CARBOHYDRATES OF *Acetabularia* SPECIES

PART II. *A. crenulata* ACID POLYSACCHARIDE

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ABSTRACT

Water-soluble acid polysaccharides containing variable proportions of galactose, rhamnose, xylose, 4-*O*-methylgalactose, glucuronic acid, and half-ester sulphate were isolated from caps and stalks of the green seaweed *Acetabularia crenulata*. Fractionation, partial desulphation, methylation, periodate oxidation, and partial hydrolysis studies established (1→3)-linked galactose 4-sulphate (major) and 6-sulphate, and (1→2)-linked L-rhamnose, as the main, structural features of the macromolecules. Glucuronic acid, galactose, and rhamnose are all present as end-groups, indicating a highly branched molecule. Glucuronic acid is linked to both rhamnose and galactose, and galactose residues are mutually linked, in the polysaccharides.

INTRODUCTION

It has previously been reported[1] that *Acetabularia crenulata*, a green seaweed, synthesises a water-soluble fructan of the inulin-type, together with complex acid-containing polysaccharides which were fractionated on DEAE-cellulose. The present paper describes a structural study of the acid polysaccharides.

RESULTS AND DISCUSSION

Before describing the results of this investigation, it is relevant to point out that *A. crenulata* is a unicellular alga, that ~50% of the dried weight consists of calcium carbonate, and that collection of gram quantities of dried alga is both difficult and tedious.

Zetsche[2] reported different proportions of sugars in hydrolysates of stalks and caps of *Acetabularia*. For this reason, in the present experiments, stalks and caps were extracted separately both with cold and hot water. The results of the analysis of the four extracts (*CS*, *HS*, *CC*, and *HC*), after separation of the fructan, are summarised in Tables I and II. They had similar properties, and each comprised the same monosaccharides, but in somewhat different proportions. They also contained different amounts of half-ester sulphate. In a second extraction of the caps, the cold-

TABLE I

WATER-SOLUBLE, ACID POLYSACCHARIDES

Extract	*Yield* (*mg*)	$[\alpha]_D$ (*degrees*)	*Content* (%)				
			Carbohydrate	*Sulphate*	*Uronic acid*		*Protein*
					CPC	*Carbazole*	
Stalks (12 g)							
Cold water (*CS*)	55	−14.8	54	24.3	—	4.0	—
Hot water (*HS*)	89	−13.6	75	17.6	—	5.0	—
Caps (30 g)							
Cold water (*CC*)	450	−4.5	57	27.8	7.6	—	—
Hot water (*HC*)	500	−13.6	56.5	21.3	6.9		7.7
Caps (11 g)							
Hot water (*HC2*)							
0.5M KCl	105	−21.6	32.4	6.5	5.1	5.7	20
M KCl	310	−6.8	53.5	27.0	10.2	13.3	4.7
M-S	53	—	88.0	6.9	10.6	—	—

TABLE II

SUGARS PRESENT IN THE WATER-SOLUBLE POLYSACCHARIDES

	Molar proportions			
	CS	HS	CC	HC
Galactose	5.0	4.75	2.09	3.0
Xylose	2.0	1.0	0.59	0.55
4-*O*-Methylgalactose	0.2	0.55	0.59	0.55
Rhamnose	1.0	1.0	1.0	1.0
Glucuronic acid[a]	0.5	0.45	0.6	0.7

[a]Calculated from the uronic acid content of the polysaccharides.

water extraction was omitted and two fractions of the acid polysaccharide were isolated from the hot-water extract, after removal of the fructan, by elution of the DEAE-cellulose column with 0.5M and M potassium chloride (Table I). A similar variation in the relative proportions of the constituents was found in these two fractions (Table III), but the major difference was the high protein-content of the 0.5M fraction (Table I). Due to lack of material, it was not possible to carry out further studies on the extracts from the stalks.

Cold- and hot-water extracts of the caps were hydrolysed and separated by preparative paper electrophoresis into three acid fractions and a mixture of neutral mono- and oligo-saccharides. From this mixture, galactose, xylose, L-rhamnose, and 4-*O*-methylgalactose were separated and characterised. This is the first time that the last-named sugar has been reported as a constituent of a green-algal polysaccharide, although it has been found in agar[3] isolated from *Gelidium amansii*. The galactose had

TABLE III

SUGARS PRESENT IN FRACTIONS OF THE WATER-SOLUBLE POLYSACCHARIDES

	Molar proportions						
	*0.5*M			M			M-*S*
	1[a]	*2*[b]	*Average*	*1*[a]	*2*[b]	*Average*	
Galactose	3.50	3.40	3.5	1.25	1.05	1.15	1.3
Xylose	0.42	0.6	0.5	0.13	0.16	0.14	—
4-*O*-Methylgalactose	0.40	0.65	0.5	0.1	0.1	0.1	—
Rhamnose	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Glucuronic acid[c]			1.1			0.9	0.3

[a]Determined by g.l.c. as the Me_3Si derivative of the alditol. [b]Determined by phenol–sulphuric acid after separation on a paper chromatogram. [c]Calculated from the uronic acid content of the polysaccharides.

