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STRUCTURAL INVESTIGATIONS OF THE EXTRACELLULAR POLYSACCHARIDE ELABORATED BY S19, A *Xanthomonas*-TYPE BACTERIUM*

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ABSTRACT

The extracellular, acidic heteropolysaccharide from *Xanthomonas* S19 consists of D-glucuronic acid, D-glucose, D-galactose, and D-mannose residues in the approximate molar ratios of 1.6:3:1:1, plus acetyl groups linked to C-2 and/or C-3 of a large proportion of the glucose residues. Methylation studies showed that the glucose is present as non-reducing end-group also as 1,2- and 1,4-linked units, the galactose residues are solely 1,3-linked, a major proportion of the mannose residues are 1,2,4-linked and the rest 1,2-linked. A high proportion of the glucuronic acid units are 1,4-linked. Periodate oxidation confirmed the presence of these linkages. The disaccharides D-Glc-(1→4)-D-Glc, D-Glc-(1→2)-D-Man, D-Glc-(1→3)-D-Gal, D-Gal-(1→2)-D-Glc, D-GlcA-(1→4)-D-GlcA, and β-D-GlcA-(1→4)-D-Man were isolated from a partial hydrolysate of the polysaccharide, and characterised. The similarities and differences between this polysaccharide and those from other *Xanthomonas* species are discussed.

INTRODUCTION

The organism was isolated from soil in Brazil and was tentatively identified as a plant pathogenic *Xanthomonas* species at the National Collection of Plant Pathogenic Bacteria, Ministry of Agriculture, Fisheries and Food Plant Pathology Laboratory, Harpenden, England. It has an orange pigment in contrast to most *Xanthomonas* species which are yellow. There are twenty-five or more different species of *Xanthomonas* bacteria, and only a few of their extracellular polysaccharides have been studied in detail. Tables I and II give details of the chemical composition and linkages present in the polysaccharides that have been investigated. In addition, the aldobiouronic acid, β-D-GlcA-(1→2)-D-Man has been isolated from hydrolysates of polysaccharides from *X. campestris*, *X. oryzae*, and *X. hyacinthi*. In contrast, *X. stewartii* extracellular polysaccharide yields β-D-GlcA-(1→4)-D-Gal on hydrolysis.

*Dedicated to the memory of Professor Edward J. Bourne.

β-D-Glc-(1→4)-D-Glc has been obtained from *X. hyacinthi* and *X. campestris* polysaccharides, and β-D-Glc-(1→4)-D-Man from the former. β-D-Glucose linked to C-3 and to C-6 of galactose, and β-D-galactose linked to C-3 of galactose have been reported as features of the extracellular polysaccharide of *X. stewartii*.

TABLE I

CHEMICAL COMPOSITION OF EXTRACELLULAR POLYSACCHARIDES METABOLISED BY *Xanthomonas* SPECIES

Species of Xanthomonas	*Molar proportion*				*Percentage*		
	GlcA	*Glc*	*Gal*	*Man*	*Pyruvic*	*Acetic*	*Ref.*
phaseoli	1	1	a[a]	1	6–7	—	1
campestris	2.0	2.8	a	3.0	3–3.5	4.7	2, 3, 4
stewartii	1	4.5	3	a	—	—	5
oryzae	2	5	a	5.4	0.3	—	6
hyacinthi	+	1	a	0.78	—	—	7
translucens	+	1	a	0.9	+	—	7
maculofoliigardeniae	+	1	a	0.95	—	—	7

[a]Key: a, absent; +, present, but proportion not determined; —, not investigated.

TABLE II

LINKAGES FOUND IN THE EXTRACELLULAR POLYSACCHARIDES OF *Xanthomonas* SPECIES

Xanthomonas *species*	*GlcA*	*Glc*	*Gal*	*Man*
campestris	1,4- branching	1,4- 1,2,4- 1,3,4-	a[a]	1,2- end-group
stewartii	+	1,6- end-group	1,3- 1,6- 1,3,4,6-	a
oryzae	1,4- end-group	1,4- 1,3,4-	a	1,2- 1,2,6-
hyacinthi, translucens, and maculofoliigardeniae	+	1,4- 1,3,4- end-group	a	1,2- end-group

[a]Key: a, absent; +, present, but linkage not determined.

