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STRUCTURAL INVESTIGATIONS OF THE EXTRACELLULAR POLYSACCHARIDES ELABORATED BY *Beijerinckia mobilis*

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ABSTRACT

The extracellular mucilage from *Beijerinckia mobilis*, a member of the Azotobacteriaceae, after removal of contaminating protein, was separated into a neutral polysaccharide (*N-2*, 10%); a neutral, dialysable fraction (*N-1*, 5%), consisting of glucose and oligosaccharides containing glucose, arabinose, and rhamnose; and an acidic polysaccharide (85%). *N-2* (mol. wt, 1900) was highly branched and comprised glucopyranose, mannopyranose, and arabinofuranose residues (1:1:1). The various linkages were determined. The acid fraction was a polymer of high molecular weight composed of L-guluronic acid (65%), D-glucose (15%), and D-*glycero*-D-*manno*-heptose (20%), together with acetic and pyruvic acids. From the results of methylation, periodate oxidation, and partial hydrolysis, a branched molecule with a backbone of guluronic acid and heptose, and side chains of glucose and guluronic acid is proposed. Pyruvic acid was found to be acetal-linked to ~25% of the heptose residues. The similarities between this polysaccharide and that from the related species *Azotobacter indicum* are discussed.

INTRODUCTION

Beijerinckia mobilis, a member of the Azotobacteriaceae, is not officially recognised by Berger's Manual of Determinative Bacteriology (1957) and is considered by some to be variant of *Beijerinckia indica* or *Azotobacter indicum*. In fact, the latter species is often[1] placed in the genus *Beijerinckia*. *B. mobilis* appears to be confined to soils of tropical regions, and has been found only in India and Burma[2], Java[3], tropical Africa[4], Northern Australia, and South America[5].

Quinnell *et al.*[6] found that the extracellular mucilage from *A. indicum* contained glucose, a uronic acid, and an aldoheptose in the ratios of 3:2:1. Later workers[7] characterised the heptose as D-*glycero*-D-*manno*-heptose, and the uronic acids as glucuronic acid with traces of galacturonic and guluronic acids. They also separated 12% of a neutral polysaccharide containing glucose, mannose, rhamnose, and arabinose. They found the uronic acid content of the acidic polysaccharide to be 17–19% and reported 25% of mannose. In a further study[8] of this extracellular

mucilage, in which the only difference in the culture conditions was the absence of buffer, only a trace of mannose in the extracellular acid polysaccharide was found together with glucose, heptose, and uronic acid in equal amounts.

Norwegian workers[9] subsequently examined the extracellular mucilage from the same organism and did not find any neutral polysaccharide. Their polymer contained glucose, an aldoheptose, thought to be D-*glycero*-D-*manno*-heptose, 30% of L-guluronic acid, and 20% of acetyl groups.

In the present studies, the organism was cultured by Alginate Industries Ltd., and a crude polysaccharide was supplied to the authors.

RESULTS AND DISCUSSION

The average percentage yield of crude polysaccharide, based on the sucrose used was 30%, and an average yield of 15 g/l of culture was obtained. The contents of the different batches varied as follows: protein 10–20%, carbohydrate (as glucose) 38–58%, moisture 12–20%. These variations depended upon the effectiveness of the initial centrifugation of the gelatinous medium in removing the bacterial cells. All the samples, after hydrolysis, gave the same pattern of spots on p.c. and peaks on g.l.c. When a 0.1% suspension of the crude polysaccharide was centrifuged, it was found that all the nitrogen, and thus all the protein, had been removed in the centrifugate (*A*).

The purified polysaccharide (*C*), isolated from the supernatant, was devoid of nitrogen and had an average carbohydrate content of 64% as glucose. However, when the exact composition of the polysaccharide was known, these values could be corrected; an average value of 70% was obtained, and of this ,~41% was uronic acid. The molar proportions of the sugars (based on two methods) found in the hydrolysate, which had been separated into neutral and acid fractions, were mannose 1, glucose 4.9–5.6, arabinose 1.05–1, rhamnose trace, heptose 1.25 (one determination).

Taking into account the uronic acid content, the approximate carbohydrate composition of the total extracellular polysaccharide, measured on a hydrolysate, is therefore uronic acid 58, glucose 22, heptose 6, arabinose 5, mannose 4.5%, and rhamnose trace. These figures do not take into account any neutral sugars in the oligouronic acids or those lost by degradation during hydrolysis; for these reasons, it is considered that the percentage of heptose is probably higher. It was found that 11% of the neutral sugars (glucose equivalent) and 30% of the uronic acid (glucuronic acid equivalent) were lost during hydrolysis.

The presence of uronic acid and at least five neutral sugars suggested the presence of more than a single polysaccharide. The high viscosity of the solution (1000 cp for a 1% solution), and the fact that the solution gelled when salt was added, made fractionation by gel filtration impossible. Fractionation on columns of Permutit Deacidite FFIP resin and DEAE-cellulose was attempted, but recovery was low (30–50%) and no fractionation occurred. Attempted fractionation by precipitation with barium hydroxide[10] or with ethanol[11] resulted in all the carbohydrate being precipitated, and with Cetrimide[7] (B.D.H), all the polysaccharide was complexed.

