606. *Structural Investigations on the Water-soluble Polysaccharide of the Green Seaweed* Enteromorpha compressa.*

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Rhamnose, xylose, and glucose have been separated and characterised from a hydrolysate of the water-soluble polysaccharide (A) extracted from *E. compressa.* A starch-type polysaccharide (3·5%) had been separated from (A) before hydrolysis. Polysaccharide (A) which also contained 18% of uronic acid and 16% of ester sulphate was unattacked by salivary α-amylase and sheep's rumen xylanase, and resisted further fractionation. Desulphated polysaccharide was isolated in 71% yield after treatment of (A) with methanolic hydrogen chloride. Periodate oxidations of the sulphated and desulphated material provided evidence that the majority of the sulphate groups are linked to rhamnose. Polysaccharide (A) is compared with similar extracts from *Ulva lactuca* and *Acrosiphonia centralis.*

Enteromorpha spp. of green seaweed belong to the class Ulvaceae, a group of algæ occurring commonly in British waters, both fresh and salt. Members of this class show a preference for estuarine growth, especially where there is an influx of sewage. Identification of the different species of this genus is difficult and the collection of pure species can only be carried out by an expert algologist.

An investigation of a mixture of *E. torto*, *E. compressa*, and *E. intestinalis* [1] has shown that a dilute-acid extract consists of at least two polysaccharides. Analysis of a hydrolysate of the mixture indicated the presence of D-glucose, L-rhamnose, D-xylose, 3-*O*-methyl-L-rhamnose, D-glucuronic acid, and sulphate residues. Chloroform extraction of the methylated material yielded a soluble fraction containing a glucan and an insoluble complex material. Fractionation of a hydrolysate of the latter gave fully methylated rhamnose 18%, partially methylated rhamnose and xylose 65%, and unmethylated rhamnose 15%. This, as far as the authors are aware, is the only structural investigation of polysaccharides of this genus.

A supply of *Enteromorpha compressa* was collected for us by Mr. H. Powell from the shore near Millport, Isle of Cumbrae, and dried at 60°, and the polysaccharides present in this material were subjected to structural investigations. After examination of a number of organic solvents it was found that 85% aqueous ethanol and dimethyl sulphoxide [2] were the most suitable for the removal of colouring matter. With the latter solvent, however, 6% of heteropolysaccharide was also extracted and for this reason 85% aqueous ethanol was used for all the large-scale extractions. Chromatographic examination of the alcoholic extract, after deproteinisation [3] and deionisation [4] showed the presence of glucose

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* Brief accounts of part of this work were given to the Biochemical Society (*Biochem. J.*, 1961, **80**, 43P) and the 4th International Seaweed Symposium, 1961.

[1] Lowe, Ph.D. Thesis, London, 1956.

[2] Hägglund, Lindberg, and McPherson, *Acta Chem. Scand.*, 1956, **10**, 1160.

[3] Laidlaw and Reid, *J. Sci. Food Agric.*, 1952, **3**, 19.

[4] Anderson and Wylam, *Chem. and Ind.*, 1956, 191.

and sucrose. Exhaustive hot-water extraction of the alcohol-treated residue yielded (16·8% of dry weight of weed) off-white polysaccharide contaminated with nitrogenous material. Subsequent treatment of the residual *E. compressa* with hypochlorite [5] followed by 4% alkali and then 18% alkali in the cold under nitrogen gave 6%, 10%, and 4·5% of crude polysaccharide. The first three extracts consisted mainly of rhamnose together with glucose and xylose, and the fourth extract and residual weed, which comprised mainly glucose, still contained small quantities of xylose and rhamnose. Other properties of these extracts are given in Table 1. No evidence for the presence of 3-*O*-methylrhamnose was obtained in any of the extracts.

Since there was no fractionation into homopolysaccharides and as the hot-water extract was obtained in highest yield, this material, designated the " water-soluble " polysaccharide, was investigated. After removal of much of the contaminating nitrogenous material, this had $[\alpha]_D$ −49°. It gave a negative test for keto- and/or 3,6-anhydro-sugars and stained the typical blue-purple colour of starch with dilute iodine solution. Treatment of a portion with salivary α-amylase removed some of the glucose-containing polysaccharide as maltose and glucose, in keeping with the presence of a starch-type polysaccharide.