RESULTS AND DISCUSSION

The purified polysaccharide (*A*) had a carbohydrate content (as glucose) of 70, ash 5, protein 9.6 (calculated from N, 1.55%), and uronic acid ~25% of the carbohydrate. Hydrolysis with acid of various concentrations caused insignificant loss of

carbohydrate, but gave different proportions of oligosaccharides, together with glucose, galactose, mannose, and uronic acid. The identity of the neutral sugars was confirmed by ionophoresis[8], and by g.l.c. of the Me_3Si derivatives (before and after reduction) and of the alditol acetates. Use of a D-glucose oxidase spray after p.c. confirmed the presence of D-glucose. Galactose and mannose were isolated by p.c. after oxidation of the D-glucose to D-gluconic acid. The former sugar was characterised as D-galactose with D-galactose oxidase and by its $[\alpha]_D$ value (+74°), while the latter was characterised as the phenylhydrazone.

Glucuronic acid was indicated by its chromatographic and electrophoretic mobilities as well as those of its lactone. Reduction of the isolated acid to the neutral sugar gave only glucose. This product was confirmed as the D sugar by oxidation with D-glucose oxidase, proving the original presence of D-glucuronic acid.

Attempted fractionation of the polysaccharide. The high viscosity of a dilute, aqueous solution made fractionation on cellulose or resin columns impossible. Precipitation with barium hydroxide left 3.4% of the material in solution and this proved to be of low molecular weight and to have the same composition as that which precipitated. Precipitation with Cetrimide[9] removed all of the carbohydrate from solution, again indicating that the polysaccharide is a single, polydisperse, acidic heteropolysaccharide.

Molar proportions of the component monosaccharides. The impossibility of completely hydrolysing the polysaccharide to the constituent monosaccharides without excessive degradation made accurate determination of their proportions difficult. Initial hydrolysis with 90% formic acid for 8 h, followed by reduction of the glucuronic acid to glucose and hydrolysis with 90% formic acid for another 4 h, gave only monosaccharides in the molar proportions of glucose:galactose:mannose of 4.5:1.0:0.9. The proportion of mannose to galactose doubled in the hydrolysate of the reduced solution compared with that of the unreduced hydrolysate, indicating that it was probably held in glucuronosyl linkage in the polysaccharide and retained in oligouronic acids on hydrolysis. The glucose was derived from both the glucose and glucuronic acid residues present in the polysaccharides. Thus, since the latter comprises ~25% of the carbohydrate, the approximate molar proportions of GlcA:Glc:Gal:Man are 1.6:3:1:1 in the polysaccharide.

The presence of acetyl groups in the polysaccharide. Infrared spectra of the present polysaccharide gave a band at 1725 cm^{-1} indicative of acetate or other acyl groups[10]. This inference was supported by the n.m.r. spectrum (D_2O) of the polysaccharide which contained a peak[3] at τ 7.73. Such esters are labile to mild, acid hydrolysis, and the n.m.r. spectrum of the polysaccharide recovered after autohydrolysis of the free-acid form was devoid of this peak. Furthermore, electrophoresis and i.r. spectroscopy of the ethereal extract of the autohydrolysate indicated the presence of a carboxylic acid. Treatment of the extract with 2,4-dinitrophenylhydrazine[11] gave a single product with R_F 0.70 (t.l.c., solvent *5*), compared with R_F 0.50 for the 2,4-dinitrophenylhydrazone of pyruvic acid, thereby indicating the absence of this acid. G.l.c. of the methyl ester of the product in the ethereal extract

gave a peak with the same retention time as that of methyl acetate. N.m.r. spectroscopy of the derived sodium salt gave a peak (τ 8.05) with the same chemical shift as that of sodium acetate. T.l.c. of the hydroxamic acid derivative[12] in each of two different solvents gave a single spot of mobility identical to that of the hydroxamic acid obtained from sodium acetate. The identity of the acetic acid was confirmed by the preparation of the *p*-nitrobenzyl derivative[13] (m.p. and mixture m.p. 77–78°).