Furthermore, the complex did not dissolve as expected[7] in 50% acetic acid. After consultation with Dr. J. E. Scott, a purified form of Cetrimide (hexadecyltrimethylammonium bromide from Eastman Kodak) and a very dilute solution of polysaccharide were used, and ~85% of the carbohydrate was then complexed. The neutral carbohydrate recovered from the sypernatant had $[\alpha]_D$ +16°, was devoid of protein and uronic acid, and contained ~55% of glucose; it was fractionated into dialysable (*N-1*) and non-dialysable (*N-2*) material.

Analysis of the neutral fractions N-1 *and* N-2. — Fraction *N-1* contained mainly glucose with some mannose and oligosaccharides. After hydrolysis, arabinose and rhamnose were also present. The amount of glucose was 60% before, and 80% after, hydrolysis. It was thought that these small fragments resulted from the presence of an enzyme which degraded the polysaccharide during the purification and fractionation. However, a sample of crude polysaccharide that had been suspended in boiling water for 20 min still contained the same oligo- and mono-saccharides. Thus, it seems that the carbohydrates of low molecular weight are present in the medium during the growth of the bacteria and are produced either because the polysaccharides are incompletely metabolised, or because of the presence in the medium of an enzyme which partially hydrolyses the polysaccharide, leaving mono- and oligo-saccharides trapped inside the gel; removal by dialysis is not possible unless a very dilute solution (0.05%) is used.

The fraction *N-2* contained 30% of glucose, and comprised approximately equal proportions of glucose, mannose, and arabinose. Each of these sugars was isolated and characterised by p.c., by g.l.c. of the Me_3Si derivatives both before and after reduction, and by the formation of crystalline derivatives. These results are in keeping with those of Jones *et al.*[7], who reported the presence of a neutral polysaccharide, which comprised glucose, mannose, rhamnose, and some arabinose, in the extracellular mucilage of *A. indicum*.

The small quantity of oligosaccharides separated from the partial hydrolysate were mixtures even after repeated p.c. Of the five fractions obtained, four contained only mannose and arabinose and had d.p. 7–15. One with d.p. 4 contained glucose and mannose. Mannose appears, therefore, to be linked to the other two sugars in the macromolecule.

Methylation studies on fraction N-2. — The large number of methyl glycosides derived from methylated *N-2* gave a complex pattern of peaks on g.l.c. It was therefore decided to rely on characterisation of the methylated alditol acetates (see Table I for retention times). The identity of each peak was confirmed by its fragmentation pattern on mass spectrometry.

From Table I, it can be seen that there is a large amount of end group, mainly arabinofuranose, and some mannopyranose, indicating a highly branched polymer probably with fairly short chains. The remainder of the arabinose is (1→2)- and (1→3)-linked furanose units, and (1→4)- and/or (1→5)-linked residues. In addition, arabinose occurs at branch points linked (1→2),(1→4) and/or (1→2),(1→5). The 2,3-di- and 3-*O*-methylarabinoses give no information as to the ring size of the units.

TABLE I

METHYLATION ANALYSIS OF THE NEUTRAL POLYSACCHARIDE[a]

Peak No.	T[b]		*Methylated derivative*
1	0.45 (m)	Arabinose	2,3,5
2	0.70		3,5
3	0.85		2,5
5	1.07 (tr)		2,3
6	1.85[c]		3
4	0.99 (tr)	Mannose	2,3,4,6
6	1.85[c]		2,4,6
11	4.5		2,4
7	2.2 (m)	Glucose	2,3,4
8	2.3		2,3,6
9	3.7 (tr)		3,6
10	4.2 (tr)		3,4

[a]G.l.c., Pye 104, column *5*. [b]Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol. [c]Column *6*: *T* 1.83 and 1.92, respectively, for these two methylated sugars. Key: m = major, tr = trace.

However, since the majority of the arabinose is furanoid, it is probably safe to assume that these latter linkages are (1→5) and (1→2),(1→5) for these residues.

The mannose, in addition to the small amount of end group, is (1→3)- and (1→3),(1→6)-linked. The glucose is (1→6)- and (1→4)-linked, and a very small proportion occurs at branch points.

Periodate oxidation stndies of N-2. — Fraction *N-2* reduced 1.3 mol of periodate per "anhydro sugar" unit, and the derived polyalcohol contained intact mannose (major), arabinose, and glucose (2%) residues. This result is in agreement with the methylation data, since the only mannose residues that are vulnerable to attack are those present as non-reducing end-units. Arabinose involved in (1→3)-links and at branch points would also be resistant to periodate. In contrast, the only glucose residues which would not be cleaved are those at branch points.

Molecular size of N-2. — The polysaccharide was subjected to gel-permeation chromatography, by Dr. A. DeBelder (Pharmacia Ltd.), on columns of Sephadex G50/G75; the values $\bar{M}_W$ 5,400 and $\bar{M}_N$ 1,900 were obtained.

Thus, it is concluded that polysaccharide *N-2* has a highly branched molecule with fairly short side-chains, and that they are held in the matrix of the gel, possibly by hydrogen bonding to the acid polysaccharides.

The acid polysaccharide had $[\alpha]_D$ +26°, and comprises ~85% of the total extracellular carbohydrate. It is completely excluded from a column of Sepharose 6B, indicating a molecular weight >5 million. On Sepharose 2B, a broader distribution is obtained, but a considerable amount was still excluded, indicating a molecular weight probably >20 million. Nevertheless, there is a possibility that molecular aggregation has occurred under the gel-like conditions, and that the apparent molecular weight is a multiple of the actual molecular weight. Against this possibility is the fact that

application of the total extracellular mucilage to a column of Sepharose 2B gave an elution pattern with fractions of high and very low molecular weight, the latter probably being the neutral material.

