1040. *The Acid Polysaccharide from the Green Seaweed* Ulva lactuca. *Part II.* The Site of the Ester Sulphate.*

By Elizabeth Percival and J. K. Wold.

From the water-soluble sulphated polysaccharide described in Part I a starch (*ca.* 1·2% of the dry weight of weed) has been separated. The residual polysaccharide, comprising rhamnose, xylose, glucose, and glucuronic acid, resisted further fractionation into homopolysaccharides, but a molecular-size separation was achieved on a diethylaminoethylcellulose column. Treatment with methanolic hydrogen chloride of the starch-free polysaccharide removed the ester sulphate, and periodate-oxidation studies of the suphated and de-sulphated material provided evidence that the majority of the sulphate groups are linked to rhamnose. Treatment of the sulphated polysaccharide with alkali led to the isolation, after hydrolysis, of D-arabinose (1·1%) and D-lyxose (trace), and 2-*O*-methyl-D-xylose resulted from hydrolysis after the action of sodium methoxide on the alkali-treated polysaccharide. Mechanisms for the formation of these sugars from xylose 2-sulphate present in the polysaccharide are advanced. From the proportion of D-arabinose formed it is calculated that *ca.* 15% of the xylose units are sulphated.

Part I of this series described the isolation and characterisation of 4-*O*-β-D-glucuronosyl-L-rhamnose from a partial acid hydrolysate of the polysaccharide extracted by cold water from *Ulva lactuca*. The present studies are concerned with similar polysaccharide material isolated from *U. lactuca* which was harvested from the lower bay of Fundy, Nova Scotia, in July 1960, and at St. Abbs, Berwickshire, during May 1961. Separate exhaustive cold- and hot-water extraction of the two samples gave overall yields of *ca.* 21% and 23% (based on the dry weight of weed), respectively, of sulphated polysaccharide. Visual examination of paper chromatograms of acid hydrolysates of the different cold- and hot-water extracts showed the same relative proportions of rhamnose, xylose, and glucose together with traces of galactose and mannose in all the extracts, indicating that this particular species of green seaweed synthesises the same water-soluble polysaccharide material irrespective of its environment and of the season of the year. Quantitative analysis of successive extracts from the Canadian weed (Table 1) showed slightly decreasing proportions of sulphate and uronic acid, but this was offset by an increase in nitrogeneous material. The cold-water extracts were combined and after conversion into the free acid had a calculated equivalent weight of 374 based on a uronic acid and sulphate content of 18·8% and 15·3%, respectively. The equivalent weight found by titration (355) was 5% lower than this calculated value.

A starch-type polysaccharide (12·0%) was separated from the combined cold-water extracts as the starch–iodine complex,[2] and the recovered sulphated polysaccharide (A)

* Part I, *J.*, 1962, 2082.

[1] McKinnell and Percival, *J.*, 1962, 2082.
[2] Love, Mackie, McKinnell, and Percival, *J.*, 1963, 4177.

TABLE 1.

	$[\alpha]_D$	Ash (%)	Nitrogen (%)	Sulphate (%)	Uronic anhydride (%) *
1st Cold-water extract	−48°	10·0	0·74	15·9	15·9
2nd ,, ,, ...	−50	13·0	0·50	15·8	15·5
3rd and 4th Cold-water extract ...	−49	10·0	0·78	13·8	15·2
1st Hot-water extract	−45 †	11·3	1·55	12·9	11·6
2nd ,, ,, ...	−42 †	5·9	2·00	10·1	10·5

* By decarboxylation. † Solution turbid and difficult to read.

(83%), although it still contained glucose, no longer gave a colour with iodine and was unattacked by salivary α-amylase. This material resisted further fractionation on a paper chromatogram,[3] by ionophoresis,[4] by fractional precipitation with barium hydroxide,[5] and by selective extraction with dimethyl sulphoxide.[6]

It has been shown [7] that polysaccharide mixtures, for example, sugar-beet araban and pectic acid can be separated by elution through columns of diethylaminoethylcellulose (D.E.A.E.-cellulose), the acidic polysaccharide being readily and the neutral polysaccharide at most only weakly absorbed. Application of the polysaccharide (A) to such a column in the phosphate form and elution with phosphate buffers of the same neutral pH value, but of increasing concentration, failed to yield any neutral polysaccharide. A low recovery of degraded acidic polysaccharide was obtained by elution with alkali. In a second experiment the D.E.A.E.-cellulose was converted into the chloride form and the elution carried out with solutions of potassium chloride of increasing concentration. The polysaccharide was eluted with this neutral medium in three distinct fractions. Each fraction contained the same sugars in approximately the same relative proportion, had similar specific rotations and sulphate contents (see Table 2), and gave identical infrared spectra.

TABLE 2.