The absence of pyruvic acid was confirmed by the failure of lactic acid dehydrogenase[14] to react with the product in the ethereal extract, the dialysate of the autohydrolysate, and the sodium salt derivative. The acetate content[9] of the polysaccharide was 18.7%. This value was confirmed by comparing the weight of the polysaccharide before (1.274 g) and after (1.037 g) deacetylation and, based on a carbohydrate content of 70%, corresponds to one acetyl group per 2 anhydro sugar residues.

Location of the O-*acetyl residues.* The sites of *O*-acetyl residues have been located by reacting the free hydroxyl groups with methyl vinyl ether[15]. Simultaneous deacetylation and methylation of the product results in the acetyl groups' being replaced by methyl groups. The insolubility of polysaccharide *A* made complete etherification impossible. However, one fraction (*C*) of the acetalated polymer was soluble in the reaction mixture and had most of its free hydroxyl groups substituted, as, after methylation and hydrolysis, the hydrolysate contained 2-*O*-methyl-, 3-*O*-methyl-, and 2,3-di-*O*-methyl-glucose. These methylated sugars were absent from the hydrolysate of the methylated polysaccharide *A*. This indicates that the *O*-acetyl residues are present on C-2 and/or C-3 of the D-glucose residues.

Methylation studies. The carbohydrate contents (as glucose[9]) of the initial polysaccharide (17.1 mg), of the derived methylated polymer (12.5 mg), and of the hydrolysate (12.0 mg) of the latter were determined in order to ensure that the hydrolysate is representative of the whole macromolecular structure and is not from a fraction of the polysaccharide.

The methylated polysaccharide was hydrolysed and the products were analysed by g.l.c. as the methyl glycosides (Table III) and the derived methylated alditol acetates (Table IV). It can be seen from both these Tables that the 3,4,6-tri-*O*-methylhexose could have the *gluco* and/or *manno* configuration. In addition, the hydrolysates contained a small proportion of oligouronic acids. From these results, it can be deduced that, in polysaccharide *A*, the galactose is solely 1,3-linked, and the glucose is present as end-group, as 1,4-linked, and possibly as 1,2-linked units. The mannose is present at branch points linked at C-2 and C-4 and possibly also present as 1,2-linked units.

No methylated glucuronic acid derivatives could be detected in the methylated hydrolysates. As the polysaccharide had been subjected to two treatments with the methylsulphinyl carbanion, this result is to be expected[16]. Such units, particularly if 1,4-linked, suffer β-elimination during the second treatment and the products are degraded on acid hydrolysis. In order to determine the linkage of the uronic acid units in the polysaccharide, the partially hydrolysed polysaccharide (*B*, see later) was

reduced[17] before methylation. Comparison of the hydrolysates of methylated *B* and reduced and methylated *B* showed that the only difference was the presence of twice as much 2,3,6-tri-*O*-methylglucose in the latter, indicating that a high proportion of the glucuronic acid residues are 1,4-linked.

TABLE III

METHYLATED METHYL GLYCOSIDES FROM THE METHYLATED POLYSACCHARIDE

Position of O-*methyl*	*Methyl glycosides of*	T[a]	
		Column 1	*Column 2*
2,3,4,6-	Glucose	1.00; 1.38	1.00; 1.35
3,4,6-	Glucose	2.90; 4.30	1.72
	Mannose	2.90	1.72
2,4,6-	Galactose	3.80; 4.30	2.20; 2.42
2,3,6-	Glucose	3.20; 4.30	1.72; 2.2
3,6-	Mannose	8.00; 9.40	3.2; 3.8

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

TABLE IV

ANALYSIS OF THE METHYLATED ALDITOL ACETATES FROM THE METHYLATED POLYSACCHARIDE

Position of O-*methyl*	*Alditol acetate derivative of*	T[a] (*Column 5*)	*Ratio of relative peak areas*
2,3,4,6-	Glucose	1.0	1.0
3,4,6-	Glucose/mannose	1.7	1.1
2,4,6-	Galactose	1.9	1.0
2,3,6-	Glucose	2.15	1.7
3,6-	Mannose	3.2	1.0

[a]Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol.