The acid polysaccharide contains uronic acid (65%), glucose (15%), and a substance (20%) which reacted as a heptose and had the same mobility in p.c. as D-*glycero*-D-*manno*-heptose, and the same retention times on g.l.c. both before and after reduction. Complete separation from glucose proved difficult. After repeated p.c., pure, syrupy material (~2 mg) was obtained which had the same $[\alpha]_D$ (+17°) as authentic D-*glycero*-D-*manno*-heptose: the literature value, $[\alpha]_D$ +21°, was recorded for crystalline material. Complete hydrolysis of the polysaccharide, necessary to obtain a quantity of the heptose, resulted in considerable degradation, suggesting that the heptose is linked to the uronic acid, and this was confirmed by partial hydrolysis.

Characterisation of the uronic acid. — The identification of the uronic acid was important because of the conflict, between Jones *et al.*[7] and Haug and Larsen[9], as to the identity of the uronic acid present in the extracellular polysaccharide from *A. indicum*. The uronic acid from *B. mobilis* had $[\alpha]_D$ +42° (*cf.* +37° for L-guluronic acid from alginic acid). It is not possible to differentiate the four common uronic acids by chromatography, and although paper electrophoresis[12] in borate buffer containing Ca^{2+} clearly indicated the presence of guluronic acid, the occurrence of slight streaking indicated the possible presence of glucuronic acid. Lactonisation[13] of the unknown acid and of authentic guluronic acid could not be achieved. After conversion of the uronic acid into the corresponding sugar, p.c. and g.l.c. indicated a major component with the mobility of gulose, together with a fast-moving component having the same mobility and retention time as 1,6-anhydrogulose. Electrophoresis of the reduced material in molybdate buffer gave 2 spots with $M_{GLUCITOL}$ 0.0 and 1.18, identical with the spots given by gulose. A sugar will complex with molybdate only if it possesses the *cis,cis* configuration of three vicinal hydroxyl groups. Of the sugars derived from the common uronic acids, β-L-gulopyranose and mannose possess this configuration, whereas 1,6-anhydrogulopyranose, glucose, and galactose do not. The ability to form a complex leads to mobility on electrophoresis, and whereas the smaller non-mobile spot might be due to glucose and/or galactose, it is more likely to be 1,6-anhydrogulose. The absence of mannose and presence of gulose was confirmed by p.c., using a borate-containing solvent. G.l.c. of the reduced acid and gulose gave identical chromatograms, which were easily distinguished from that of glucose. The absence of D-glucose, and hence of D-glucuronic acid, was also confirmed by the negative reaction to a D-glucose oxidase spray after p.c. of the reduced acid. Thus, it appears that the uronic acid present in *B. mobilis* is L-guluronic acid, the same as that found by the Norwegian workers for *A. indicum*.

The presence of pyruvic acid in the acid polysaccharide. — An n.m.r. spectrum (D_2O) of the polysaccharide contained peaks at τ 7.8–8.5 for uncoupled methyl protons, indicating the presence of pyruvic acid[14,15], although this was not reported[7,9] for the *A. indicum* polysaccharide.

Mild, acid hydrolysis of the acid polysaccharide and ether extraction yielded a

syrup which was indicated to be pyruvic acid by its mobility on p.c. and on electrophoresis, and by the retention time on g.l.c. of the methyl ester; this identification was confirmed by preparation of the crystalline 2,4-dinitrophenylhydrazone. Using the method of Sloneker and Orentas[16], the acid polysaccharide was found to contain 2.6% of pyruvic acid *i.e.*, 1 pyruvic acid residue per 20 sugar residues.

The presence of acetyl groups in the acid polysaccharide. — The Norwegian workers[9] reported the presence of one acetyl group per two sugar units in the *A. indicum* polysaccharide; *cf.* 1 acetyl group per four sugar units (AcO, 8.5%) for the *B. mobilis* polysaccharide[17]. A parallel experiment on *A. indicum* polysaccharide gave AcO, 15%, in agreement with the results of the earlier workers.

An attempt to discover the site of the acetyl groups by blocking the free hydroxyl groups with methyl vinyl ether, followed by simultaneous deacetylation and methylation[18], was unsuccessful, mainly due to the insolubility of the polysaccharide in methyl sulphoxide in the presence of toluene-*p*-sulphonic acid.

Analysis of the acid polysaccharide and the depyruvylated, deacetylated material gave identical carbohydrate compositions, namely, uronic acid 65, heptose 20, and glucose 15%.

Methylation studies. — Methylation of uronic acid-containing polysaccharides is difficult, and hydrolysis of the methylated material leads to considerable degradation. For this reason, and also because the fragmentation pattern of acetates of partly methylated hexuronic acids had not been fully established, the uronic acid residues in the partly methylated polysaccharides were reduced to the corresponding sugar.

The methylated sugars, characterised from the hydrolysates of the variously methylated polysaccharides, are given in Tables II and III; the gulose derivatives have been described previously[19].