	Wt. (g.)	$[\alpha]_D$	Sulphate (%)	$[\eta]$
Fraction 1	0·25	−67°	16·4	1·50
Fraction 2	0·15	−82°	17·9	1·10
Fraction 3	0·16	−75°	17·9	0·95

Viscosity measurements (Table 2) indicated that the three fractions had different molecular weights, the order of elution being in agreement with the fact that compounds of higher molecular weight are eluted first from an ion-exchange column.[8]

Fraction 3 and polysaccharide (A) were examined in the ultracentrifuge by Dr. C. T. Greenwood. The sedimentation patterns indicated that the latter is heterogeneous, whereas the former gave a single sharp peak and was apparently homogeneous. In view of these results it was decided, until evidence to the contrary is obtained, to regard polysaccharide (A) as a single polydisperse heteropolysaccharide. It had $[\alpha]_D$ −70·7° and contained glucose : xylose : rhamnose in the molar proportions of 1 : 3·4 : 4·8 (after 6 hours' hydrolysis) and 1 : 3·5 : 6·9 (after 16 hours' hydrolysis), together with traces of mannose and galactose, sulphate 18%, and uronic anhydride 14·1%, and gave negative tests for the presence of ester lactone,[9] keto-sugar, 3,6-anhydro-sugar,[10] and amino-sugar. A modification [11] of the Elson–Morgan test for 2-amino-2-deoxy-sugars in which amino-acids

[3] Spolter and Marx, *Biochim. Biophys. Acta*, 1960, **38**, 123.
[4] Foster, Newton, Hearn, and Stacey, *J.*, 1956, 30.
[5] Meier, *Acta Chem. Scand.*, 1958, **12**, 144.
[6] Hägglund, Lindberg, and McPherson, *Acta Chem. Scand.*, 1956, **10**, 1160.
[7] Neukom, Deuel, Heri, and Kündig, *Helv. Chim. Acta*, 1960, **43**, 64.
[8] Jones, Wall, and Pittet, *Canad. J. Chem.*, 1960, **38**, 2285.
[9] Bell, " Modern Methods of Plant Analysis," Vol. II, p. 13, Springer Verlag, 1955.
[10] Yaphe, *Analyt. Chem.*, 1960, **32**, 1327.
[11] Cessi and Piliego, *Biochem. J.*, 1960, **77**, 508.

do not interfere was used to test for amino-sugars. The initial Elson–Morgan test [12] on polysaccharide (A) was positive.

Desulphation of polysaccharide (A) was attempted in several ways. The free-acid form, which was slightly soluble in dioxan, was desulphated with lithium aluminium hydride [13] in this medium. Extensive degradation of the polysaccharide occurred and the degraded material, recovered in 15% yield (sulphate 6·5%), contained all the sugars present in the original polysaccharide. Autodesulphation of the free-acid form of the polysaccharide in dry methanol again caused degradation; the residual insoluble polysaccharide recovered in 53% yield contained 2·4% of sulphate and glucose : xylose : rhamnose in the molar proportions of 1 : 1·2 : 1·5; the methanol-soluble material contained these sugars in the molar ratio of 1 : 6·3 : 14·8, respectively.

As for *Enteromorpha compressa*,[14] dry methanolic hydrogen chloride was found to be the best agent for removal of the sulphate groups from polysaccharide (A). After two treatments the sulphate in the residual polysaccharide (B), recovered in 50% yield, had been reduced to 0·3%. Comparison (Table 3) of the relative molar proportions of the sugars in

TABLE 3.

	Glucose	Xylose	Rhamnose	Arabinose
Polysaccharide (A)	1·0	3·4	5·0	—
,, (B)	1·0	2·3	3·2	—
,, (D)	1·0	1·6	2·0	0·1

hydrolysates from polysaccharides (A) and (B) revealed the essential similarity of the two materials and indicated that desulphation had cleaved a proportion of the rhamnose and xylose units.

Oxidation of the sulphated (A) and partially desulphated (C) (SO_4^{2-} 5·0%) polysaccharides with periodate in buffered solution at 2° stopped after *ca.* 70 hr. (Table 4),

TABLE 4.

Moles of periodate reduced per C_6 anhydro-unit at 2°.

Time (hr.)	4	20	44	70	120
Polysaccharide (A)	0·04	0·15	0·18	0·20	0·20
,, (C)	0·[illegible]8	0·31	0·36	0·40	0·40

and the reduction of periodate was then 0·20 and 0·40 mole for each " C_6 anhydro-unit " in (A) and (C), respectively. Parallel oxidation experiments were carried out at room temperature in the absence of buffer on the above two samples and also on desulphated polysaccharide (B) (see Table 5) and, when reduction was complete, the respective oxo-

TABLE 5.

Moles of periodate reduced per C_6 anhydro-unit at room temperature.

Time (hr.)	4	9	25	43	80	100	125
Polysaccharide (A)	0·10	0·20	0·28	0·30	0·30	0·30	0·30
,, (B)	0·22	0·50	0·60	0·62	0·65	0·65	0·65
,, (C)	0·14	0·42	0·48	0·52	0·52	0·52	0·52

polysaccharides were isolated. The true reduction of periodate for every sugar residue in a heterogeneous polymer consisting of variable amounts of sulphate, ash, and protein in addition to the carbohydrate material is very complicated to determine accurately, and the reduction of periodate in terms of " C_6 anhydro-unit " is chosen for simplicity and is calculated on the assumption that the polysaccharide is a homohexan. Care is necessary in the interpretation of such results, but they are of value for comparison with those of oxidation studies on substances of similar composition.

[12] Elson and Morgan, *Biochem. J.*, 1933, **27**, 1824.
[13] Grant and Holt, *Chem. and Ind.*, 1959, 1492.
[14] McKinnell and Percival, *J.*, 1962, 3141.