$[\alpha]_D$ +58°, and literature values of +80° for D- and −78° for L-galactose[4] indicate a mixture of 86% of D- and 14% of L-galactose. It must, however, be pointed out that the concentration of galactose in the rotation solution was measured by the phenol–sulphuric acid method[5], and it is possible that experimental error could, in part, account for the low rotation. The fact that the 2,4-dinitrophenylhydrazone derivative formed very readily and gave no depression of melting point when mixed with the corresponding standard D-derivative is evidence that the galactose in question is solely the D isomer.

Four neutral oligosaccharides (**1–4**) were separated: **1** had R_{Gal} 0.23 and 0.17 in solvents (*A*) and (*C*), respectively, a d.p.[6] of 2.7, and gave galactose and a trace of xylose on hydrolysis; **2** had R_{Gal} 0.18 (*A*) and 0.12 (*C*), a d.p. 1.8, and gave galactose and traces of xylose and rhamnose on hydrolysis; **3** had R_{Gal} 0.39 (*A*) and 0.43 (*C*), values identical with those of the (1→6)-linked galactose disaccharide, although on hydrolysis it gave traces of xylose in addition to galactose; **4** had the same mobility as the (1→3)-linked galactose disaccharide, namely R_{Gal} 0.57 (*A*) and 0.61 (*C*), a d.p. of 2.04, and gave galactose (major), xylose (trace), and 4-*O*-methylgalactose (trace) on hydrolysis.

The fastest moving acid-fraction was a complex mixture and was discarded. The second acid-fraction (M_{GlcUA} 0.87) comprised mainly D-glucuronic acid which was characterised, after purification, by its chromatographic and ionophoretic mobility and by reduction to D-glucose.

The slowest moving acid-fraction (M_{GlcUA} 0.69) contained three components (*A*, *B*, and *C*) which were separated and examined (Table IV).

Oligouronic acid A had the chromatographic mobility of a trisaccharide, but attempts to measure its d.p. failed to distinguish between the structures, GlcUA→GlcUA→Gal and GlcUA→Gal→Gal. It gave a pink colour with triphenyltetrazolium hydroxide[7] and a bluish spot with the phenylamine–aniline reagent[8], indicating the

TABLE IV

PROPERTIES OF OLIGOURONIC ACIDS SEPARATED FROM A PARTIAL HYDROLYSATE

Oligouronic acid	*Yield (mg)*	$[\alpha]_D$ *(degrees)*	R_{Glc} *(solvent B)*	R_{GlcUA} *(solvent G)*	M_{Glc} *(borate buffer)*	M_{GlcUA} *(borate buffer)*	*Sugar constituents*
A	6	+2.3 (*c* 0.3)	0.38	0.77	0.89	0.75	Galactose Glucuronic acid
B	8	+6.0 (*c* 0.4)	0.75	1.13	0.95	0.78	Galactose Glucuronic acid
C	8	−27 (*c* 0.4)	0.89	1.39	—	0.67	Rhamnose Glucuronic acid

absence of a (1→2)-linkage and the possible presence of a (1→4)-linkage. After esterification and reduction, hydrolysis gave glucose and galactose, confirming that it comprised glucuronic acid and galactose. It was methylated both before and after reduction, and from the respective hydrolysates 2,3,4-tri-*O*-methylglucuronic acid and 2,3,4,6-tetra-*O*-methylglucose were identified by paper chromatography and g.l.c., indicating that the glucuronic acid residue occupies the non-reducing end. However, in each case, 2,3,4,6-tetra-*O*-methylgalactose was obtained, indicating a branched trisaccharide. Although the methylated hydrolysates gave three spots on paper chromatography and a number of unidentified peaks on g.l.c., it proved impossible, with the material available, to characterise the third component and *A* is tentatively identified as a GlcUA→(Gal)→Gal.