TABLE II

METHYLATED METHYL GLYCOSIDES FROM THE ACID POLYSACCHARIDE

T^a			
Column 1	*Column* 2		*Methylated derivative*
(*1*) *Acid polysaccharide, methylated, reduced, re-methylated, and hydrolysed*			
4.3; 4.7	3.3	Heptose	3,4,6,7
1.0; 1.36	1.0; 1.32	Glucose	2,3,4,6
3.0; 4.1	1.65; 2.3		2,4,6
1.53	1.37	Gulose	2,3,4,6
2.1; 3.7	1.5, 2.1		2,4,6
	3.1		2,6
(*2*) *Acid polysaccharide, methylated, reduced, and hydrolysed*			
4.4; 4.7	3.3	Heptose	3,4,6,7
1.0; 1.36	1.0; 1.32	Glucose	2,3,4,6
3.0; 4.1	1.65; 2.3		2,4,6
3.2; 4.5	1.37; 1.93	Gulose	2,3,4
	2.46; 3.2		2,4

[a]Retention time relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside.

TABLE III

ANALYSIS OF THE METHYLATED ALDITOL ACETATES FROM THE ACID POLYSACCHARIDE

Peak No.	T[a] (*Column 5*)		*Methylated derivative*
(1) Acid polysaccharide, methylated, reduced, and remethylated			
4	3.1	Heptose	3,4,6,7
3	1.75	Glucose	2,4,6
1	1.03	Gulose	2,3,4,6[b]
2	1.68 (m)		2,4,6
5	3.8		2,6
(2) Acid polysaccharide, methylated and reduced			
4	3.1	Heptose	3,4,6,7
7	7.5		3,6
1	1.0	Glucose	2,3,4,6
2	1.75		2,4,6
3	1.89	Gulose	2,3,4
5	4.0		2,4
6	6.0		2
(3) Acid polysaccharide, methylated			
1	1.0	Glucose	2,3,4,6
2	1.75		2,4,6
3	3.1	Heptose	3,4,6,7
4	7.3 (tr)		3,6

[a]Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol. [b]May contain 2,3,4,6-tetra-*O*-methylglucose. Key: m = major, tr = trace.

It can be seen that the glucose is mainly (1→3)-linked. The presence of 2,3,4,6-tetra-*O*-methylglucose shows that glucopyranose is also present as end group. Although no methylated heptoses were available for comparison, two peaks were identified from their mass spectra as 3,4,6,7-tetra-*O*-methyl- (major fragment ions at *m/e* 43, 45, 87, 89, 99, 129, 189, and 205) and 3,6-di-*O*-methyl-heptose (major fragment ions at *m/e* 43, 87, 99, 117, 129, and 189), indicative of (1→2)- and 2,4,7-linked or substituted heptose.

Since stereoisomers cannot be distinguished by mass spectrometry, it was not possible to determine the origin of the methylated hexitol (*i.e.*, from glucose or the reduced uronic acid). Incorporation of deuterium at C-6 of the uronic acid by reduction with potassium borodeuteride made this distinction possible. Alkaline conditions (*e.g.*, Hakomori methylation) must subsequently be avoided, as exchange would then occur. Methylation of HO-6 of the reduced uronic acid, without loss of deuterium, was possible with diazomethane.

The recorded[20] fragmentation patterns for partially methylated alditol acetates containing deuterium enabled the characterisation of 2,3,4,6-tetra-*O*-methyl-, 2,4,6-tri-*O*-methyl-, and 2,6-di-*O*-methyl-guloses, confirming that the guluronic acid is mainly (1→3)-linked, with some end-group and some (1→3),(1→4)-linked units. No deuterium was found in the 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol peak

obtained by reduction of 2,4,6-tri-*O*-methylglucose with borodeuteride and remethylation, and this fact also indicates that no glucuronic acid is present in the polysaccharide.

The g.l.c. peak-areas of the end groups were 18.5% of those of the total gulose. If gulose comprises 65% of the molecule, the end groups are 12%.

The site of pyruvic acid. — The presence of 2,4,7-linked or substituted heptose suggests that it is these units which carry the pyruvic acid at positions 4 and 7. In other polysaccharides[14,15], pyruvic acid is acetal-linked to positions 4 and 6 of either glucose, galactose, or mannose thus forming a 6-membered ring. A model of the heptose with pyruvic acid acetal-linked to positions 4 and 7 was strain-free.

The acetal linkage should be acid-labile and base-stable. Thus, the pyruvic acid residues should not be removed from the polysaccharide by methylation or by periodate oxidation. Nevertheless, the di-*O*-methylheptose derivative disappeared during reduction and remethylation, indicating that the pyruvic acid is lost during these experiments. N.m.r. spectroscopy after each stage of the procedure showed that pyruvic acid was lost during dialysis after reduction of the methylated polysaccharide. It was concluded that the pyruvic acid was cleaved by the IR-120(H^+) resin used to remove the inorganic cations.

Periodate-oxidation studies. — The acid polysaccharide reduced 0.59 mol of periodate and released 0.15 mol of formaldehyde per "anhydro sugar" unit, and the polyalcohol was recovered in 73% yield. Formaldehyde is only released by oxidation of the heptose residues and the yield indicates that not all of the heptose had been oxidised. Indeed, a hydrolysate of the polyalcohol contained intact heptose possibly derived from units carrying pyruvic acid. The depyruvylated polysaccharide reduced 0.65 mol of periodate and yielded 0.19 mol of formaldehyde, and the polyalcohol was recovered in 63% yield. The formaldehyde release is in good agreement with the heptose content (20%) estimated by g.l.c. Hydrolysis of the polyalcohol gave no intact heptose.