It can be seen from Table 4 that the partially desulphated material reduced more periodate than the more highly sulphated material. Consequently it may be deduced that removal of sulphate ester groups from the polysaccharide leads to formation of additional α-glycols. It is known that, in buffered solution at low temperature, vicinal *cis*-hydroxyl groups in sugars are selectively oxidised by periodate. Since at 2° polysaccharide (C) reduced twice as much periodate as polysaccharide (A) it may be concluded that desulphation furnishes *cis*- rather than *trans*-glycol groupings. The only sugar present in the polysaccharide in which *cis*-glycol groupings occur is L-rhamnose at positions 2 and 3, and it may be presumed therefore that sulphate groups are linked either to C-2 or C-3 in rhamnose. Infrared examination of polysaccharide (A) revealed a broad peak at 1240 cm.$^{-1}$, characteristic of the S=O stretching vibration, and a second peak at 850 cm.$^{-1}$, corresponding to the C–O–S vibration. Both these peaks were missing from the desulphated polysaccharide (B).

Model experiments on galactose sulphates [15] have shown that the latter peak is characteristic for axial ester sulphate. A large proportion of the ester sulphate groups are therefore tentatively assigned to C-2 of the rhamnose residues on the assumption that the infrared findings for galactose can be applied to all monosaccharides and that the L-rhamnose is present in the polysaccharide in its most stable 1C conformation in which only C-2 carries an axial hydroxyl group.

The overall pattern of periodate oxidation closely resembles that for the water-soluble polysaccharide from *Enteromorpha compressa* [14] and emphasises again the essential similarity of these two polymers; although the overall reduction of periodate at 2° (0·38 and 0·68 mole for *Enteromorpha* for the sulphated and desulphated polysaccharide) is considerably less for *U. lactuca*, in spite of the fact that for the *Ulva* polysaccharides a considerably higher concentration of sodium periodate was used. This may be due to the apparently higher proportion of rhamnose and lower proportion of sulphate present in the *E. compressa* polysaccharide.

Comparison of the proportions of uncleaved sugars in the oxopolysaccharides from the sulphated and the desulphated polysaccharides showed (Table 6), as was expected, that

TABLE 6.

Molar proportions of uncleaved sugars in the oxopolysaccharides.

Polysaccharide	Glucose	Xylose	Rhamnose
(A_0)	1·0	3·3	8·3
(B_0)	1·0	2·7	1·8
(C_0)	1·0	2·0	3·9

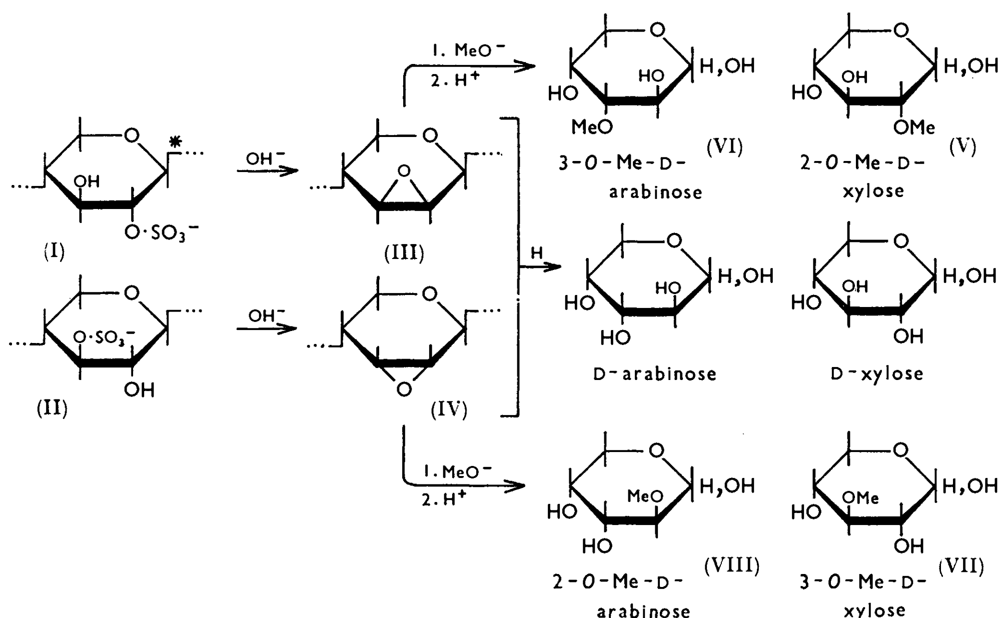
there was considerably less rhamnose in the latter and that the ratio of xylose to glucose was less than in the sulphated oxopolysaccharide, indicating the presence of some sulphated xylose.

Confirmation of the presence of sulphated xylose units was obtained from the action of alkali on the original cold-water extract. N-Sodium hydroxide at 80° in the presence of borohydride [16] was used, the latter to prevent degradation of the carbohydrate by conversion of the reducing end groups into primary alcohol groups. The polysaccharide alcohol (D) was recovered in 71% yield with the sulphate content reduced from 14·1% to 12·5%. Visual examination on a paper chromatogram of an acid hydrolysate of this product (D) revealed the presence of arabinose and apparently lyxose (trace), in addition to the sugars found in the original extract, and quantitative determination [17] of the relative molecular proportions gave glucose : xylose : rhamnose : arabinose = 1 : 1·6 : 2·0 : 0·1. Since the sugars in the original extract had only been tentatively identified by chromatography the opportunity was taken, while characterising the arabinose, of separating and

[15] Lloyd, Dodgson, Price, and Rose, *Biochem. Biophys. Acta*, 1961, **46**, 108.
[16] Rees, *J.*, 1961, 5158.
[17] Wilson, *Analyt. Chem.*, 1959, **31**, 1199.

characterising the other sugars. L-Rhamnose, D-xylose, D-mannose, and D-arabinose were characterised as crystalline materials or by crystalline derivatives. The separated syrupy lyxose had a negative rotation and was therefore probably D-lyxose. The proportion of galactose was too small for isolation and was considered to be structurally insignificant. D-Glucose was separated as a syrup from a hydrolysate of the starch-free polysaccharide and was characterised as the crystalline phenylosazone and by conversion into gluconic acid by glucose oxidase [18] (paper chromatography).