Periodate-oxidation studies. Periodate oxidation of polysaccharide *A* for 50 h followed by borohydride reduction[9] resulted in the recovery of 72% by weight of polyalcohol having a carbohydrate content of 83%. Similar treatment of the deacetylated polysaccharide gave a 60% recovery of polyalcohol, but the carbohydrate content had decreased to 55%. Although both polyalcohols contained uncleaved glucose residues, the proportion from the deacetylated polysaccharide was considerably smaller. Furthermore, the proportion of erythritol in the hydrolysate from the latter polyalcohol was considerably larger, showing an increased oxidation of the 1,4-linked glucose residues in the deacetylated polysaccharide, and confirming that it was these units which carried the acetyl groups in the initial material. The hydrolysates of both polyalcohols contained glucose, galactose, mannose, and glucuronic acid, together with glycerol, erythritol, and erythronic acid. The presence of the last three compounds demonstrates that some end-group hexose, 1,4-linked glucose, and

1,4-linked glucuronic acid residues, respectively, had been oxidised. The mannose that occurs at branch points and the 1,3-linked galactose would both be immune to periodate oxidation. The presence of uncleaved 1,4- and 1,2-linked units in the polyalcohols is probably due to the formation of hemiacetal linkages between adjacent cleaved and uncleaved residues[18] which hinder further oxidation. Reduction destroys these linkages. Thus, a second periodate-oxidation should result in further oxidation of the polyalcohol. Three sequential oxidations and reductions showed this to be so; the final polyalcohol was devoid of glucose and glucuronic acid residues.

Partial hydrolysis studies. After preliminary studies, it was found that hydrolysis of polysaccharide *A* with 0.25M oxalic acid for 5 h gave the highest yield of oligosaccharides. The residual polysaccharide (*B*, ~21% of the initial carbohydrate) comprised 100% of carbohydrate, of which 23% was uronic acid. A hydrolysate of *B* contained glucuronic acid, glucose, galactose, and mannose. The alcohol-soluble carbohydrate (73% of the initial carbohydrate) in the hydrolysate contained monosaccharides, oligosaccharides, and oligouronic acids. An aliquot was converted into the free-acid form and fractionated into neutral sugars (460 mg) and acidic material (710 mg). The components in each fraction were isolated by preparative p.c., and some of their properties are given in Table V.

TABLE V

OLIGOSACCHARIDES FROM A PARTIAL HYDROLYSATE OF THE POLYSACCHARIDE

Neutral fraction	*Carbohydrate (mg)*	R_{Glc}[a]	*D.p.*	*Components*
Monosaccharides	325			
F1	26	0.56	2	Glc>Gal = Man
F2	3	0.44	2	Glc:Gal = 1:1
F3	10	0.36	2	
F4	4	0.25	3	Glc>Gal>Man
F5	7	0.14	5	Glc,Gal,Man
F6	8	0.08	8	Glc,Gal,Man
	383			
Acidic oligosaccharides		R_{GlcA}[b]		
G1	4	1.0	1.9	GlcA
G2	25	0.4	2.2	GlcA:Man = 1:1
G3	500	0.05	13	GlcA,Glc,Gal,Man
	529			

[a]1-Butanol–ethanol–water (40:11:19). [b]Ethyl acetate–acetic acid–formic acid–water (18:3:1:4).