TABLE 1.

Extract	$[\alpha]_D$	Ash (%)	SO_4^{2-} (%)	N_2 (%)	Uronic acid (%)
1	−41°	13·0	9·6	3·0	17·0
2	−79	23·4	11·9	2·86	12·8
3	−46	10·0	8·3	2·49	16·6
4	—	40·0	—	—	—

The addition of an iodine–potassium iodide solution to a starch-containing polysaccharide mixture preferentially precipitates the starch.[6] A 3·5% yield of a pure starch was separated by this means from the " water-soluble " polysaccharide and the residual water-soluble polysaccharide (A) recovered from the supernatant solution in 64% yield had $[\alpha]_D$ −87°, ash 13·7, sulphate 16, uronic anhydride 18·3, and nitrogen 0·86%; it still contained rhamnose, xylose, and glucose units. The relative increase in the percentage of sulphate (11·9 ⟶ 16%) and decrease in nitrogen (1·4 ⟶ 0·86%) suggests that considerable purification of the polysaccharide had occurred during the dispersion and iodine precipitation, and this would partly account for the loss of *ca.* 30% of the material. The equivalent (301) [7] of polysaccharide (A) calculated from the above analytical figures agrees reasonably with that found by direct titration of the free acid polysaccharide (310).

The individual sugars present in a hydrolysate of polysaccharide (A) were separated on thick paper and characterised. An acidic fraction was also separated which had a comparable rotation, and $R_{\text{Galacturonic acid}}$ 1·07, and M_G 0·80, identical with those of the 4-*O*-β-D-glucuronosyl-L-rhamnose isolated from hydrolysates of the water-soluble polysaccharides of *Acrosiphonia centralis* [7] and *Ulva lactuca*.[8] Reduction of the methyl ester methyl glycoside of this acidic fraction and chromatographic examination of an acid hydrolysate of the reduced material indicated the presence of equal proportions of glucose and rhamnose, evidence that the acidic fraction was a glucuronosylrhamnose. Material separated was insufficient for further characterisation.

Since polysaccharide (A) was immune to the action of α-amylase it is unlikely that the glucose in the hydrolysate is derived from unfractionated starch-type polysaccharide. It is more probable that these residues are involved in other than α-1,4-linkage in the polysaccharide.

Fractional precipitation with cetyltrimethylammonium bromide [9] or hydroxide at

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[5] Wise, *Ind. Eng. Chem. Analyt.*, 1945, **17**, 63.
[6] Steiner and Guthrie, *Ind. Eng. Chem. Analyt.*, 1944, **16**, 736.
[7] O'Donnell and Percival, *J.*, 1959, 2168.
[8] McKinnell and Percival, *J.*, 1962, 2082.
[9] Bera, Foster, and Stacey, *J.*, 1955, 3788.

various pH's,[10,11] Fehling's solution,[12] saturated barium hydroxide,[13] or with potassium chloride[14] of polysaccharide (A) in an attempt to isolate a homopolysaccharide was unsuccessful. Paper chromatography has been employed to separate mixtures of sulphated mucopolysaccharides.[15] Paper chromatography of polysaccharide (A) gave an apparent separation but this appeared to be a fractionation of two polysaccharides of different molecular weights rather than an actual separation of differently constituted polymers since both fractions contained the same sugars and ester sulphate.[16]

Evidence that the xylose units were not present as a separate β-1,4′-xylan was deduced from the immunity of the material to attack by sheep's rumen xylanase.[17]

Johnston and Percival[18] reported the removal of a proportion of the sulphate groups from carragheen by the action of 1% methanolic hydrogen chloride and the isolation of 15% of degraded polysaccharide with a low sulphate content (1·5%). Similar treatment of fucoidin with different concentrations of methanolic hydrogen chloride[19] caused concomitant desulphation and degradation, and the recovered polysaccharide, isolated in low yield, had a slightly higher sulphate content than the starting material. Polysaccharides which contain the more acid-stable uronosyl linkages, such as chondroitin sulphate, have been desulphated by this method[20] with very little if any degradation of the polysaccharide. Polysaccharide (A) was therefore kept for 48 hr. with 0·09M-methanolic hydrogen chloride, 71% of desulphated polysaccharide (A) was recovered in which the sulphate content had been reduced to 0·75%. Comparison of the $[\alpha]_D$ and the percentage monosaccharide composition before and after desulphation (Table 2) revealed that little if any change in the fundamental structure of the polysaccharide had occurred during the desulphation.