It has been established [19] from experiments on model monosaccharide sulphates that the ester sulphate groups are cleaved by alkali only if they are linked to carbon atoms which are adjacent to carbon atoms carrying free hydroxyl groups *trans* to the sulphate residues. The arabinose units formed as a result of alkali-treatment of the polysaccharide can only have arisen from xylose since glucose, rhamnose, and glucuronic acid could not possibly have been converted into this sugar under the conditions of the experiment. Their formation can be explained if the polysaccharide contains 1,4-linked xylose residues which are sulphated on C-2 (I) or C-3 (II). Either of these residues under the action of alkali yields epoxide derivatives (III) and (IV) which on acid hydrolysis of the polysaccharide give D-arabinose and/or D-xylose units. With correction for the ash, protein, sulphate, and uronic acid content it was estimated that 1·1% of arabinose was formed. The decrease in sulphate content of 1·6% corresponds to 2·5% of pentose, of which 1·1% is arabinose and consequently 1·4% is xylose. This means that cleavage of the epoxide ring yields 44% of arabinose and 56% of xylose, provided that the sulphated rhamnose is not affected during the alkali-treatment—indeed no evidence for the formation of a



* β-Linkage is indicated because of the negative rotation of the polysaccharide.

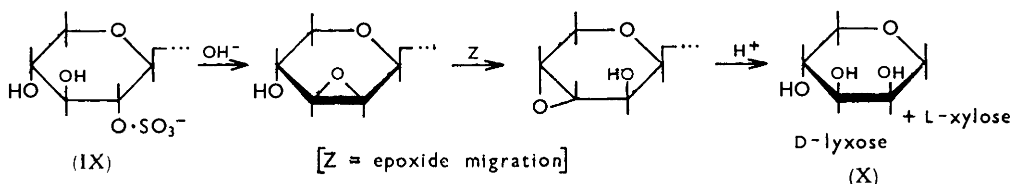
second 6-deoxyhexose was obtained. The quantity of lyxose formed was so small that it scarcely affects this calculation. The removal of about a tenth of the sulphate by alkali corresponds to monosulphation of about 15% of the xylose units.

The formation of D-arabinose does not distinguish between 2- and 3-sulphated xylose units. However, cleavage of epoxide rings with sodium methoxide results in the formation

[18] Keilin and Hartree, *Biochem. J.*, 1948, **42**, 230.
[19] Percival, *Quart. Rev.*, 1949, **3**, 369.

of methyl derivatives, the methoxyl group entering the sugar units at the site of attack. Xylose 2-sulphate should yield 2-*O*-methylxylose (V) and 3-*O*-methylarabinose (VI), whereas in the products from 3-sulphated xylose (VII and VIII) the position of the methyl groups is reversed. In the present experiments the action of sodium methoxide on the alkali-treated polysaccharide, followed by acid hydrolysis, led to the isolation and characterisation of 2-*O*-methylxylose. It had the same chromatographic and ionophoretic mobility as 2-*O*-methylxylose run as control, failed to give a spot with triphenyltetrazolium-hydroxide spray,[9] gave only xylose on demethylation, and was characterised as the crystalline anilide. It is difficult to explain the absence of a spot with the mobility of 3-*O*-methylarabinose in the hydrolysis products since the expected diaxial opening [20] would yield the 3-*O*-methylarabinose. It is probable that in the complex polysaccharide other steric factors are important. Aspinall and Ross,[21] on cleavage of a pentose disaccharide 2,3-epoxide with sodium methoxide, also obtained only the methylated xylose derivative.

The apparent presence of D-lyxose in the hydrolysate of the alkali-treated polysaccharide can be explained by epoxide-ring migration, a phenomenon that occurs under



alkaline conditions if there is a free *trans*-hydroxyl group adjacent to the epoxide ring. The *trans*-hydroxyl anion acts as a nucleophilic agent and attacks from the opposite side to the anhydro-ring oxygen, with Walden inversion and the formation of a new anhydro-ring. Any end group 2-sulphated xylose (IX) could give rise to D-lyxose (X) by this mechanism. Ricketts and Overend [22] observed the migration of a 2,3-epoxide to the 3,4-epoxide during the alkaline desulphation of a dextran sulphate. Further evidence that the lyxose was formed in this way was derived from the absence of lyxose in the hydrolysate of the polysaccharide which had been oxidised by periodate before alkali-treatment. The last experiment rules out the presence of any 3-sulphated end-group xylose units in the polysaccharide. Such units on alkali-treatment should yield L-lyxose and in view of the incomplete characterisation of the derived lyxose their possible presence cannot be ignored. However, such units would be immune to periodate and therefore yield lyxose on alkali-treatment after oxidation. It should be noted that a smaller quantity of arabinose was obtained in the hydrolysate of the alkali-treated oxidised polysaccharide. This indicates that not all the xylose 2-sulphate is present as end group.