Each residue of heptose that is available for periodate oxidation reduced 0.30 mol of periodate. Thus, the remaining 0.29 mol of periodate was presumably reduced by end-group units, each of which should reduce 2 mol. This corresponds to a degree of branching of 14% which is in reasonable agreement with the value (12%) found from methylation studies. The amount of intact uronic acid and glucose in the polyalcohol indicated a loss of 11% of uronic acid and 3% of glucose, confirming a 14% degree of branching. Thus, the acid polysaccharide is a branched polymer with L-guluronic acid at branch points, and D-glucose and L-guluronic acid at the non-reducing ends. The remainder of the D-glucose, L-guluronic acid, and heptose form the chains, with the latter two sugars mutually linked.

Similar measurements on the depyruvylated polysaccharide indicated a 4% loss of glucose. This increase in the oxidation of glucose might indicate degradation during the removal of pyruvic acid, thereby exposing glucose to attack by periodate. However, the heptose in the depyruvylated polysaccharide accounts for the reduction of 0.38 mol of periodate, and thus the remaining 0.27 mol of periodate is presumably

used to oxidise the non-reducing end-group, corresponding to 13.5% of branching. It follows that very little, if any, degradation had occurred during depyruvylation. This conclusion was further confirmed by measurement of the g.l.c. peak-areas for the components of the hydrolysate of the methylated, depyruvylated polysaccharide, which gave a degree of branching of 13%.

Mild, acid hydrolysis of the polyalcohols from the acid polysaccharide, before and after depyruvylation, released only glycerol and glyceric acid, the expected fragments from oxidised glucose and guluronic acid end-groups, respectively. Increase in the concentration of the acid or of the temperature released monosaccharides, but all attempts to isolate larger fragments were unsuccessful. It appears that the acyclic acetal linkages of the oxidised heptose residues were stabilised by the adjacent guluronic acid residues.

The methylation results for the polyalcohol, apart from a decrease in the proportion of 3,4,6,7-tetra-*O*-methylheptose, were substantially the same as those from the initial polysaccharide. The methylated, depyruvylated polyalcohol was devoid of methylated heptose.

Attempts[21] to isolate a mono- or di-saccharide, carrying the pyruvic acid acetal, by partial hydrolysis before and after methylation of the acid polysaccharide were unsuccessful. Other workers[14] have found the same difficulty where a glycuronosyl residue is linked to the sugar carrying the pyruvic acid residue.

Oligosaccharides obtained by partial, acid hydrolysis. — The oligosaccharides (Table IV) separated from a partial, acid hydrolysate of the acid polysaccharide are devoid of glucose and consist of mixtures of guluronic acid and heptose, apart from **3** which contains only guluronic acid.

TABLE IV

ANALYSIS OF OLIGOSACCHARIDES FROM THE ACID POLYSACCHARIDE

No.	*Wt. (mg of carbohydrate)*	*D.p.*	*Uronic acid (%)*	*Other constituents*	*Reducing end*	$[\alpha]_D$ *(degrees)*
1	167	6	63	Heptose	Heptose	+50
2	107	3	67	Heptose	Heptose	+47
3	116	2	98	—	Uronic acid	+64
4	165	2	50	Heptose	Heptose	+75
5[a]	25	1.1	50	Heptose	—	—

[a]Mixture of heptose and uronic acid, which was not separated.

After methylation, reduction, and remethylation of these oligosaccharides, the following methylated sugars were characterised in the hydrolysates. 2,3,4,6-Tetra-*O*-methylgulose was a constituent of all of the hydrolysates, proving that guluronic acid was present as the non-reducing end-group. 2,4,6-Tri-*O*-methylgulose was also present, except in oligosaccharide **4**, and 3,4,6,7-tetra-*O*-methylheptose was a constituent of the hydrolysates of oligosaccharides **1**, **2**, and **4**, From these facts, it can be

deduced that the structures of these oligosaccharides are as follows: **4**, GulA-(1→2)-heptose; **3**, GulA-(1→3)-GulA; **2**, GulA-(1→3)-GulA-(1→2)-heptose; and that the hexasaccharide comprises two molecules of **2**. This was further confirmed by further hydrolysis of **1**, which gave (p.c.) **2**, heptose, and guluronic acid. A possible repeating-unit for the polysaccharide is therefore:

→3)-L-GulA-(1→3)-L-GulA-(1→2)-D-*gly*-D-*man*-Hept-(1→

The location of the glucose residue is not revealed from these studies, as no oligosaccharides containing glucose were detected. It is known that the (1→3)-linkage is acid-labile, and it is considered that the glucose residues are present as (1→3)-linked side-chains terminated by glucose. Guluronic acid also probably occurs as side chains. Both types of side chain are attached to the uronic acid in the backbone through position 4, this being the only unit which occurs at branch points.