TABLE 2.

	$[\alpha]_D$	Glucose	Xylose	Rhamnose	Sulphate	Uronic acid
Polysaccharide (A) (%)	−87°	5·7	15·0	45·0	16·0	18·3
Desulphated polysaccharide (A) (%)	−88	9·75	15·0	51·0	0·75	23·5

The equivalent weight of desulphated polysaccharide (A) determined by titration of the free acid polysaccharide with standard alkali was 655. Calculation of the equivalent based on the uronic acid and sulphate content gave a value of 642, in reasonable agreement with the experimental value.

Oxidation of the sulphated and desulphated polysaccharides with periodate stopped after *ca.* 48 hr. at 2° (Table 3) and the reduction of periodate was 0·38 mole and 0·68 mole, respectively. After 72 hr. at 2° the amount of periodate reduced was still constant and the solutions were allowed to warm to room temperature; oxidation was then complete after a further 5 hr. The reduction of periodate was then 0·48 mole and 0·79 mole for every C_6 sugar residue for polysaccharide (A) and desulphated polysaccharide (A), respectively.

TABLE 3.

Moles of periodate reduced per C_2 anhydro-unit.

Time (hr.)	0·5	1·75	3	5	24	48	72
Polysaccharide (A)	0·170	0·214	0·242	0·322	0·355	0·382	0·382
Desulphated polysaccharide (A)	0·425	0·580	0·630	0·660	0·680	0·680	0·680

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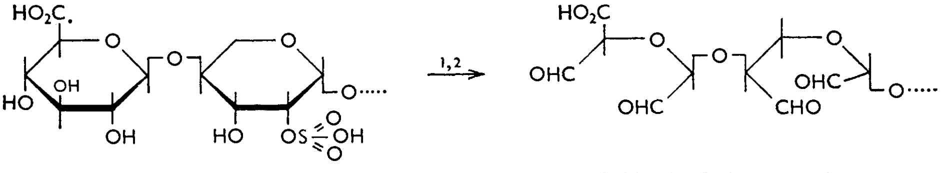
[10] Scott, *Chem. and Ind.*, 1955, 168; *Methods Biochem. Analysis*, 1960, **8**, 145.
[11] Bouveng and Lindberg, *Acta Chem. Scand.*, 1958, **12**, 1977.
[12] Chanda, Hirst, and Percival, *J.*, 1951, 1240.
[13] Mackie and Percival, *J.*, 1961, 3010.
[14] O'Neill, *J. Amer. Chem. Soc.*, 1955, **77**, 2837.
[15] Spolter and Marx, *Biochem. Biophys. Acta*, 1960, **38**, 123.
[16] Lloyd, *Biochem. J.*, 1960, **75**, 478.
[17] Howard, *Biochem. J.*, 1957, **67**, 643.
[18] Johnston and Percival, *J.*, 1950, 1994.
[19] Elizabeth Percival, unpublished work.
[20] Kantor and Schubert, *J. Amer. Chem. Soc.*, 1957, **79**, 152.