The present work fully supports earlier findings that the water-soluble polysaccharides of *U. lactuca*,[1] *E. compressa*,[14] and *A. centralis* [23] closely resemble each other. Like other green algae, each of these weeds metabolises a small proportion of a starch. Apart from the separation of this glucose polymer, the water-soluble extracts of these three genera have persistently resisted fractionation into homopolysaccharides and appear to constitute a single heteropolysaccharide or a family of closely related heteropolysaccharides, since they appear to differ among themselves in the relative proportions of sugars present and in their sulphate content. The low reduction of periodate by all three materials indicates a high proportion of 1,3-linked units and the aldobiouronic acid, 4-*O*-glucuronosyl-L-rhamnose, is present in each, possibly as an end unit. All the evidence so far available is

[20] Mills, see Newth and Homer, *J.*, **1953**, 989.
[21] Aspinall and Ross, *J.*, 1961, 3674.
[22] Ricketts, *J.*, 1956, 3752; Overend and Ricketts, *Chem. and Ind.*, 1957, 632.
[23] O'Donnell and Percival, *J.*, 1959, 2168.

in agreement with the presence of both rhamnose and xylose in the 2-sulphated form in these polysaccharides.

EXPERIMENTAL

The analytical methods used have been described by McKinnell and Percival.[14] In addition, the following chromatographic solvent (9) was used: butan-1-ol–pyridine–water (6 : 4 : 3).

Air-dried *Ulva lactuca* (360 g.), collected in Nova Scotia, was moistened with an equal weight of water, and the colouring matter extracted from the moist weed at room temperature with acetone containing 25% of dimethyl sulphoxide. The residual off-white weed was dried and subjected to four cold-water extractions, each with 4 l. for 14 hr. with stirring. The weed residue was then extracted twice more as before, but with hot water on a boiling-water bath and under nitrogen. After filtration through muslin, the aqueous extracts were centrifuged, and the supernatant liquid was filtered at the pump and concentrated to 500 ml. The derived solution was then dialysed against running water for 3—4 days, concentrated, and freeze-dried. Yields of off-white polysaccharide from each of the extractions were 13·8 g., 13·0 g., 14·0 g. (3rd and 4th extracts combined), 26 g., 16·5 g., respectively. Chromatographic examination (solvents 2 and 3) of hydrolysates (N-sulphuric acid for 5 hr. at 100°) from each of the extracts revealed spots corresponding to glucose, xylose, rhamnose (strongest), galactose (very faint), and mannose (very faint) (spray a). The main acid spot (spray c) had the mobility of 4-*O*-β-D-glucuronosyl-L-rhamnose [1] (this material is also revealed by spray a). The extracts had the rotations and percentage composition given in Table 1. Aqueous solutions of each extract gave a blue colour with iodine. Cuprimetric titration [24] of a hydrolysate (2N-sulphuric acid at 100° for 12 hr.) of the weed residue, which contained 3% of nitrogen gave *ca.* 43% of carbohydrate (calculated as glucose). The cold-water extract (1 g.), after conversion into the free acid (0·77 g.) by treatment with Amberlite IR-120 (H^+) resin had SO_4^{2-} 15·3,[25] uronic acid 18·8%, and equiv. wt. 355.

A sample of *U. lactuca* (250 g. dry wt.) from Scotland was extracted under the same conditions and gave a 21% yield of similar polysaccharide material, $[\alpha]_D$ −49°. After treatment with salivary α-amylase the residual polysaccharide had $[\alpha]_D$ −70·7°.

Fractionation of the Cold-water Extract.—A starch-type polysaccharide (1·2 g.) was fractionated from the cold-water extract (10 g.) as the starch–iodine complex,[2] and the residual starch-free polysaccharide (A) (8·3 g.) was isolated from the supernatant liquid by freeze-drying after dialysis and concentration. It had $[\alpha]_D$ −70·7° [Found: SO_4^{2-}, 18; uronic anhydride 14·1% (by carbazole method, corrected value)] and ν 1240 and 850 cm.$^{-1}$ (Nujol mull; Perkin-Elmer " Infracord " spectrophotometer). The relative proportions of the sugars in two different hydrolysates [N-sulphuric acid (a) for 6 hr. at 100° and (b) for 16 hr. at 100°] were determined.[17] Further fractionation was attempted as follows:

(*a*) *By chromatography.*[3] Elution of the cold-water extract and of polysaccharide (A) on paper chromatograms with ammonium formate buffer (0·04M; pH 4·3)–propan-2-ol (65 : 35) for 24 and 48 hr. and development with Azur II (at pH 3·3—5·8) gave sulphated polysaccharide as a long dark blue streak with no apparent fractionation of polysaccharide (A). In the cold-water extract the starch was retained on the starting line (0·001M-iodine spray). Elution of the cold-water extract (100 mg.) on Whatman 3MM paper with this solvent, and extraction, with water, of the parts of the paper containing the front and tail of the sulphated material and the material on the starting line, gave, from the first two, sulphated polysaccharide (with a negative iodine reaction) containing xylose, rhamnose, and glucose (paper chromatography of a hydrolysate), and from the starting-line polysaccharide comprising only glucose (paper chromatography of a hydrolysate). The combined starch-free fraction had $[\alpha]_D$ −70° (*c* 1·2).