Oligosaccharide B had the chromatographic mobility of a slow disaccharide or a fast trisaccharide, and its d.p. was intermediate between these two; it must be remembered that the accurate measurement of d.p. for mixed oligosaccharides depends on an exact knowledge of the constituents. Methylation studies gave similar results to those obtained for *A*, again indicating a branched trisaccharide. On the other hand, the presence of tetra-*O*-methylgalactose in the hydrolysates of the methylated oligosaccharides can equally well be explained by the presence of a very alkali-labile linkage between the glucuronic acid/glucose and the galactose residues which is cleaved during the methylation, releasing galactose for complete methylation.

Oligosaccharide C comprised glucuronic acid and rhamnose and had a d.p. of 2. The uronic acid was identified by its chromatographic and ionophoretic mobilities, and as glucose after reduction. The disaccharide gave no colour with triphenyltetrazolium reagent, indicating a (1→2)-linkage. Methylation and hydrolysis of the reduced oligosaccharide gave 2,3,4,6-tetra-*O*-methylglucose and 3,4-di-*O*-methylrhamnose, confirming the (1→2)-linkage. These results, together with the negative rotation, characterised this material as β-D-GlcUA-(1→2)-L-Rha.

Analytical gel-electrophoresis of the unfractionated, hot-water extract (*HC*) separated a slow-moving band of sulphated polysaccharide, and a small band of fast-moving material which appeared to comprise sulphated polysaccharide and protein, and could be glycoprotein (Fig. 1). An attempt to fractionate these two materials by

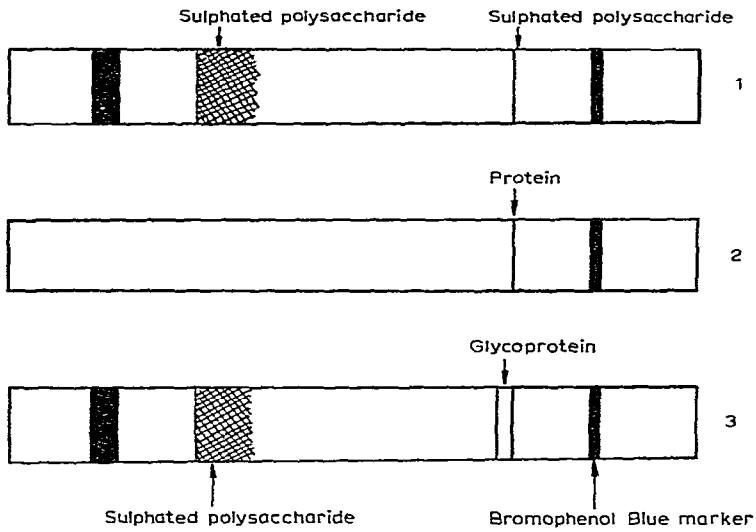

Fig. 1. Gel Electrophoresis of hot-water-soluble, acid polysaccharide (*HC*). *1*, Stained for sulphate; *2*, stained for protein; *3*, stained for charged material.

preparative gel-electrophoresis was unsuccessful. A protein fraction was obtained after elution of the Bromophenol Blue marker. The sulphated polysaccharide appeared to react with the polyacrylamide gel, since no carbohydrate was eluted. It was only after extrusion of the gel and its extraction with water that any carbohydrate could be detected, and even after dialysis the aqueous extract was contaminated with polyacrylamide.

After treatment of the cold-water extract (*CC*) with alkali, it was found that the ratio of carbohydrate to sulphate remained unchanged. From this, it is deduced that the sulphate groups are not located on any carbon atom in the sugar molecule where there is a vicinal, *trans*, free hydroxyl-group, or on either C-3 or C-6 of galactose residues where HO-6 or HO-3 are unblocked, that is where 3,6-anhydride formation could occur on removal of the sulphate.

Desulphation of the M-fraction of *HC2* with methanolic hydrogen chloride reduced the sulphate from 27 to 6.9%, with a 10% loss of carbohydrate. Analysis of the molar proportion of the sugars in the partly desulphated material (M-S) showed (Table III) a small reduction in rhamnose relative to galactose, and only trace quantities of xylose and 4-*O*-methylgalactose could be detected.

Infrared examination of this fraction before desulphation showed a broad band at 1240 cm^{-1} characteristic of S=O stretching vibration, and a moderate-sized band at 850 cm^{-1} characteristic of axial sulphate[9]. The former band was much reduced and the latter had virtually disappeared in the partially desulphated material. A small band at 820 cm^{-1} indicated sulphated, primary hydroxyl-groups[9] unchanged after desulphation. From these results, it can be deduced that a large proportion of the sulphate residues substitute axial hydroxyl groups, that is C-4 of galactose and C-2 of

TABLE V

PERIODATE OXIDATION OF SULPHATED AND PARTLY DESULPHATED POLYSACCHARIDES

	Polysaccharide		
	0.5M	M	M-S
Starting material (mg)	7.4 (46 μmoles)	16.4 (0.1 mmole)	16.7 (0.1 mmole)
$NaIO_4$ added (mg)	21.4 (0.1 mmole)	53.5 (0.25 mmole)	53.5 (0.25 mmole)
Buffer (ml)	25	25	25
Recovered carbohydrate (mg)	5.1	12	11
Theoretical[a] reduction of IO_4^- (mole/C_6 unit)	0.30	0.27	0.34
Experimentally found reduction (mole/C_6 unit)	0.25	0.52	0.52

[a]Based on the loss of carbohydrate.