Fraction F1. — This gave a single spot on p.c. which stained red with the tetrazolium spray and greenish blue with the aniline–diphenylamine spray, indicating

linkages other than (1→2) and the presence of (1→4)-linkages, respectively[19]. The identities of the three sugars in the hydrolysate were confirmed as glucose and galactose by reaction with D-glucose and D-galactose oxidases, respectively, and mannose by paper electrophoresis in molybdate buffer[8]. Methylation of *F1*, followed by hydrolysis, gave 2,3,4,6-tetra-*O*-methylglucose (major), 3,4,6-tri-*O*-methylhexose, 2,4,6-tri-*O*-methylgalactose, and 2,3,6-tri-*O*-methylglucose. The derived disaccharide alditols (obtained by reduction with borodeuteride) had $M_{GLUCITOL}$ values of 0.0, 0.50, and 0.66 on electrophoresis in molybdate buffer, indicating the presence of (1→3)-, (1→4)-, and (1→2)-linked units[8]. Bearing in mind the linkages found from the methylation analysis of polysaccharide *A*, these products are therefore 3-*O*-glucosylgalactitol, 4-*O*-glucosylglucitol, and 2-*O*-glucosylmannitol/glucitol, respectively. Further evidence for the presence of the three disaccharides in fraction *F1* was obtained by methylating the disaccharide alditols and analysing the mixture by g.l.c.–m.s. The three g.l.c. peaks had mass-spectral peaks characteristic for (1→3)- (*m/e* 101, 133, 172), (1→4)- (*m/e* 102, 134, 172), and (1→2)- (*m/e* 101, 133, 172, 177) linked disaccharide alditols, respectively. Fraction *F1* is therefore a mixture of D-Glc-(1→3)-D-Gal, D-Glc-(1→4)-D-Glc, and D-Glc-(1→2)-D-Glc/Man.

Fraction F2. — This is a disaccharide containing equal proportions of glucose and galactose. The derived disaccharide aditol, on electrophoresis in molybdate buffer, gave a single spot ($M_{GLUCITOL}$ 0.66) indicating a (1→2)-linked disaccharide alditol. Since the galactose residues in the polysaccharide are solely 1,3-linked, *F2* must be D-galactosyl-(1→2)-D-glucose. This provides evidence of 1,2-linkages for glucose.

Fraction F3 was a mixture and was not examined further.

Fraction F4. — This was a mixture of trisaccharides which, on partial hydrolysis, gave (p.c.) glucose, galactose, and mannose, together with spots having the mobilities of *F1*, *F2*, and unhydrolysed material. After borohydride reduction to the alditols and hydrolysis, the proportions of reducing sugars were Glc > Gal > Man. Chromatography on tungstate-impregnated paper[21] (5% aqueous sodium tungstate dihydrate adjusted to pH 8 with 2.5M H_2SO_4) showed, in addition to the reducing sugars, spots for glucitol (major) and mannitol (minor). The derived alditols gave two spots on electrophoresis in molybdate. These components were isolated, and the major alditol, after partial acid hydrolysis, gave equal amounts of glucose and galactose, together with glucitol and a disaccharide having the chromatographic mobility of *F1*. Thus, the major spot is derived from a trisaccharide consisting of glucose (2 mol) and galactose (1 mol) with glucose as the reducing unit. To account for the proportion of glucose and the presence of mannose after reduction, the minor trisaccharide is composed of 2 mol of mannose and 1 of glucose, one of the former units being the reducing end-group.

Fractions F5 *and* F6. — Partial hydrolysis of these two fractions gave products with the mobilities (p.c.) of *F1* → *F5*, together with the three monosaccharides in proportions Glc > Gal > Man.

Acidic fraction G1. — On electrophoresis at pH 6.7, this fraction gave two

spots, M_{GlcA} 0.97 and 1.0. Hydrolysis with formic acid failed to yield any neutral sugars. After reduction, followed by esterification and further reduction, the product contained glucitol[21] and no reducing carbohydrate. The same product, after hydrolysis, gave glucose and glucitol. Thus *G1* is a mixture of glucuronic acid and diglucuronic acid.

Acidic fraction G2. — This had $[\alpha]_D$ −17.5° {*cf.* β-D-GlcA-(1→2)-D-Man[6], $[\alpha]_D$ −28°} and M_{GlcA} 0.57 (pH 6.7). Hydrolysis of *G2* gave glucuronic acid and mannose, and hydrolysis of the derived neutral disaccharide gave glucose and mannose in equimolar proportions. The permethylated, neutral disaccharide alditol derived from *G2* gave a single peak on g.l.c. (*T* 0.95, relative to permethylated maltitol), the mass spectrum of which contained peaks characteristic of a (1→4)-linked disaccharide alditol[20]. G.l.c. of the hydrolysed, methylated, reduced material as the methylated alditol acetates gave two peaks corresponding to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylmannitol which were clearly distinguishable from 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol. Thus, Fraction *G2* is β-D-GlcA-(1→4)-D-Man.