The comparatively low reduction of periodate by these polysaccharides indicates the presence of a proportion of 1,3′-linked residues. The higher consumption of periodate by the desulphated material provides evidence that the removal of sulphate produced additional units with adjacent free hydroxyl groups. If the sulphate is mainly attached to a particular sugar residue then determination of the relative proportions of the unattacked monosaccharides in the hydrolysates of the two periodate-oxidised polysaccharides should provide evidence of the site of the sulphate groups. The following percentage proportions of unattacked units were obtained: oxopolysaccharide (A) (81% yield), glucose 26, xylose 16, rhamnose 58; desulphated oxopolysaccharide (69% yield), glucose 40, xylose 14, rhamnose 44. The lower proportion of rhamnose units in the oxidised desulphated polysaccharide was confirmed by determination of the absolute amount of rhamnose in each polysaccharide and its oxidised derivative.[21] It was considered that the free aldehydic groups present in the oxopolysaccharides might interfere in the estimation. For this reason a second series of determinations was carried out on the products after reduction with borohydride of the different polysaccharides (Table 4). All the reduced materials gave lower results and this difference is greater in the two oxopolysaccharides. Estimations on the reduced materials are considered therefore to be the more accurate. Calculation of the percentage of rhamnose in the reduced desulphated polysaccharide and sulphated (A) from the analytical figures and the molar proportion of sugars present gave 45·2 and 34·5%, respectively, in reasonable agreement with the values, 48 and 35%, determined directly on these materials.

TABLE 4.

	Rhamnose (%)		Rhamnose (%)
Polysaccharide (A)	37	Reduced polysaccharide (A)	35
Desulphated polysaccharide (A)	51	,, ,, (DSA)	48
Oxopolysaccharide (A)	31·7	,, oxopolysaccharide (A)	27
Desulphated oxopolysaccharide (A) ...	37	,, ,, (DSA) ...	29

The higher cleavage of rhamnose units in the desulphated polysaccharides proves that the sulphate groups are mainly attached to this sugar residue. Infrared analysis of polysaccharide (A) revealed a peak at 850 cm.$^{-1}$ and, it being assumed that results obtained for glucose and galactose sulphates[22] can be applied to rhamnose, this indicates the presence of axial sulphate in polysaccharide (A). This peak is absent in the desulphated polysaccharide. It appears therefore, L-rhamnose being assumed to be present in the polysaccharide in its stable 1C conformation, that position 2 of rhamnose carries some of the sulphate residues since this is the only carbon atom in rhamnose (1C) to carry an axial hydroxyl group. This agrees with the isolation of a 4-*O*-glucuronosylrhamnose fragment from a hydrolysate of the polysaccharide. Such a unit in the sulphated polysaccharide would be immune to oxidation by periodate, and, after removal of the sulphate, would be cleaved by this reagent. Apart from the galactan sulphate of *Porphyra umbilicalis*[23]



4-O-Glucuronosyl-L-rhamnose 2-sulphate units

Oxidised 4-O-glucuronosyl-L-rhamnose units

1. MeOH, HCl. 2. NaIO$_4$.

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[21] Dische and Shettles, *J. Biol. Chem.*, 1948, **175**, 595; 1949, **181**, 379.

[22] Lloyd, Dodgson, Price, and Rose, *Biochim. Biophys. Acta*, 1961, **46**, 108.

[23] Turvey and Rees, *Nature*, 1961, **189**, 831.

this is the first direct evidence of the site of the sulphate group in algal sulphated polysaccharides.

From the analytical figures it is possible to calculate that while *ca.* 77% of the rhamnose units remained unoxidised in the sulphated polysaccharide only 60% was unattacked in the desulphated material. The increase in periodate reduction (0·38—0·68 mole) is not fully accounted for by this increase in free hydroxyl groups and it is therefore probable that residues other than rhamnose carry sulphate. A comparison of the proportion of unattacked glucose and xylose in the oxopolysaccharides indicates a loss of xylose units in the desulphated material and it is possible that these units also carry sulphate groups.

A decision concerning the oxidation by periodate of the uronic acid units in the polysaccharide is difficult. Only traces of uronic acid could be detected by paper-chromatographic analysis of the hydrolysates of the oxopolysaccharides. Determination of the uronic acid colorimetrically [24] gave results which, although they bore no relation to those obtained by decarboxylation of the unoxidised materials (Table 5), were similar and considerably lower for both oxidised materials. While this cannot be regarded as proof that these units are oxidised by periodate, it as least indicates that this is probable and that the uronic acid residues are present as end groups.

TABLE 5.