L-rhamnose (in the *1C* conformation), and that a smaller proportion, resistant to hydrolysis with methanolic hydrogen chloride, are located on C-6 of the galactose residues. The sulphate content (27%) of the M-fraction is too high to be accommodated as galactose monosulphate, and it is possible that a proportion of the galactose residues are disulphated at C-4 and C-6.

The 0.5M-fraction of *HC2* reduced 0.25 mol. of periodate per sugar unit which corresponded approximately to 1 mol. of periodate for every sugar unit cleaved (Table V). From the molar ratios of the sugars before and after oxidation (Table VI),

TABLE VI

MOLAR PROPORTIONS OF SUGARS IN THE 0.5M-FRACTION BEFORE AND AFTER PERIODATE OXIDATION

	Before oxidation			*After oxidation*		
	1[a]	*2*[b]	*Average*	*1*[a]	*2*[b]	*Average*
Galactose	3.5	3.40	3.5	6.0	5.65	5.8
Xylose	0.5	0.6	0.5	0.92	0.8	0.9
4-*O*-Methylgalactose	0.6	0.5	0.5	0.95	0.8	0.9
Rhamnose	1.0	1.0	1.0	1.0	1.0	1.0

[a]Determined by g.l.c. as the Me_3Si derivative of the alditol. [b]Determined by phenol–sulphuric acid after separation on a paper chromatogram.

it can be seen that a higher proportion of rhamnose is cleaved than the other residues. The major fragment in the hydrolysate of the polyalcohol was glycerol, and this was followed by threitol and a little glycolaldehyde. No propylene glycol could be detected. The threitol would be derived from either (1→4)-linked galactose or end-group galactose 4-sulphate, glycerol from end-group galactose and/or (1→4)-linked xylose, and glycolaldehyde from the reducing end of the cleaved units. In the absence of propylene glycol, the rhamnose that is cleaved must be present as end-group. This fraction has a higher proportion of xylose than the M-fraction (Table III), and in contrast to the latter, the fact that all the units of this sugar are not oxidised in the 0.5M-fraction may be due to acetal formation between the cleaved and uncleaved units[10], or it may be due to the presence of (1→3)-linked xylose.

The M and M-S materials each reduced periodate equivalent to 0.52 mol. per hexose residue (Table V); the calculated values, based on loss of carbohydrate by the oxidation, if 1 mol. of periodate was reduced for every cleaved sugar unit, was found to be 0.27 and 0.34 mol. per hexose residue, respectively. From the difference in the calculated and determined figures, it may be deduced that a large proportion of the vulnerable sugar units each reduce 2 mol. of periodate and are therefore end-units or (1→6)-linked galactose residues.

The reduction of the same amount of periodate by the initial and partly desulphated material can only be explained if the hydroxyl groups carrying the labile sulphate in the initial material are not adjacent to a free hydroxyl-group, which would be the case with (1→3)-linked 4-sulphated galactose. Furthermore, the loss of rham-

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TABLE VII

METHYLATED SUGARS PRESENT (G.L.C.[a]) IN THE HYDROLYSATES OF THE METHYLATED POLYSACCHARIDES

Column 1	*Column* 2	*Column* 5	*Corresponding to*	
4.10 3.61	(2.47) 2.14	1.90	Galactose	2,4,6-tri-*O*-methyl (*M*)
6.20	3.18 (2.47)	(2.96)		2,3,4-tri-*O*-methyl (*M*)
1.78 (1.65)	1.63 1.53	1.18		2,3,4,6-tetra-*O*-methyl
—	(4.8)	5.17		2,4-di-*O*-methyl
—	—	3.90		6-*O*-methyl (?)
—	—	6.55		2-*O*-methyl
—	—	8.85	Free galactose[b]	
1.65 1.02	(0.63)	0.86	Rhamnose	3,4-di-*O*-methyl (*M*)
0.50	0.46	0.46		2,3,4-tri-*O*-methyl (*M*)
—	(1.01)	1.63		4-*O*-methyl
—	—	1.03		2,3- or 2,4-di-*O*-methyl
0.58 (0.50)	(0.46)	0.64	Xylose	2,3,4-tri-*O*-methyl
1.35 (1.65)	(0.76) 0.71	—		3,4-di-*O*-methyl
1.42 (1.65) (1.75)	(0.63) (0.76)	—		2,3-di-*O*-methyl (?)
1.35 (1.01)	—	—		2-*O*-methyl[b]
2.37 3.02	(2.47) (4.8)	—	Glucuronic acid	2,3,4-tri-*O*-methyl
—	(4.8)	(2.96)		2,3-di-*O*-methyl
0.73 0.96 1.17 2.04 2.42 2.90	2.25	7.78	Unidentified peaks	