Acidic Fraction G3. — This had M_{GlcA} 0.3 (pH 6.7). After further hydrolysis, it gave spots with the mobilities of *F1* (major), *F2* → *F6*, *G1*, and *G2* (major), as well as unhydrolysed material.

CONCLUSION

The partial hydrolysis studies confirm the methylation and periodate-oxidation results. In addition, evidence that some of the glucose units are 1,2-linked was also obtained. The relative proportion of 3,4,6-tri-*O*-methylhexose in the hydrolysis of the methylated polysaccharide *A* indicates that part of this methylated derivative also results from 1,2-linked mannose residues.

In the macromolecule *A*, the glucose, present as end-group, 1,2- and 1,4-linked units, is linked to glucose, to galactose, and to mannose; the galactose is solely 1,3-linked, and the mannose which occurs at branch points is linked through C-2 to glucose and through C-4 to glucuronic acid. Glucuronic acid is also linked through C-4 to other glucuronic acid residues.

Thus, S19 *Xanthomonas* extracellular polysaccharide has many structural features that are similar to those of other *Xanthomonas* polysaccharides (Table II). It differs in that it contains both mannose and galactose residues. Mannose is the only sugar that occurs at branch points in this polysaccharide, whereas in the polysaccharides from other species, glucose occurs most commonly at branch points. In *X. oryzae*, mannose as well as glucose occurs at branch points, but here the mannose is 1,2,6-linked in contrast to that of the present polysaccharide where it is 1,2,4-linked. Mannose also occurs as end-group in a number of the other polysaccharides, but only glucose end-group was detected in the present polysaccharide.

EXPERIMENTAL

General. — The general methods[22], and methods for methylation of polysaccharides and of oligosaccharides and their analyses have been described previously[9] In addition, the following solvent system for paper chromatography (p.c.) was used: *K*, water-saturated butanone+3% cetylpyridinium chloride. Detection was also effected with *12*, Tetrazolium spray[23]; and *13*, aniline–diphenylamine spray[19]; both for glycosidic linkages. Ionophoresis was carried out as before[9]. T.l.c. was performed on Kodak precoated-cellulose or polygram Sil G plates with *1*, ethyl acetate; *2*, water-saturated phenol; *3*, water-saturated 1-butanol; *4*, 1-pentanol–formic acid–water (4:1:5) (organic layer), and *5*, ethyl acetate–light petroleum (b.p. 60–80°)–acetic acid[24] (50:50:7). In addition to the columns previously described for g.l.c.[9], glass columns (3 m × 5 mm) packed with 20% of Carbowax 1500 on Chromosorb P at 50° (column *7*) and 3% of OV 17 on Chromosorb W at 220° (column *8*) were used. Uronic acid was determined by a modified carbazole reaction[9] and by the 3-hydroxybiphenyl method[25]. Acetate was determined as before[9].

Culture of S19 Xanthomonas *bacterium.* — The culture solution contained sucrose, acid-hydrolysed casein, dipotassium hydrogen phosphate, and magnesium sulphate. The crude polysaccharide was obtained as a fawn powder by precipitation from the culture solution with acetone, followed by filtration, air-drying, and milling. This was supplied by Alginate Industries Ltd., Girvan, Scotland.

Purification of the polysaccharide. — The polysaccharide (22 g) was purified by agitation in water (4 l) for 24 h. The derived thick gel was treated with ethanol (5–6 vol.), and a solution of the recovered precipitate in water was subjected to prolonged dialysis, and then freeze-dried to a white, hydrophilic, fluffy solid (*A*, 16 g).