(*b*) *Ionophoresis in borate buffer* (pH 10). This was effected at 1200 v for 4 hr. on polysaccharide (A). Immersion of the dried paper in Azur II failed to yield distinct spots but gave a 2-inch streak extending from the starting line.

(*c*) *Extraction with dimethyl sulphoxide.*[6] Polysaccharide (A) (1 g.) was stirred with dimethyl sulphoxide (50 ml.) for 16 hr. at 30°. The undissolved material (0·74 g.) and that precipitated (0·21 g.) by acetone from the supernatant liquid contained the same sugars in the same relative proportions (visual examination of a paper chromatogram of the hydrolysates).

[24] Somogyi, *J. Biol. Chem.*, 1952, **195**, 19.
[25] Jones and Letham, *Chem. and Ind.*, 1954, 662.

(*d*) *Fractionation on DEAE-cellulose.* A column (45 × 2·8 cm.) containing DEAE-cellulose in the OH^- form was converted into the phosphate form by irrigation with 0·5M-sodium dihydrogen phosphate (pH 6; 500 ml.). Washing with water was continued until the eluate gave a negative test for phosphate ions. Polysaccharide (0·3 g.) in water (8 ml.) was applied to the column, and the latter was then stepwise eluted with phosphate buffer (pH 6; 250 ml. each step) of increasing concentration, (1) 0·025M, (2) 0·05M, (3) 0·10M, and (4) 0·25M. This was followed by gradient elution with sodium hydroxide (water ⟶ 0·3N-sodium hydroxide; 1 l. each). The eluate was collected in 25 ml. fractions which were analysed for their carbohydrate content.[26] The phosphate eluate was devoid of carbohydrate, but two distinct fractions were eluted with alkali. These were isolated by freeze-drying after dialysis and concentration: (1) 130 mg., $[\alpha]_D$ −57°, SO_4^{2-} 10·6%; (2) 30 mg., SO_4^{2-} 9·0%.

A second column (60 × 3·5 cm.) containing the OH^- form of DEAE-cellulose (60 g.) was converted into the chloride form by elution with 0·5M-potassium chloride (500 ml.), followed by water until a negative chloride reaction of the eluate was attained. Stepwise elution with potassium chloride of increasing concentration in six steps (0·025 ⟶ 1·5M; 500 ml. each) gave three fractions of polysaccharide material (Table 2). The three fractions gave identical infrared spectra.

Solutions of polysaccharide (A) and fraction 3 from the DEAE-cellulose column (1·0 ml., containing 0·5 g. in 100 ml. of 0·5M-sodium chloride) were examined in a Spinco model E analytical ultracentrifuge at a speed of about 50,000 r.p.m.

Removal of Sulphate from Polysaccharide A.—(*a*) *By reductive fission.* The acid form of the polysaccharide (0·37 g.), after storage for 12 hr. over phosphorus pentoxide, was shaken with pure, dry dioxan (80 ml.) for 0·5 hr. Lithium aluminium hydride (0·4 g.) was added and the mixture refluxed for 14 hr. A further 0·2 g. of hydride was then added and heating continued for a further 9 hr. After cooling, the excess of hydride was destroyed with ethyl acetate, hydrochloric acid added to pH 5, and the mixture vigorously shaken. The whole was then filtered and the dioxan removed from the filtrate by evaporation after addition of water. The derived solution was dialysed against distilled water, changed at intervals. The dialysate gave a strong positive test for sulphate. From the concentrated solution from the dialysis sac, polysaccharide (55 mg. after drying) was precipitated with ethanol (Found: SO_4^{2-}, 6·5%). Hydrolysis and paper chromatography revealed xylose, rhamnose, glucose, and glucuronic acid (trace). Refluxing with lithium aluminium hydride for 48 hr. gave a 2% yield of desulphated polysaccharide (Found: SO_4^{2-}, nil).

(*b*) *Auto-desulphation.* The acid form of the polysaccharide (1·72 g.; $[\alpha]_D$ −80°; SO_4^{2-} 19%) was shaken with dry methanol (100 ml.) at room temperature for 30 hr. The undissolved polysaccharide was washed with methanol and dissolved in water. After dialysis of the aqueous solution the polysaccharide was isolated by freeze-drying (0·91 g.). It had $[\alpha]_D$ −55·9° (*c* 0·2) (Found: SO_4^{2-}, 2·4%). From the supernatant liquid a syrup (0·70 g.) was obtained. Hydrolysates of the residual polysaccharide and of the syrup from the supernatant liquid contained glucose, xylose, and rhamnose in the relative molar proportions [17] of 1 : 1·2 : 1·5 and 1 : 6·3 : 14·8, respectively.

(*c*) *With methanolic hydrogen chloride.* The neutral polysaccharide (1·12 g.; $[\alpha]_D$ −70·7°; SO_4^{2-} 18%) was shaken with 0·09M-methanolic hydrogen chloride (150 ml.) at room temperature for 48 hr. Undissolved material was centrifuged off, and the supernatant liquid, after neutralisation with silver carbonate gave a mixture of syrupy glycosides (80 mg.). Paper chromatography of an acid-hydrolysate revealed spots of glucose (trace), xylose, rhamnose, and uronic acid.