[a]*M* = major peak; figures in brackets indicate incompletely resolved peaks.[b]Not present in M-S.

nose, probably as end-group during the desulphation, must have produced a new end-group which was equally vulnerable to periodate oxidation.

The ratio of rhamnose to galactose, 1:1.5 in the polyalcohol from the M-fraction, and 1:2 in that from M-S material, is smaller than in the respective starting-materials, indicating that rhamnose was oxidised preferentially to galactose in both samples. Xylose and glucuronic acid were both completely cleaved by the oxidation and must therefore be present as end-groups or (1→4)-linked units.

Paper chromatography, t.l.c., and g.l.c. of the fragments obtained on hydrolysis of the two polyalcohols revealed threitol, propylene glycol, glycerol, and glycolaldehyde in both hydrolysates. Again, threitol would be derived from either (1→4)-linked galactose or end-group galactose 4-sulphate, propylene glycol from (1→2)-linked rhamnose, glycerol from end-group galactose and/or (1→4)-linked xylose, and glycolaldehyde from the reducing end of the cleaved units except from (1→2)-linked rhamnose which gives glyceraldehyde. This has the same chromatographic mobility as glycerol and would therefore be masked.

Methylation of the M-fraction was attempted but, due to the high sulphate content, complete methylation proved impossible. However, after partial desulphation, permethylation of M-S was achieved. G.l.c. analysis of the derived glycosides and methylated alditol acetates (Table VII) revealed that (1→3)-linked galactose was a major structural feature of the macromolecule and that end-group and (1→6)-linked galactose were also present. The 2,4-di-*O*-methylgalactose is probably derived from (1→3)-linked 6-sulphated units, the 2-*O*-methylgalactose from (1→3)-linked units sulphated at C-4 and C-6, and the 6-*O*-methylgalactose from (1→3)-linked 4-sulphated units branched at C-2. No hexa-*O*-acetylgalactitol could be detected, proving the absence of free galactose in the methylated polysaccharide. Rhamnose is present mainly as end-group and (1→2)-linked or 2-sulphated units (Table VII). Di-*O*-methylrhamnose derivatives are difficult to separate and characterise, and the presence of (1→3)-linked units is not ruled out from these results. End-group glucuronic acid and xylose are present, and evidence for (1→4)- or (1→2)- and triply linked xylose was obtained.

The results from the methylation of the 0.5M-fraction were similar to those from the partly desulphated, M-S fraction. The major differences were the presence of free galactose, indicating that some of these units were either linked or sulphated at all the hydroxyl groups. There was also a relatively greater amount of mono- and di-*O*-methylgalactoses which would serve to accommodate the higher proportion of sulphate.

The most-surprising fact that emerges from both the periodate and methylation studies is the high proportion of end-units, indicating a large number of short branches. Although the periodate and methylation results are in general agreement, no 2,3,6-tri-*O*-methylgalactose (derived from end-group galactose 4-sulphate) was obtained, but the presence of small amounts of this methylated sugar might well be masked by other compounds present.

The methylation results are also in agreement with the partial hydrolysis

studies, and in particular confirm the presence of (1→3)- and (1→6)-linked galactose units only tentatively characterised in the partial hydrolysates.

Although the amount of polysaccharide from the stalks did not permit extensive structural studies, the investigation on the cold- and hot-water extracts of the caps indicated that, although they differed in the proportion of their constituents, they had essentially similar structures, and it is believed that all the extracts and fractions discussed in this paper represent extremes of a polydisperse family of highly branched polysaccharides which differ only in the fine details of structure.

Polysaccharides containing this mixture of monosaccharides have not previoulsy been reported for any alga. Although rhamnose and glucuronic acid are the major sugars in the sulphated polysaccharides of the Ulvales, they are devoid of galactose.