Analysis of the purified polysaccharide A. — The nitrogen, ash, and uronic acid contents of aliquots of the polysaccharide were determined, and a further aliquot (10 mg) was dissolved in water (350 ml) by shaking for 24 h. The carbohydrate content of the solution was determined from a standard glucose graph. Separate, weighed aliquots of the polysaccharide were hydrolysed with (*i*) 0.5M sulphuric acid (in triplicate), (*ii*) 0.25M sulphuric acid, and (*iii*) 90% formic acid. Each hydrolysate was assayed for the content of carbohydrate and examined by (*a*) p.c. (solvents *A*, *B*, *C*, and *G*; spray reagents *1*, *4*, *6*, and *10*), (*b*) ionophoresis in borate buffer and in pyridine–acetic acid buffer, and (*c*) g.l.c. as the Me_3Si-sugar and Me_3Si-alditol derivatives (column *4*), and alditol acetate (column *6*) derivatives.

Characterisation of the constituent sugars. — The polysaccharide (1.13 g; 789 mg of carbohydrate) was hydrolysed with 0.25M sulphuric acid (100 ml) at 100° for 9 h. After neutralisation ($BaCO_3$), the filtrate was concentrated to 15 ml (680 mg of carbohydrate), and oligouronic acids were precipitated with ethanol (5–6 vol.) and removed. The filtrate was concentrated to 5 ml, and additional oligouronic acids were precipitated as before (total barium uronates, 968 mg). The final filtrate was concentrated to dryness. The residue (112 mg of carbohydrate) was freed from inorganic ions with Amberlite IR-120(H^+) and IR-45(HO^-) resins.

The D-glucose in an aliquot (30 mg of carbohydrate) was converted into D-gluconic acid with D-glucose oxidase (Glucostat X1), and the residual galactose and mannose were separated on 3MM paper (solvent *B*) and determined[9].

An aliquot of a formic acid hydrolysate of the polysaccharide in the free-acid form was fractionated into neutral and acidic material on Deacidite FFIP (SRA 67) ($HCOO^-$) resin[9]. The acid fraction was further fractionated by p.c., and the mono-uronic acid and its lactone were isolated. Both were examined on p.c. (solvents *G* and *K*) and by ionophoresis in borate buffer containing Ca ions[26]. Each product was then esterified, reduced, and hydrolysed, and the derived neutral sugar was examined by p.c. (solvents *A*, *B*, *C*; sprays *1*, *4*, and *6*), and by g.l.c. of the Me_3Si-sugar, Me_3Si-alditol, and alditol acetate derivatives.

Autohydrolysis of the free-acid polysaccharide. — The polysaccharide (1.274 g) in water (300 ml) gave a homogeneous gel after shaking for 3 days. This was shaken with Amberlite IR-120(H^+) resin (300 ml) for 12 h. After centrifugation, the solution of free-acid polysaccharide and the washings were heated in an atmosphere of nitrogen at 100° for 10 h. The solution was then extracted with ether (100 ml) for 5 h. The ethereal extraction was repeated twice with fresh ether, and the combined ethereal extracts were concentrated to 3 ml. The residual aqueous solution was dialysed and the deacetylated polysaccharide (1.037 g) was recovered by freeze-drying.

The ethereal extract obtained above was examined by ionophoresis (buffer *c*, pH 6.8; spray *8*).

Detection and characterisation of acetyl groups. — The dried polysaccharide *A* (50 mg) in M methanolic hydrogen chloride (1 ml) in a sealed tube was boiled for 30 min and the resulting mixture analysed[27] by g.l.c. on column *7*. Sodium acetate and sodium pyruvate were treated similarly.

To a second autohydrolysate of the free-acid polysaccharide (300 mg), ether (25 ml) was added with stirring, and the residual polysaccharide was precipitated with ethanol (2 vol.). The filtered solution was neutralised to pH 7.0 with 0.1M sodium hydroxide. An aliquot was converted into the hydroxamate derivative[12] which was examined by t.l.c. (solvents *2* and *4*; detection with 1% ferric chloride and 0.1% conc. HCl in 95% aqueous ethanol).