	Uronic acid (%)		Uronic acid (%)
Polysaccharide (A) (*a*)	18·3	Desulphated polysaccharide (A) (*a*)	23·5
,, (A) (*b*)	6·0	,, ,, (A) (*b*)	10·2
Oxopolysaccharide (A) (*b*)	2·7	Desulphated oxopolysaccharide (A) (*b*)	3·3

(*a*) By decarboxylation. (*b*) Colorimetric determinations.

Comparison of the water-soluble polysaccharides of *E. compressa, U. lactuca,* and *A. centralis* (Table 6) reveals many similarities. The main sugar residues in each case are rhamnose, xylose, and glucose together with about 20% of glucuronic acid and sulphate ester groups. For each of the polysaccharides evidence has been obtained that at least a high proportion of the uronic acid units are attached to position 4 of rhamnose since 4-*O*-glucopyranosyl-L-rhamnose has been separated from each of the acid hydrolysates. A considerable portion (*ca.* 45%) of the hydrolysate from *A. centralis* comprised glucuronosyl-rhamnose fragments which probably accounts for the low proportion of free rhamnose in this hydrolysate.

TABLE 6.

	Enteromorpha compressa (*a*)	*Acrosiphonia* [7] *centralis* * (*b*)	*Ulva* [5] *lactuca* * (*c*)
Rhamnose	8·1	1·4	4·2
Xylose	2·7	1·6	1·3
Glucose	1·0	1·0	1·0
Mannose	—	0·2	—
Galactose	—	0·1	—
Uronic acid (%)	18·3	20·3	20·8
SO_4^{2-} (%)	16	7·8	15·9
Ash (%)	13·7	10·0	19·4
$[\alpha]_D$ (in water)	−87°	−31°	−47°

(*a*) After hydrolyses with N-sulphuric acid for 8 hr. at 100°. (*b*) After hydrolyses with N-sulphuric acid for 7 hr. at 100°. (*c*) After hydrolyses with 0·3N-sulphuric acid for 12 hr. at 100° and N-sulphuric acid for 15 hr. at 100°; hydrolysates being combined.

* Recent work has revealed that these two polysaccharides contained a small proportion of a starch-type polysaccharide.

Examination of each of the residual polysaccharides after periodate oxidation reveals that a fairly high proportion of all the neutral sugars are unattacked, evidence for the

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[24] McCombe and McCready, *Analyt. Chem.*, 1952, **24**, 1630.

presence of many 1,3′-linked residues and/or branch points. Furthermore earlier work has shown the presence of both 1,4-linked xylose and rhamnose in *U. lactuca*,[25] *A. centralis*,[7] and mixed *Enteromorpha* spp.[1]

Attempts to fractionate each of these polysaccharides have failed, and at present it must be assumed that each is a single heteropolysaccharide.

There are minor differences, such as proportion of sulphate, in the polysaccharides synthesised by these three genera of green algæ and it is probable that as information accumulates it will be found that instead of a single polysaccharide there is a family of polymers with the same basic structure, but which differ in the proportions of the different units and in their sulphate content. Such a picture is gradually emerging in the case of the galactan sulphates synthesised by the red algæ [26] and the hemicelluloses of the land plants.[27]

Partial hydrolysis and methylation studies on the desulphated polysaccharides of these three genera are in progress.

EXPERIMENTAL

The analytical methods used have been described by O'Donnell and Percival.[7] Paper chromatograms were sprayed with (*a*) saturated aqueous solution of aniline oxalate; (*b*) 1% (saturated) solution of silver nitrate in acetone followed by 0·5N-sodium hydroxide in 70% ethanol followed by 2% sodium thiosulphate (the papers were dipped in each solution and dried between each operation); (*c*) 0·1% Bromocresol Green in ethanol, and then with alkali until just blue, the solution being filtered if necessary.