EXPERIMENTAL

The general methods were as described in Part I[1]. In addition, uronic acid was determined by the C.P.C. method[11] and a modified carbazole method[12], and sulphate by the C.P.C. method[11]. Acidic materials were run in solvents (*B*) and (*G*) [ethyl acetate–pyridine–acetic acid–water (5:5:1:3)]. G.l.c. of the methylated alditol acetates was effected on Chromosorb W coated with 3% ECNSS-M (column *6*) and the retention times (*T*) are expressed relative to that of 2,3,4,6-tetra-*O*-methylglucitol 1,5-diacetate. The concentrations of the solutions used in the determination of specific rotations were determined by the phenol–sulphuric acid method[5].

Isolation of the polysaccharide. — Acid polysaccharide was extracted from the stalks and caps of *Acetabularia crenulata*[1] with cold and hot water, and the acidic polysaccharide from each extract was separated from the neutral fructan as described previously[1]. The yield and properties of each extract are given in Table I.

A second batch of acid polysaccharide was prepared from caps (11 g) by exhaustive extraction with hot water (steam-bath) after extraction with hot 80% ethanol. The combined, aqueous extracts were centrifuged, and the supernatant was dialysed, concentrated, and freeze-dried (1.8 g).

Fractionation of the hot-water-soluble polysaccharide on DEAE-cellulose. — The above extract (1.8 g) was fractionated into neutral and acidic polysaccharides, as for the previous extracts, except that in this experiment, after elution of the neutral material with water and 0.3M KCl, the acidic polysaccharides were eluted with 0.5M KCl (*HC2*, 0.5M) and M KCl (*HC2*, M). The yields and properties of the two fractions are given in Table I.

Hydrolysis of the polysaccharides. — (*a*) An aliquot (5 mg) of each of the polysaccharides (Table I) was hydrolysed and analysed by paper chromatography. A portion of the hydrolysate was reduced, and the derived mixture of sugar alcohols, as their Me_3Si derivatives, was analysed by g.l.c. on column (*4*). The proportion of each sugar was determined by measurement of the peak area relative to a standard graph of the particular sugar. The relative proportions of the sugars in the two

fractions from *HC2* were determined also by separation on a paper chromatogram (solvent *A*) and elution of the appropriate area of the paper, filtration of the eluate through a Millipore filter, and determination of the weight of sugar in each filtrate by the phenol–sulphuric acid method[5].

(*b*) A second aliquot (100 mg) of the cold-water extract from the caps (*CC*) was hydrolysed, and the constituents were separated into acidic and neutral materials by preparative ionophoresis in pyridine–acetic acid buffer (pH 6.7) at 3000 volts and 25 mamp for 2 h. Four bands were located by means of guide strips (spray *1*) with M_{GlcUA} 1.06 (*1*); 0.87 (*2*); 0.69 (*3*); and 0.0 (*4*), respectively. The four bands were eluted from the appropriate strips of paper and the following freeze-dried yields were obtained: (*1*) 5 mg, (*2*) 5 mg, (*3*) 10 mg, and (*4*) 35 mg.

Separation and characterisation of the neutral sugars. — The neutral material (band *4*, 35 mg) eluted from the starting line of the ionophoretogram in (*b*) above contained 4 sugars and oligosaccharides which were separated by preparative paper chromatography (solvent *B*). *Fraction 1* was a syrup with chromatographic mobility of galactose (solvents *A–D*). It had $[\alpha]_D$ +58° (*c* 0.3). The Me_3Si derivatives of the sugar and the derived alcohol had retention times (*T*, 2.06, 2.7 and 2.84, respectively) on g.l.c. (column *4*), identical to those of authentic D-galactose and galactitol. The derived 2,4-dinitrophenylhydrazone[13] had m.p. and mixed m.p. 117–118°. *Fraction 2* was a syrup with the chromatographic mobility of xylose (solvents *A–D*), and the retention times (*T* 1.43, 2.07 and 1.0) of the Me_3Si derivatives of the sugar and the derived alditol on g.l.c. (column *4*) were the same as those of authentic xylose and xylitol. *Fraction 3* was a syrup which gave a brown spot with spray *1* on a paper chromatogram and had R_{Gal} 1.75 (solvent *A*), 1.39 (solvent *B*), and 1.42 (solvent *C*), identical with the values for 4-*O*-methyl-D-galactose, prepared from methyl 2,3,6-tri-*O*-benzoyl-D-galactopyranoside by methylation[14], removal of the benzoyl groups[15], and hydrolysis. Demethylation[16] gave only galactose (paper chromatography). The behaviour of the Me_3Si derivatives of the sugar (*T* 1.66, 1.87) and that of the derived alditol (*T* 2.05) on g.l.c. (column *4*) was identical with that of the standard materials. *Fraction 4* was a syrup with the mobility of rhamnose (solvents *A–D*) and $[\alpha]_D$ +7.9° (*c* 0.33). The Me_3Si derivative on g.l.c. (column *4*) of the sugar had *T* 0.85 and 1.25, and of the derived alditol 1.45, values identical with those of L-rhamnose and rhamnitol, respectively. The derived 2,4-dinitrophenylhydrazone[13] had m.p. and mixed m.p. 165°. *Fraction 5* was a syrup which was separated by preparative paper chromatography in solvent (*B*) into four fractions. The d.p. and chromatographic mobility of each was measured, and the constituent sugars were determined by paper chromatography of a hydrolysate.