The polysaccharide (2 g) was deacetylated with M sodium hydroxide in the presence of KBH_4, and the mixture extensively dialysed. The combined dialysates were concentrated, brought to pH 4.5 with 2.5M sulphuric acid, and steam distilled. The distillate was neutralised with 0.1M sodium hydroxide and concentrated to small volume. The *p*-nitrobenzyl derivative[13] of the derived sodium acetate was prepared.

Location of O-acetyl residues. — The dried polysaccharide *A* was homogenised in dry methyl sulphoxide by shaking for 48 h, and then treated with methyl vinyl ether as described by De Belder and Norrman[15]. After 3 h at −15°, the reaction mixture was kept at 4° for a further 14 days and then centrifuged. The supernatant solution and acetone washings were concentrated (10 ml), kept at 4° for 2 days, and then centrifuged. The supernatant solution was poured into water, dialysed, and concentrated to dryness, and the derived solid (*C*) as well as each of the centrifugates

were methylated separately. Each of the methylated products was examined in the usual way, and the methylated sugars were characterised.

Partial hydrolysis. — The polysaccharide (4.8 g of carbohydrate) was hydrolysed with 0.25M oxalic acid for 5 h at 100°. The mixture was poured into ethanol (6 vol.), and the precipitate was removed by centrifugation. The supernatant solution was neutralised ($CaCO_3$) and the filtrate analysed for carbohydrate content by p.c. (solvent *A*; sprays *1*, *4*, and *6*) and by electrophoresis. The precipitate (*B*, 1 g), after dialysis, was analysed for carbohydrate and uronic acid contents, and by p.c. and electrophoresis after hydrolysis. An aliquot was methylated.

Carboxyl-reduction of the polysaccharide B. — An aliquot of the partially hydrolysed polysaccharide (*B*, 233 mg) was complexed with ethyl(3-dimethylaminopropyl)carbodiimide hydrochloride, and the complex was reduced with borohydride[17]. This process was repeated. Separate aliquots of the recovered neutral polymer were (*a*) hydrolysed, and the hydrolysate analysed by p.c. and by g.l.c. (column *6*) of the hexa-acetates; and (*b*) methylated.

Characterisation of the oligosaccharides in the partial hydrolysate. — An aliquot of the neutralised supernatant (2.2 g of carbohydrate) was converted into the free-acid form by shaking with Amberlite IR-120(H^+) resin, and the recovered solution (1.17 g of carbohydrate) was fractionated into a neutral fraction and an acidic fraction by elution from a column of Deacidite FFIP resin[9]. Both fractions (the acidic fraction as the ammonium salts) were subjected to preparative paper chromatography (the neutral with solvent *A*, and the acidic with solvent *B*), and the isolated oligosaccharides are detailed in Table V.

Each of the neutral oligosaccharides *F1*, *F2*, and *F4* was analysed for purity by p.c., and their rotation and d.p. were measured. They were reduced to the respective alditols and the latter subjected to electrophoresis in molybdate buffer[8]. They were each hydrolysed and the hydrolysates analysed by p.c. Fraction *F1* was also methylated (modified Kuhn method[9]) before and after reduction. The unreduced, methylated material was hydrolysed and the methylated sugars were characterised by g.l.c.–m.s. The methylated, reduced material was analysed directly by g.l.c.–m.s.[20] (column *8*).

The three acidic oligosaccharides were examined for d.p. and mobility on electrophoresis, and subjected to further hydrolysis. Fraction *G1* was also reduced with borohydride, esterified, and again reduced. The product was examined both before and after hydrolysis by p.c. and by ionophoresis (pH 6.7). Fraction *G2* was esterified with methanolic hydrogen chloride, and reduced, and an aliquot was hydrolysed. A second aliquot was methylated (modified Kuhn[9]), and a hydrolysate examined as the methylated alditol acetates by g.l.c. A further portion of fraction *G2* was reduced with potassium borodeuteride, and the product was methylated, reduced with borohydride, and remethylated. The derived permethylated alditol was examined by g.l.c.–m.s.[20] (column *8*).

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