The insoluble polysaccharide (0·78 g.), isolated as in (*b*), contained 3·8% of sulphate. Repetition of the above treatment gave a 69% yield of polysaccharide (B) (Found: SO_4^{2-}, 0·3%). The two peaks recorded in the infrared spectrum of polysaccharide (A) were absent. Increasing the strength of the methanolic hydrogen chloride to 0·15M resulted, after a single treatment, in a 68% yield of polysaccharide, $[\alpha]_D$ −80° (Found: SO_4^{2-}, 2·8%). In a third experiment with 0·10M-methanolic hydrogen chloride at 14—16° polysaccharide (C) was obtained (Found: SO_4^{2-}, 5·0%).

Periodate Oxidation of Polysaccharides (*A*), (*B*), *and* (*C*).—(*a*) *At 2° in buffer solution.* Polysaccharides (A) and (C) (1 mmole = $1C_6$ anhydro-unit; 0·16 g.) were dissolved separately in

[26] Dubois, Gilles, Hamilton, Rebers, and Smith, *Analyt. Chem.*, 1956, **28**, 350.

0·2M-acetate buffer (pH 3·6; 25 ml.) [27] and made up to 50·0 ml. with 0·10M-sodium metaperiodate in the same buffer, and the mixtures were set aside at 2° in the dark. At intervals aliquot parts (0·10 ml.) were removed and the reduction of periodate was measured [28] (Table 4).

(*b*) *At room temperature*. Each of the polysaccharides (A, B and C) (0·5 mmoles, 80 mg.) was oxidised separately with sodium metaperiodate (2·5 mmoles, 0·53 g.) in water (50 ml.) at room temperature. The reduction, followed as in (*a*), was found to be complete in 80 hr. (Table 5), and the excess of periodate was then removed by the addition of ethylene glycol. The oxopolysaccharides (A_0, 65 mg.; B_0, 60 mg.; and C_0, 63 mg.) were isolated from the residual solutions by freeze-drying after dialysis and concentration.

Estimation of the Relative Molar Proportions of the Residual Sugars in the Oxopolysaccharides.—Each of the oxopolysaccharides (A_0, B_0, and C_0) (20 mg.) was hydrolysed separately with N-sulphuric acid at 100° for 4 hr., and the derived syrups were analysed [17] for their glucose xylose, and rhamnose content (Table 6)

Alkali-treatment of the Aqueous Extract.—To the cold-water extract (3·0 g.; $[\alpha]_D$ −49°; SO_4^{2-}, 14·1%) dissolved in water (500 ml.) was added sodium borohydride (0·4 g.), and the solution was stored for 48 hr. at room temperature. Sodium hydroxide (20 g.) and sodium borohydride (3·0 g.) were then added and the loosely stoppered flask was maintained at 82° ± 2°.[16] After 4 hr. further sodium borohydride (3·0 g.) was added and after 10 hr. the solution was cooled and made slightly acid with concentrated hydrochloric acid (60 ml.). The mixture was dialysed until free from chloride ions (3 days), concentrated, and freeze-dried. The recovered polysaccharide (D) (2·15 g.) obtained as a white solid, $[\alpha]_D$ −68° (*c* 0·54) (Found: SO_4^{2-}, 12·5%) still gave a blue colour with iodine. Paper chromatography of an acid-hydrolysate in solvents 2, 3, and 9 [14] showed spots corresponding to rhamnose, xylose, arabinose, glucose, mannose (trace), lyxose (trace), and uronic acid.

Separation and Characterisation of the Sugars in the Alkali-treated Polysaccharide (D).—The polysaccharide (3·0 g.) was hydrolysed with N-sulphuric acid (100 ml.) for 6 hr. at 100°. The derived syrup (2·35 g.) was eluted from a column (4 × 45 cm.), containing Amerlite CG-45 resin (acetate form) with water until the eluate gave a negative test for carbohydrate.[26] The combined aqueous eluate was concentrated to a syrup (1·47 g. after drying over P_2O_5 *in vacuo*). The column was then eluted with 10% acetic acid (2 l.). Concentration of the eluate and removal of acetic acid by repeated additions of water and subsequent evaporations were followed by neutralisation of the uronic acid with barium carbonate. Concentration gave the barium uronates as a light brown solid (0·93 g.). Paper chromatography (solvent 3 [14]) of this material, after conversion into the free acid with Amberlite IR-120 (H^+) resin, revealed acid spots only [sprays (*a*), (*c*)], the main spot having the mobility of 4-*O*-β-D-glucuronosyl-L-rhamnose.

The neutral fraction was separated on Whatman No. 3 MM paper (*ca.* 20 sheets; 23 × 45 cm.) with solvent 2. This gave a satisfactory separation of xylose, rhamnose, glucose, and lyxose; arabinose and mannose had to be re-separated with solvent 3. The sugars were eluted from the appropriate portions of the papers with water until the eluates gave a negative test [26] for carbohydrate. Subsequent treatment of the aqueous eluates with charcoal, filtration, and concentration gave the respective sugars. The glucose fraction was not isolated as it had arisen from two sources, namely, the starch as well as the sulphated pohysaccaride. This sugar was separated in the same way from a deionised hydrolysate of the starch-free polysaccharide (A). To each of the syrupy sugars a few drops of ethanol were added. Rhamnose crystallised readily, xylose and arabinose after some days, and the others resisted crystallisation:

L-Rhamnose hydrate (275 mg.), m. p. and mixed m. p. 89—90°, $[\alpha]_D$ +9·0° (*c* 1·09), m. p. and mixed m. p. after removal of hydrate water 122—123°; the benzoylhydrazone [29] had m. p. and mixed m. p. 185—189° (decomp.).