Separation and characterisation of the acidic fragments [*see* (b) *above*]. — Paper chromatography (solvent *B*, spray *1*) of *Fraction 1* (M_{GlcUA} 1.06) revealed spots with R_{Glc} 0, 0.13, 0.36, 0.89, and 1.75. The slowest of these gave a positive test for sulphate[17]. This fraction was not examined further. Chromatographic analysis (Solvent *B*, spray *1*) of *Fraction 2* (M_{GlcUA} 0.87) revealed 3 spots with R_{Glc} 0.22, 0.92 (major), and 1.61. The major constituent had the same chromatographic mobility as glucuronic

acid in solvents (*B*) and (*G*). It was separated by preparative paper chromatography and had M_{GlcUA} 1.0 on ionophoresis in borate buffer. After conversion of an aliquot into the methyl ester methyl glycosides, reduction, and hydrolysis, analysis by paper chromatography (D-glucose oxidase spray[18]) gave a single, pink spot with the mobility of glucose. This was confirmed by g.l.c. (column *4*) of the Me_3Si derivative of the reduced material. Paper chromatography (solvent *B*) of *Fraction 3* (M_{GlcUA} 0.69) revealed 3 spots with R_{Glc} 0.33, 0.70, and 0.92. A fresh portion of this fraction, obtained from the M-KCl fraction (200 mg) of polysaccharide *HC2*, was separated by preparative paper chromatography (solvent *B*) into 3 oligouronic acids (*A*, *B*, *C*), and the respective yields, properties, and constituent monosaccharides of each are given in Table IV. Paper chromatograms of the three acids were sprayed with the triphenyltetrazolium[7] and diphenylamine–aniline[8] reagents.

Constitution of the oligouronic acids A–C. — The degree of polymerisation (d.p.) of each was determined[6] on small, measured aliquots. Standard phenol–sulphuric acid graphs were made on synthetic mixtures of GlcUA:Gal = 1:2 2:1, and 1:1. If *A* has the ratio 1:2, then the aliquot contained 41 μg of carbohydrate, and the derived alcohol 27.2 μg of glucuronic acid and galactose. It was found experimentally to be 26 μg. If the ratio is 2:1, then the aliquot contained 56 μg, for which the corresponding diuronic acid should contain 38.9 μg of uronic acid. Experimentally, the value obtained was 39 μg.

From its chromatographic mobility, *B* could be either an aldotri- or aldobiouronic acid. When the above reasoning was applied to this acid, assuming a 1:2 ratio, the calculated and experimentally found carbohydrate contents of the derived alditol were very different. A 2:1 ratio gave a theoretical content of 25.3 μg of diuronic acid, and the experimentally found value was 21 μg. The 1:1 ratio gave a theoretical content of 17.1 μg of glucuronic acid, and the value determined experimentally was 20 μg.

Acid *C* had the mobility of an aldobiouronic acid. From a standard graph of GlcUA:Rha = 1:1, the amount present in the unreduced aliquot was 79 μg which should theoretically contain 43 μg of glucuronic acid. The experimentally found amount was 46 μg.

Conversion of A–C *into neutral oligosaccharides and methylation.* — Each of the oligouronic acids (*A*–*C*) was converted into the respective methyl ester methyl glycosides[19] and then reduced with potassium borohydride. An aliquot of each derivative was hydrolysed and analysed by paper chromatography (solvent *C*, spray *1*). A second aliquot was subjected to three Purdie methylations[14]; it was necessary to add a little methanol in the first methylations in order to dissolve the oligosaccharides. After the third methylation, t.l.c. of the products (solvent *F*) gave a single spot for each oligosaccharide with R_F 0.47, 0.75, and 0.65, respectively. After hydrolysis, the mixtures were subjected to paper chromatography in solvents (*A*) and (*D*).

Aliquots (*ca.* 1 mg) of the oligouronic acids *A* and *B* were separately methylated by the method of Bishop and Perila[20].