EXPERIMENTAL

General. — The general methods have been described previously[22]. In addition, the following solvent systems for paper chromatography (p.c.) were used: *G*, ethyl acetate–pyridine–acetic acid–water (5:5:1:3); *H*, 1-butanol–ethanol–water (3:1:1); *J*, butanone–acetic acid–water (9:1:1) saturated with boric acid. Detection was also effected with *5*, *p*-anisidine hydrochloride; *6*, D-glucose oxidase[23]; *7*, heptose spray[7]; *8*, Bromophenol Blue; *9*, *o*-phenylenediamine for keto acids[24]; *10*, D-galactose oxidase; and *11*, aniline oxalate in glacial acetic acid. Ionophoresis was carried out on Whatman No. 3 paper in (*a*) 0.1M borate buffer at pH 10.0 (the non-migrating marker was 2,3,4,6-tetra-*O*-methylglucose), (*b*) molybdate at pH 5.0 (the non-migrating marker was glycerol), (*c*) pyridine–acetic acid at pH 4.0 and 6.8 (glucose was the non-migrating marker), (*d*) ammonium carbonate (pH 8.9), (*e*) borate with calcium ions[12]. T.l.c. was performed on Kodak precoated-cellulose in solvent *B* or ethyl acetate. In addition to the g.l.c. outlined previously for methylated methyl glycosides, a Pye 104 gas chromatograph was used with nitrogen carrier-gas, flame-ionisation detector, and glass columns (3 m × 5 mm) packed with Gaschrom Q, with 7.5% of Apiezon K (column *4*) for the Me_3Si derivatives of sugars and alditols, and 3% of OV 225 (column *5*) for the methylated alditol acetates. A Perkin–Elmer F11 gas chromatograph with flame-ionization detector and helium gas, and a glass column (4 m × 1.6 mm) packed with 3% of OV 225 on Gaschrom Q at 205–210° (column *6*), was coupled *via* an all-glass system through a Watson–Biemann separator to a Hitachi RSM-4 mass spectrometer. Mass spectra of partially methylated alditol acetates were obtained at 50 eV with the ion source at 230° and an 80-μA target current.

Uronic acid was determined by a modified carbazole reaction[25] (read of a guluronic acid graph), and D-glucose with D-glucose oxidase. The degree of polymerisation (d.p.) of oligosaccharides was measured by the Timell modification[26] of the Peat method[27]. Alditol acetates were prepared from the methylated sugars by the method of Björndal *et al.*[28].

Esterification and glycosidation of uronic acid was achieved by refluxing with

2% methanolic hydrogen chloride for 12 h. The acetyl content of the acid polysaccharide was determined by the method of Hestrin[17]. Unless otherwise stated, reductions were carried out with potassium borohydride in aqueous solution. Samples of polysaccharide for i.r. spectra were prepared by spreading a thin paste with water on a microscope slide, allowing to dry, and supporting the resulting thin film in a photographic slide mount. Spectra were obtained using a Perkin–Elmer 257 grating spectrometer.

Culture of Beijerinckia mobilis. — The culture (strain number NCB 9879) was obtained from the National Collection of Industrial Bacteria, Torry Research Station, 135 Abbey Road, Aberdeen. After culture for 5–7 days on Burks agar slopes, the resulting slime (1.5 ml) was transferred to Burks medium supplemented with ammonium citrate (200 ml), and incubated at 26° for 2–3 days. The seed-stage culture (20 ml) was then inoculated into Burks medium supplemented with potassium nitrate (200 ml), and incubated for 4 days at 26° and 220 r.p.m. on a shaker.

Analysis of the extracellular mucilage. — The mixture was centrifuged and the crude polysaccharide was obtained as an off-white powder by precipitation with acetone.

Each crude sample was analysed for protein, carbohydrate, and moisture, and then hydrolysed, and the hydrolysate was analysed by p.c. (solvents *A–C*), and by g.l.c. of the Me_3Si derivatives before and after reduction.

Purification of the mucilage. — The crude material (1 g, 380 mg of carbohydrate) as a 0.1% suspension was dispersed in water, using a Stilson high-speed stirrer, and then centrifuged at 10,000 r.p.m. for 60 min. The centrifugate (*A*) was freeze-dried to give a light, flocculent, cream-coloured solid (0.3 g; carbohydrate content, 11%). The supernatant solution was concentrated by freeze-drying, since it frothed badly on conventional concentration. The addition of acetone (5 vol.) to the concentrated solution gave purified polysaccharide as a white precipitate (*C*, 0.46 g; carbohydrate content, as glucose, 64%). The filtrate, after removal of acetone, was freeze-dried to give a sticky, yellow substance (*B*, 0.2 g; carbohydrate content, 18%).

Analysis of the purified polysaccharide. — A portion of *C* (1 g) was hydrolysed and the hydrolysate was applied to a column of Deacidite FFIP (SRA 67) ($HCOO^-$) resin (200 g). The neutral sugars were eluted with water, and the acid components were obtained by gradient elution (0 → 2M formic acid). The molar proportions of the neutral sugars were measured by the peak areas on g.l.c. of the Me_3Si ethers of the derived alditols. Microgram quantities of the sugars were obtained by p.c. (solvent *A*). Where this resulted in mixtures, the carbohydrate content of the mixture was measured and the mixture was fractionated by preparative p.c. (solvent *B*). The carbohydrate content was calculated from the individual standard graphs. All eluates were passed through millipore filters.

Measurement of the extent of degradation caused by hydrolysis. — The polysaccharide (100 mg), of known content of carbohydrate and of uronic acid, was hydrolysed, the hydrolysate was freeze-dried, and the product was assayed for carbohydrate and uronic acid.

Fractionation of the polysaccharide. — A solution of the purified polysaccharide (20 g) in water (4 l) was treated with 10% aqueous Cetrimide (hexadecyltrimethylammonium bromide, Eastman Kodak) (100 ml). The complex was collected on nylon cloth, washed with water, and stirred with 2M sodium chloride (2 l) for 48 h. The polysaccharide (hereinafter called the acid polysaccharide) was recovered from the resulting solution by precipitation with ethanol. Purification was effected by repeated dissolution in water and reprecipitation with ethanol, and the polysaccharide was finally recovered as a white solid (14 g) by freeze-drying.