Soxhlet extraction of dried *Enteromorpha compressa* with 85% aqueous alcohol removed most of the colouring matter. Chromatographic examination of the alcoholic extract, after deproteinisation [3] and deionisation [4] indicated the presence of glucose and sucrose. The residual dried weed (170 g.) was extracted successively with the following solvents: (1) distilled water at 70° in an atmosphere of nitrogen until further extraction gave a negative Molisch test; (2) water (1000 ml.) at 70° containing glacial acetic acid (4 c.c.) and sodium chlorite (20 g.),[5] added in 5-g. lots at hourly intervals; (3) after being thoroughly washed with water the residual weed was treated under nitrogen with 4% sodium hydroxide solution at room temperature for 24 hr.; (4) the weed was further extracted with 18% sodium hydroxide under the same conditions as in (3); (5) the residual weed was washed free from alkali and kept for 36 hr. in 72% sulphuric acid.[28]

In all cases except (5) the polysaccharide was isolated by freeze-drying of the solution obtained after dialysis and concentration. Each of the extracts and the residual weed were hydrolysed, and chromatographic analysis revealed rhamnose with smaller proportions of glucose and xylose in the first three extracts, and glucose with small quantities of xylose and rhamnose in extract (4) and in the residual weed. Other properties of these extracts are given in Table 1.

Since there was no fractionation into homopolysaccharides and since the hot-water extract was obtained in highest yield, this material, which is designated the " water-soluble polysaccharide," was selected for investigation. The nitrogen-containing contaminant was reduced to 1·4% by precipitation with trichloroacetic acid,[29] and the purified material, isolated in 91% yield by freeze-drying, after dialysis, had $[\alpha]_D$ −49° (*c* 1·1) [Found: ash (direct), 11·0, (as sulphate) 13·2; SO_4^{2-}, 11·45; uronic anhydride [30] (decarboxylation), 17·2%]. It gave a negative Seliwanoff test, and was stained blue-purple with dilute iodine solution.

The Action of α-Amylase on the Water-soluble Polysaccharide.—Incubation of the " water-soluble " polysaccharide (45·2 mg.) dissolved in distilled water (10 ml.) with fresh salivary α-amylase at 37° overnight was followed by the introduction of more enzyme and a further incubation for 24 hr. The enzyme was deactivated at 70° for 10 min. and the solution dialysed. Chromatographic analysis of a hydrolysate of the undialysed material indicated a decrease in

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[25] Brading, Georg-Plant, and Hardy, *J.*, 1954, 319.
[26] Percival, 4th International Seaweed Symposium, in the press.
[27] Hirst, *Proc. Roy. Soc.*, 1959, *A*, **252**, 287.
[28] Monier-Williams, *J.*, 1921, **119**, 803.
[29] Fisher and Percival, *J.*, 1957, 2666.
[30] McCready, Swenson, and Maclay, *Ind. Eng. Chem. Analyt.*, 1946, **18**, 290.

the glucose content of the polysaccharide. The concentrated dialysate contained maltose with a trace of glucose.

Fractionation of a Starch-type Polysaccharide.[6]—The " water-soluble " polysaccharide (15 g.) mixed with Celite (18 g.) and dispersed in 0·03N-ammonium carbonate (500 ml.) was heated at 117—120° under nitrogen for 30 min. with stirring. The starch was then precipitated by addition of 20% aqueous sodium chloride solution (200 ml.) and 12% solution of iodine in 20% potassium iodide solution (25 ml.) after cooling. After being vigorously shaken and kept for 5 min. the precipitate was removed by centrifugation. Residual polysaccharide (A) (9·6 g.) was recovered from the supernatant liquid, after dialysis and concentration, by freeze-drying. The precipitated iodine complex was immediately redispersed in 20% sodium chloride (200 ml.) and destroyed by dropwise addition of 0·5N-sodium thiosulphate solution (*ca.* 25 ml.), excess of the reagent being avoided. The Celite was removed by centrifugation and N-hydrochloric acid (60 ml.) added to the supernatant liquid. Precipitation of the starch–iodine complex was repeated and the precipitate suspended in 95% ethanol before the dropwise addition of thiosulphate solution. When the blue colour had disappeared distilled water was added. Evaporation of the ethanol and dialysis permitted isolation of the starch-type polysaccharide (0·55 g., 3·5%) ($[\alpha]_D$ +187°) by freeze-drying. Structural investigations of this material will be reported elsewhere.