The methyl glycosides of the hydrolysates from all the methylations were prepared[19] and analysed by g.l.c. on columns (*1*) and (*2*).

Gel electrophoresis of the hot-water, acidic extract[21]. — (*a*) *Analytical.* The hot-water-soluble, acidic polysaccharide (*HC*) from the caps was subjected to gel-electrophoresis (Shandon analytical polyacrylamide apparatus, Model SAE2734) using Tris—glycine buffer (pH 8.3). Three separate tubes of gel were run for 1 h at 250 volts and 3 mamp. One gel was stained for sulphate[17], one for protein with amido black, and a third for any charged material with 1-ethyl-2,3-(1-ethylnaphtho-[1,2-*d*]thiazolin-2-ylidene)-2-methylpropenylnaphtho[1,2-*d*]thiazolium bromide (Serva, Feinbiochemica, Heidelberg), by leaving the extruded columns in the reagent for 1 h and then washing to remove excess of stain. Bromophenol Blue was used as a marker (Fig. 1).

(*b*) *Preparative.* The polysaccharide used above (15 mg) was applied to the top of the column (Shandon model SAE2782) which was eluted with Tris–glycine buffer (pH 8.3). Fractions (2 ml) were collected and the absorptions measured at 280 nm for protein; fractions were tested for carbohydrate with phenol–sulphuric acid[5].

Desulphation of the polysaccharide. — (*a*) The cold-water extract from the caps (*CC*) (25 mg) in water (5 ml) was treated with sodium borohydride (6 mg) for 48 h at room temperature. Sodium hydroxide (200 mg) and sodium borohydride (30 mg) were added[22] and the loosely stoppered flask was held at 80° for 4 h. More borohydride (30 mg) was then added and the heating was continued for 10 h. Polysaccharide (24 mg) was recovered after dialysis and freeze-drying (Found: carbohydrate, 49.3; SO_4^{2-} 24.0; uronic acid, 11.9%).

(*b*) The polysaccharide (M-KCl fraction *HC2*, Table I, 97.5 mg) was shaken with 0.08M methanolic hydrogen chloride (20 ml) for 48 h at room temperature. The insoluble material (M-S) was centrifuged off and washed with methanol and ether [yield, 53.3 mg (Found: carbohydrate, 88; sulphate, 6.9; uronic acid, 10.6%)]. The ratios of the sugars present in this material are given in Table III. The alcoholic supernatant was hydrolysed, and paper chromatography revealed rhamnose as the major sugar.

Periodate oxidation of the 0.5M, M, and M-S polysaccharides. — Each of the polysaccharides was dissolved separately in 0.2M acetate buffer (pH 3.6). An excess of sodium metaperiodate (see Table V) was added and the reaction allowed to proceed in the dark at room temperature. The reduction of periodate was measured at intervals[23]. The reactions were stopped after 24 h by the addition of excess ethylene glycol (1 ml). After standing for 2 h, the solutions were dialysed, and the polyaldehydes were then reduced to the respective polyalcohols by addition of potassium borohydride (20 mg). After neutralisation with acetic acid and dialysis, the polysaccharides were recovered by freeze-drying. The oxidation results are given in Table V.

An aliquot of each polyalcohol was hydrolysed and the hydrolysate examined by t.l.c. (solvent *E*, sprayed with periodate–benzidine[24]) and paper chromatography (solvents *B*, *C*, and *G*). The molar ratios of the neutral sugars in the hydrolysate of the 0.5M-polyalcohol and of the galactose to rhamnose in the hydrolysates of the M and M-S polyalcohols were determined as for the hydrolysates of *HC2*.

Mild hydrolysis of the M-S polyalcohol (5 mg) (0.5M H_2SO_4, 1 ml, room

temperature for 5 h), followed by neutralisation ($BaCO_3$) and addition of ethanol to the concentrated filtrate, failed to yield a precipitate. The ethanol was removed and the aqueous solution analysed by paper chromatography.

Methylation of polysaccharide (HC2) *and characterisation of the methylated sugars.* — The 0.5M polysaccharide (15 mg) and the desulphated, M-S polysaccharide (10 mg) were subjected separately to a modified[25] Hakomori[26] methylation. T.l.c. (solvent *F*) of the methylated polysaccharides revealed a single spot[27] from each. After hydrolysis with formic acid, the methylated sugars in half of each hydrolysate were converted into the corresponding methyl glycosides and those in the remaining halves into the alditol acetates. Both were analysed by g.l.c.: the glycosides on columns (*1*) and (*2*) at 175°, and the acetates on column (*6*) at 165° (Table VII).

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