An aliquot of the supernatant from the Cetrimide precipitation, when re-treated with Cetrimide, gave no further complex. The Cetrimide was removed from the supernatant with Biodeminrolit (CO_3^{2-}) resin, and the solution was freeze-dried to give a yellow solid (2.3 g). The solid was dissolved in water, and ethanol (5 vol.) was added. The resulting precipitate (*N-2*) was redissolved in water and the solution was freeze-dried (yield, 1.5 g). The supernatant solution was freeze-dried to yield *N-1* (0.75 g), which was assayed for glucose and analysed by p.c. (solvents *A–C* and sprays *1*, *4*, and *6*), before and after hydrolysis.

Examination of the neutral material N-2. — *N-2* was assayed for glucose. A portion was hydrolysed and the molar proportions of the constituent sugars were estimated from the peak areas on g.l.c. of the Me_3Si ethers of the derived alditols. A second portion of *N-2* was partially hydrolysed with 0.25M sulphuric acid for 8 h at 100°. The neutralised ($BaCO_3$) hydrolysate was concentrated to small volume, applied to a column of Whatman CF11 cellulose, and eluted with 1-propanol–ethyl acetate–water (7:2:1). Fractions (5 ml) were collected, concentrated, and analysed by p.c. This method is less sensitive than the phenol–sulphuric acid method, in which 1-propanol interferes. The fractions were combined, as appropriate, into 8 groups. Those which comprised mixtures were fractionated by preparative p.c. (solvent *B*). Three monosaccharides were separated and their solutions were passed through millipore filters (mesh size, 0.45 μm). The volume was adjusted to 10 ml, and the carbohydrate content of each was measured. Aliquots of each fraction, before and after reduction, were converted into the Me_3Si derivatives and analysed by g.l.c. An aliquot of the arabinose (10 mg) was converted into the benzoylhydrazone[29], m.p. and mixture m.p. 184°. An aliquot of the mannose (10 mg) was converted into the phenylhydrazone[30], m.p. and mixture m.p. 199°. The glucose crystallised as the β-D anomer, m.p. 147°, $[\alpha]_D$ +22→+53°. In addition, a small quantity (total, ~6 mg) of 5 oligosaccharides was separated. The d.p. of each was determined, and the constituent sugars were identified by p.c. and g.l.c. after hydrolysis.

Methylation of N-2. — *N-2* (50 mg) was subjected to a modified Hakomori methylation[31]. T.l.c. (solvent *F*) of the methylated product revealed a single spot. The hydrolysate of the methylated polysaccharide (38 mg, freeze-dried weight) was partly converted into the methylated methyl glycosides and partly into the methylated alditol acetates. Both products were analysed by g.l.c., the former on columns *1* and *2*, and the latter on column *5* and by g.l.c.–m.s. (column *6*).

Periodate oxidation of N-2. — *N-2* (50 mg, equivalent to 31.75 mg of carbo-

hydrate) was dissolved in water (50 ml) and added to 0.3M sodium metaperiodate (50 ml). The oxidation, which was followed spectrophotometrically[32], was complete after 24 h and was stopped by the addition of ethylene glycol (1 ml). The mixture was stirred for 1 h and then reduced, and the derived polyalcohol (14 mg, equivalent to 7 mg of uncleaved sugars), isolated by freeze-drying, was hydrolysed and analysed by p.c. (solvents *A–C*), and the glucose content was measured.

Analysis of the acid polysaccharide. — The acid polysaccharide was assayed for the content of carbohydrate, uronic acid, and glucose. I.r. analysis revealed absorption bands at 1420 (carboxylate) and 1735 cm^{-1} (C=O stretching), which were also present as small bands in the spectra of the total mucilage, but absent from the spectrum of the neutral polysaccharide. The polysaccharide (1 g) was hydrolysed, and the molar proportions of the resulting neutral sugars were determined as for *N-2*. The hydrolysate was fractionated on 3MM paper into neutral and acidic material by elution for 48 h with solvent *C*. The acidic fraction was then applied to 3MM paper and eluted with solvent *B* to give pure uronic acid, which was analysed as follows: (*1*) t.l.c. (solvent *B*), the plates were double-developed and stained with spray *5*; (*2*) p.c. (solvent *C*); (*3*) paper electrophoresis in buffers (*c*) and (*e*), and stained with spray *5*; (*4*) reduction to the neutral sugar after esterification. The derived neutral sugar was analysed by electrophoresis in buffer (*b*), by p.c. (solvents *A–C*, and *J*), and spray reagents *4*, *6*, and *10*. An aliquot was converted into the Me_3Si derivative and analysed by g.l.c. (column *4*), both alone and in admixture with Me_3Si-gulose.

The polysaccharide (10 g) was partially hydrolysed with 0.25M sulphuric acid for 4 h at 100°. The neutralised ($BaCO_3$) hydrolysate was concentrated to small volume, and ethanol (5 vol.) was added. The precipitated barium uronates were removed by centrifugation and converted into their ammonium salts by removal of Ba^{2+} with Amberlite IR-200(H^+) resin and neutralisation with aqueous ammonia. The neutral supernatant was deionised with Amberlite IR-120(H^+) and IR-45B(HO^-) resins, and then separated on a column of cellulose (Whatman CF11, 500 g) as for the neutral polysaccharide. Impure components were refractionated by preparative p.c. (solvent *B*). Two monosaccharides were isolated. Glucose was characterised as described above. The mobility of the second monosaccharide (p.c., solvents *A–C*, and *H*; sprays *1*, *4*, *6*, *7*, and *10*) was closely similar to that of D-*glycero*-D-*manno*-heptose (kindly donated by Dr. M. B. Perry), as were the retention times on g.l.c. of the Me_3Si derivatives, both before and after reduction.