Properties of the Starch-free Polysaccharide.—Polysaccharide (A) was soluble in water and did not give a colour with iodine. It had $[\alpha]_D$ −87° (*c* 0·89) [Found: ash (direct), 13·7; SO_4^{2-} in ash, 7·8; total SO_4^{2-}, 16·0; uronic anhydride, 18·3 (by decarboxylation); N (Kjeldahl), 0·86%; equiv., of free-acid polysaccharide, 310 (by titration)]; ν 1240 and 845 cm.$^{-1}$ [22] (Nujol mull, Perkin–Elmer " Infracord " spectrophotometer). Chromatographic analysis of a hydrolysate (N-sulphuric acid for 8 hr. at 100°) revealed the presence of rhamnose, xylose, and glucose, and quantitative analysis by a colorimetric method [31] gave the molar proportions as 8·1 : 2·7 : 1·0. A portion of the hydrolysate isolated as a syrup (520 mg.) after neutralisation and deionisation was separated on a Whatman No. 17 paper chromatogram (20 × 40 cm.). After elution with solvent (1) for 48 hr. the following fractions were separated, overlap fractions being discarded.

Fraction 1. This was L-rhamnose (281 mg.), $[\alpha]_D$ +7·8° (*c* 0·98), m. p. and mixed m. p. 92°; the phenylosazone [32] had m. p. and mixed m. p. 190°.

Fraction 2. This was D-xylose (68 mg.), $[\alpha]_D$ +19·4° (*c* 1·0), m. p. and mixed m. p. 143°; the dibenzylidene dimethylacetal [33] had m. p. and mixed m. p. 211°.

Fraction 3. This was syrupy D-glucose (42 mg.), $[\alpha]_D$ +52° (*c* 0·6). Incubation of a portion with D-glucose oxidase (notatin) caused complete conversion into D-gluconic acid,[34] as revealed by chromatographic resolution in solvent (3), spray *c*.

Fraction 4. This acidic syrup (42 mg.) had $[\alpha]_D$ −12° (*c* 0·84) (cf. $[\alpha]_D$ −22° [8] and −6° [7] recorded for 4-*O*-D-glucuronosyl-L-rhamnose), R_G 0·29 (solvent 1), $R_{\text{Galacturonic acid}}$ 1·07; M_G 0·80 in borate buffer (pH 10).[7] It was converted into the methyl ester methyl glycoside and reduced with potassium borohydride in 0·4M-boric acid.[35] Hydrolysis of the syrup so obtained gave an equimolar mixture of rhamnose and glucose.

Attempted Fractionation of Polysaccharide (*A*).—(*a*) Treatment of the material (A) with cetyltrimethylammonium bromide in aqueous solution,[9] in buffer at pH 0·5,[10] with cetyltrimethylammonium hydroxide in boric acid solution,[11] and with " Arquad " (16—50%) (a hexadecyltrimethylammonium chloride), failed to achieve fractionation.

(*b*) Addition of Fehling's solution [12] to a solution of the polysaccharide (500 mg.) in water gave a precipitate (60 mg.) and addition of ethanol to the supernatant liquid in two steps, first to 40% concentration and then to 60% gave precipitates (205 mg. and 180 mg., respectively). Chromatographic examination of hydrolysates of each of these precipitates revealed rhamnose, xylose, and glucose in the same relative proportions as those of the original polysaccharide (visual estimation).

(*c*) Treatment of appropriate solutions of the polysaccharide with saturated barium hydroxide [13] and with potassium chloride [14] failed to yield precipitates.

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[31] Wilson, *Analyt. Chem.*, 1959, **31**, 1199.
[32] Freudenberg and Raschig, *Ber.*, 1929, **62**, 373.
[33] Breddy and Jones, *J.*, 1945, 738.
[34] Keilin and Hartree, *Biochem. J.*, 1948, **42**, 230.
[35] Frush and Isbell, *J. Amer. Chem. Soc.*, 1956, **78**, 2844.

(*d*) Polysaccharides (A) (100 mg.) in the minimum of water was applied to a Whatman 3MM chromatogram paper (15 × 40 cm.), which was then eluted for 24 hr. with an ammonium formate buffer–propan-2-ol mixture (65 : 35). Polysaccharide material carrying charged groups was located by spraying control strips with Azure II.[15] Two dark blue areas were revealed; the appropriate sections of paper were extracted with distilled water. The two derived syrups gave positive tests for sulphate.[16] Chromatographic analysis of hydrolysates revealed identical sugars, rhamnose, xylose, and glucose, in the two syrups.

