuffered solution and (*b*) in sodium hydrogen carbonate buffer (0·05M, pH 7·5), and the quantity of formaldehyde released was measured.[33]

TABLE 9

Moles of formaldehyde released per mole

S_A (*a*) Nil (96 hr.)	(*b*) Nil (24 hr.)
S_B (*a*) 0·48 (96 hr.)	(*b*) Trace (1 hr.), 0·63 (3 hr.), 0·79 (24 hr.)
Glucose 3-sulphate (*a*) 0·38 (96 hr.)	(*b*) ,, 0·60 (3 hr.), 0·81 (24 hr.)

Rate of Sulphate Ester Hydrolysis.—Samples of fractions (S_A), (S_B), and glucose 3-sulphate (6 of each containing *ca.* 50 μg. esterified sulphate) in 0·25N-hydrochloric acid (0·250 ml.) were heated separately in sealed tubes and the rate of ester sulphate hydrolysis measured.[16] Graphical plots were made from the experimental data and the half-life values found from these plots were (S_A) 1·61, (S_B) 0·80, glucose 3-sulphate 0·41.

Fraction (S_C) had M_{GS} 0·80 and gave a pink colour with spray (*a*). It had DP [30] 2·03 and on acid hydrolysis (HCl) gave rise to arabinose [5] and sulphate [3] in the molar ratio of 2 : 1. It had $[\alpha]_D$ +165° (*c* 0·14) (calc. for the ammonium salt of a mono-sulphated arabinobiose).

Methylation [31] of a portion (1—2 mg.), methanolysis, and gas–liquid-chromatographic examination [26] gave peaks characteristic of methyl 2,4- and 2,3-di-*O*-methylarabinosides. Desulphation of a portion (1—2 mg.) with 3% methanolic hydrogen chloride (0·5 ml.) overnight at room temperature was followed by methylation, methanolysis and gas–liquid-chromatographic examination. Peaks corresponding to methyl 2,3,4-tri- and 2,3-di-*O*-methylarabinosides were revealed.

Examination of the Neutral Fraction (R).—After repeated separation of fraction (R) (2·7 g.) on paper two pure disaccharides were isolated:

Fraction 1 (2·3 mg.) had DP [30] 2·05, $[\alpha]_D$ +32° (*c* 0·23) and gave rise to galactose on acid hydrolysis (solvents 2 and 9, spray *b*). It did not migrate on ionophoresis at pH 7 and had the same chromatographic mobility, R_{gal} 0·34 (solvent 2), 0·17 (solvent 3), as authentic 6-*O*-β-D-galactopyranosyl-D-galactose.

A portion (1 mg.) was methylated [31] and methanolysed. Gas–liquid chromatography [26] of the product revealed peaks corresponding to the methyl glycosides of 2,3,4,6-tetra- and 2,3,4-tri-*O*-methylgalactose. The pattern of peaks was identical with that produced by authentic 6-*O*-β-D-galactopyranosyl-D-galactose after the same treatment.

Fraction 2 (2·3 mg.) had DP [30] 1·90, $[\alpha]_D$ +72° (*c* 0·23) and gave galactose on hydrolysis. It had the same mobility R_{gal} 0·49 (solvent 2), 0·24 (solvent 3), as authentic 3-*O*-β-D-galactopyranosyl-D-galactose. It gave a red colour with triphenyltetrazolium reagent [29] and a grey-green colour with aniline diphenylamine phosphoric acid reagent.[34] Methylation and methanolysis of a portion (1 mg.) revealed on gas–liquid-chromatographic analysis, peaks characteristic of the methylglycosides of 2,3,4,6-tetra- and 2,4,6-tri-*O*-methylgalactose. All attempts to separate other pure oligosaccharides from this neutral material were unsuccessful.

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