Characterisation of the acidic oligosaccharides. — The acidic fraction was separated on paper (solvent *B*) into 5 components. Each fraction was passed through a millipore filter (mesh size, 0.45 μm) and assayed for uronic acid, carbohydrate, and glucose, and its d.p. was measured. Aliquots of each fraction were (*a*) hydrolysed, and (*b*) esterified, glycosidated, reduced, and hydrolysed. Aliquots of oligosaccharides **1**, **2**, and **4** (see Table IV) were also separately reduced and hydrolysed. Each of the above hydrolysates was analysed by p.c. (solvents *B* and *C*; sprays *1*, *4*, *6*, and *7*). Further aliquots of the first 4 fractions (see Table IV) were dried, then moistened with dry methanol, and methylated with diazomethane[33]. The methylating reagents were

removed by distillation, and the resulting, yellow syrup was dissolved in aqueous methanol and reduced. The partially methylated, reduced oligosaccharides were remethylated by a modified[34] Kuhn[35] method. Each methylated oligosaccharide was hydrolysed, and the hydrolysate was reduced and acetylated. The derived methylated alditol acetates were analysed by g.l.c–m.s. An aliquot of oligosaccharide **1** (Table IV) was hydrolysed with 0.5M sulphuric acid for 4 h at 100°. After neutralisation ($BaCO_3$), the hydrolysate was analysed by p.c. (solvents *A–C*; spray *4*).

Periodate oxidation of the acid polysaccharide. — A solution of the polysaccharide (2.0 g, equivalent to 1.34 g of carbohydrate) in water (250 ml) was oxidised with periodate, as for the neutral polysaccharide except that aliquots were withdrawn after 3 and 18 h for the determination of formaldehyde[36]. After reduction, the polyalcohol (1.39 g containing 0.85 g of intact sugars) was isolated by freeze-drying and assayed for glucose. An aliquot was hydrolysed, and the hydrolysate was analysed by p.c. (solvents *A–C*; sprays *1*, *4*, *6*, and *7*).

Hydrolyses with 0.25M and 0.5M sulphuric acid at room temperature for 1–8 h and p.c. of the hydrolysates revealed only glycerol and glyceric acid. 0.25M Sulphuric acid at 50° for 1 h produced the same fragments, together with glucose.

Removal of pyruvic acid from the acid polysaccharide. — A solution of polysaccharide (1.5 g) in water (500 ml) was shaken with 3 batches of Amberlite IR-120(H^+) resin and then filtered, and the resin was washed. The filtrate and washings (pH 2.5) were refluxed for 6 h, cooled, and extracted 3 times with ether in a liquid–liquid extractor. The aqueous layer was freeze-dried, and the residue (0.8 g) was oxidised by periodate as for the acid polysaccharide. The ethereal extract was concentrated to dryness and the residue was dissolved in water. Analysis by p.c. (solvent *A*; sprays *8* and *9*) and by electrophoresis [buffers (*c*) and (*d*), and spray *9*]. An aliquot was taken to dryness, and methanol and benzene were evaporated from the residue, which was then converted into the methyl ester and analysed by g.l.c. (columns *5* and *6*). A mixture of an aliquot of the aqueous solution with ethanolic 2,4-dinitrophenylhydrazine[37] (0.5 ml) was kept at 100° for several min and then at 0° for 18 h to give pyruvic acid 2,4-dinitrophenylhydrazone, m.p. 218°.

Quantitative estimation of pyruvic acid. — The method was essentially that described by Sloneker and Orentas[16], except that the polysaccharide (100 mg) was hydrolysed at room temperature (3 h) in 2M hydrochloric acid (40 ml).

Analysis of the depyruvylated polysaccharide. — The depyruvylated polysaccharide (0.8 g) was assayed for carbohydrate, uronic acid, and glucose. An aliquot (131 mg, equivalent to 102 mg of carbohydrate) in water (50 ml) was oxidized with periodate as for the neutral polysaccharide, and the derived polyaldehyde was reduced to the polyalcohol (36.2 mg, equivalent to 30.8 mg of uncleaved sugars) and assayed for uronic acid and glucose. An aliquot was hydrolysed and the hydrolysate was analysed by p.c. (solvents *A–C*; sprays, *1*, *4*, *6*, and *7*).

Methylation studies. — The acid polysaccharide (20 mg), the depyruvylated polysaccharide (20 mg), and the depyruvylated polyalcohol (20 mg) were methylated separately by the modified Hakomori method[31]. After dialysis and freeze-drying, an

aliquot of each product was hydrolysed and the hydrolysate was analysed by p.c. The remaining polysaccharides were reduced and the products were isolated by freeze-drying. Aliquots were hydrolysed, and the rest of the polysaccharides were methylated as before and hydrolysed. One part of the hydrolysate was converted into the methyl glycosides, and the other was reduced to the methylated alditols and then acetylated.

The polysaccharide (50 mg) was methylated, as described above, and the partly methylated polysaccharide, isolated by freeze-drying, was dissolved in D_2O and reduced with potassium borodeuteride. The product was again isolated by freeze-drying and methylated with diazomethane[33]. The product was hydrolysed, reduced, and acetylated as in the previous experiments.

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