The Action of Enzymes on Polysaccharide (A).—(*a*) *α-Amylase.* A sample of polysaccharide (A) (50 mg.) dissolved in distilled water (10 ml.) was incubated with fresh salivary α-amylase under the conditions used for the " water-soluble " polysaccharide. No evidence was obtained of the presence of maltose or glucose in the dialysate, and the polysaccharide isolated by precipitation by alcohol from the solution in the dialysis sac appeared to contain the same proportion of glucose as polysaccharide (A) (visual examination of a paper chromatogram of an acid hydrolysate).

(*b*) *Xylanase.* The polysaccharide (51·5 mg.) in a buffer solution at pH 6·8 [0·2M-disodium hydrogen phosphate (15·45 ml.) and 0·1M-citric acid (4·55 ml.)] was incubated for 26 hr. at 37° with sheep's rumen xylanase [17] kindly given by Dr. B. H. Howard of the Rowett Research Institute. The resulting solution was treated in the same way as the α-amylase incubate. No evidence was obtained for the presence of xylose in the dialysate, and the relative proportions of sugars in the recovered polysaccharide were the same as in the initial polysaccharide.

Desulphation of Polysaccharide (A).—The neutral polysaccharide (900 mg.) was shaken with 0·09M-methanolic hydrogen chloride (50 ml.) for 48 hr. at room temperature, after which the undissolved polysaccharide was removed and washed with dry methanol. It was dissolved in water (50 ml.) and dialysed against running water for 3 days. Concentration and freeze-drying of the material in the dialysis sac gave desulphated polysaccharide (A) (545 mg.), $[\alpha]_D$ −88° (*c* 1·0) [Found: ash, 2·6; total SO_4^{2-}, 0·75; uronic anhydride (decarboxylation), 23·5; N, 0·86%; equiv. (by titration), 655]. The two peaks recorded in the infrared spectrum of polysaccharide (A) were absent. Quantitative analysis [31] of a hydrolysate (N-sulphuric acid, 8 hr. at 100°) gave rhamnose : xylose : glucose in the relative molar proportions of 8 : 2·3 : 1·6.

Periodate Oxidation of Polysaccharide (A) and of Desulphated Polysaccharide (A).—Solutions of polysaccharides (A) and desulphated polysaccharide (A) (46·7 mg. and 43·8 mg.) in 0·2M-sodium acetate buffer (pH 3·6; 25 ml.) were made up to 50 ml. with 0·03M-sodium periodate in the same buffer.[36] The samples were kept at 2° for 72 hr. and thereafter at room temperature in the dark, and the reduction of periodate [37] measured on portions (1 ml.) withdrawn at intervals (see Table 3).

The reactions were stopped by passing sulphur dioxide through the solutions for 15 min., and the oxopolysaccharides were isolated by freeze-drying after dialysis and concentration. Yields of oxopolysaccharide (A) and desulphated oxopolysaccharide (A) were 37·6 mg. and 30·2 mg., respectively. Quantitative determination [31] of the relative molar proportions of the sugars in a hydrolysate of the two oxopolysaccharides gave:

	Rhamnose	Xylose	Glucose
Oxopolysaccharide (A)	3·6	1·0	1·6
Desulphated oxopolysaccharide (A)	2·75	1·0	2·5

Quantitative determination of rhamnose [21] was carried out on polysaccharide (A), desulphated polysaccharide (A), and their respective oxopolysaccharides in aqueous solutions containing 100·6, 98·5, 98·6, and 88·66 μg/ml., and on these solutions after treatment with potassium borohydride solution for 12 hr. The average results of duplicate experiments are given in Table 4.

Determination of the Uronic Acid Content.—The four polysaccharides were examined for uronic acid by McCombe and McCready's colorimetric method.[24] Glucuronic acid was used to prepare a standard graph. The results are given in Table 5.

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