D-Xylose (190 mg.), m. p. and mixed m. p. 144—145°, $[\alpha]_D$ +18·3° (*c* 1·01).

D-Arabinose (18 mg.), m. p. and mixed m. p. 156—158°, $[\alpha]_D$ −100·6° (*c* 0·52); benzoylhydrazone,[29] m. p. and mixed m. p. 204—208° (decomp.) (m. p. depressed on admixture with L-arabinose benzoylhydrazone).

D-Mannose (<5 mg.). Chromatographically pure syrup; phenylhydrazone,[29] m. p. and mixed m. p. 190—192°.

[27] Hough and Perry, *Chem. and Ind.*, 1956, 768; Cantley, Hough, and Pittet, *ibid.*, 1959, 1126.
[28] Aspinall and Ferrier, *Chem. and Ind.*, 1957, 1216; McComb and McCready, *Analyt. Chem.*, 1952, **24**, 1630.
[29] Hirst, Jones, and Woods, *J.*, 1947, 1048.

D-Lyxose (<5 mg.). Chromatographically and ionophoretically pure syrup. D-Configuration was assumed because of its negative rotation. D-Lyxose has $[\alpha]_D$ $-14°$. It failed to yield a crystalline phenylosazone.[30]

D-Glucose. A syrup, $[\alpha]_D$ $+48\cdot5°$ (*c* 2·7); the phenylosazone had m. p. and mixed m. p. 208—210°. Incubation with the specific enzyme, glucose-oxidase, at 35° for 24 hr. converted the sugar completely into gluconic acid,[18] as revealed by paper chromatography in solvent 3, with sprays (*a*) and (*c*).

Determination of the Relative Molar Proportions of the Sugars in Polysaccharides (*A*), (*B*), *and* (*D*).—Each of the polysaccharides (50 mg.) was hydrolysed separately with 1·3N-sulphuric acid (2 ml.) for 5 hr. at 100°, and the molar proportions of the sugars present in the neutralised, deionised solutions were determined [26] (Table 3).

Treatment of Polysaccharide (*D*) *with Sodium Methoxide.*—Polysaccharide (D) (2·0 g.) (dried over P_2O_5 at 60° *in vacuo* for one week) was soaked in dry methanol with occasional shaking for a further 2 days. The dried material, after rapid filtration, was added to a solution of lithium borohydride (0·2 g.) and sodium (6 g.) in dry methanol (250 ml.), and the mixture was refluxed for 24 hr. The insoluble polysaccharide (2·0 g.), after filtration and washing with methanol, was hydrolysed (N-sulphuric acid at 100° for 4 hr.). Neutralisation, deionisation with resin, and several additions of methanol with subsequent evaporations to remove the last trace of borate, gave a syrup (X) (1·37 g.). Separation of syrup (X) on Whatman 3MM paper in solvent 7 gave chromatographically pure 2-*O*-methylxylose (*ca.* 5 mg.). It moved with the mobility of authentic 2-*O*-methylxylose in several solvent systems and gave spots of the same colour with spray (*a*). When a similar paper was sprayed with triphenyltetrazolium chloride,[9] neither the sugar derived from polysaccharide (D) nor authentic 2-*O*-methylxylose was revealed, while authentic 3-*O*-methylxylose and 3-*O*-methylarabinose eluted on a similar paper readily gave pink spots with this spray. No spot with the mobility of 3-*O*-methylarabinose could be detected on chromatographic analysis of syrup (X). Demethylation [31] of syrup (X) gave xylose and a trace of unchanged 2-*O*-methylxylose (paper chromatography). Conversion into the anilino-derivative yielded brownish crystals, m. p. 110—120° (decomp.), insufficient for recrystallisation but undepressed in m. p. on admixture with authentic 2-*O*-methyl-*N*-phenylxylosylamine.

Periodate-oxidation followed by Alkali-treatment.—The cold-water extract (1 mmole, 0·16 g.) and sodium metaperiodate (2·5 mmoles, 0·53 g.) in water (50 ml.) was kept in the dark at room temperature. After 100 hr. the reduction of periodate [28] was complete and corresponded to 0·54 mole for every C_6 anhydro-unit. The excess of periodate was destroyed by ethylene glycol, and the solution was dialysed and then concentrated to *ca.* 25 ml. To this solution sodium borohydride (0·10 g.) was added and the mixture set aside for 24 hr., after which sodium borohydride (0·15 g.) and sodium hydroxide (1 g.) were added and the mixture was heated at $80° \pm 2°$ for 6 hr. The cooled solution was neutralised with hydrochloric acid to pH 4—5, dialysed, and evaporated to dryness. The residue obtained on hydrolysis (N-sulphuric acid 6 hr. at 100°) showed the presence of xylose, rhamnose, glucose, mannose (trace), and arabinose, but no trace of lyxose (paper chromatography).

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[30] Hamilton, *J. Amer. Chem. Soc.*, 1934, **56**, 487.
[31] Bonner, Bourne, and McNally, *J.*, 1960, 2929.