CONSTITUTIONS OF POLYSACCHARIDES FROM SERRATIA MARCESCENS[1]

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ABSTRACT

Constitutional studies of three polysaccharides prepared from *Serratia marcescens* cells by a sequence of phenol extraction, ultracentrifugation, and fractionation by Cetavlon are described. Methylation of the polysaccharides followed by acid hydrolysis yielded 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-mannose, 4,6-di-*O*-methyl-D-glucose, 2,6-di-*O*-methyl-D-glucose, and an unidentified di-*O*-methyl-glucose. The acidic components found were: 2,3,4-tri-*O*-methyl-D-mannuronic acid, 3-*O*-(2,3,4-tri-*O*-methyl-D-mannuronosyl)-2,4,6-tri-*O*-methyl-D-glucose, and *O*-2,3,4-tri-*O*-methyl-D-mannuronosyl-(1 → 3)-*O*-2,4,6-tri-*O*-methyl-D-glucosyl-(1 → 3)-2,4,6-tri-*O*-methyl-D-glucose. The polysaccharides are composed of a main chain of D-glucose and D-mannose residues joined by 1,3-glycosidic bonds. Some glucose residues carry branches at C_2 and C_4 which terminate in either D-glucose or D-mannuronic acid residues. The three polysaccharides studied had similar chemical structures but varied in the amounts of component sugars and degree of branching.

Because of the tumor-necrotizing properties of the polysaccharides of *Serratia marcescens* (*Bacillus prodigiosus*), much work has been carried out during the last two decades on isolation from their culture filtrates as well as from the cells, on analysis, and on biological properties (1–8). However, only one attempt has been made to investigate the chemical structure of the carbohydrate polymer. Thus, Rathgeb and Sylven (9) carried out structural studies on a glucan isolated by fractionation of Shear's polysaccharide (1) with trichloroacetic acid and ethanol followed by treatment with hot picric acid (7). On the basis of methylation and periodate oxidation, data suggested that the polysaccharide was composed of glucose residues combined mutually by alternating 1,4- and 1,6-glycosidic bonds. However, a weakness in this work is that identification of the methylated sugar fragments was based solely upon paper chromatographic analysis. It is now known that methyl ethers of different sugars having the same degree of methylation can have the same R_f value in a particular solvent. It is rather speculative to assign any structural significance to the periodate-oxidation data unless some reasonable idea about the inter-sugar linkages in the polysaccharide is available. The present communication describes constitutional studies on polysaccharides (or lipopolysaccharides) isolated from the cells of *S. marcescens* by phenol–water extraction followed by fractionation of the polysaccharide mixture by (i) high-speed centrifugation and (ii) by complex formation with cetyltrimethylammonium bromide (Cetavlon) (10). Figure 1 shows an abbreviated scheme of the preparative procedures for polysaccharides used in the present study (for details see ref. 10).

Graded hydrolysis of a main polysaccharide fraction (6NR) with *N* sulphuric acid produced three uronic acid components (A, B, and C), which were separated from neutral sugars by ion-exchange resins and from each other by chromatography on thick filter paper sheets. Component A was identified as D-mannuronolactone. Component B had an equivalent weight of 356 and proved to be an aldobiouronic acid. It turned dark rapidly, thus precluding optical rotation measurements. The aldobiouronic acid was converted to its methyl ester methylglycoside and the latter was reduced with lithium aluminum

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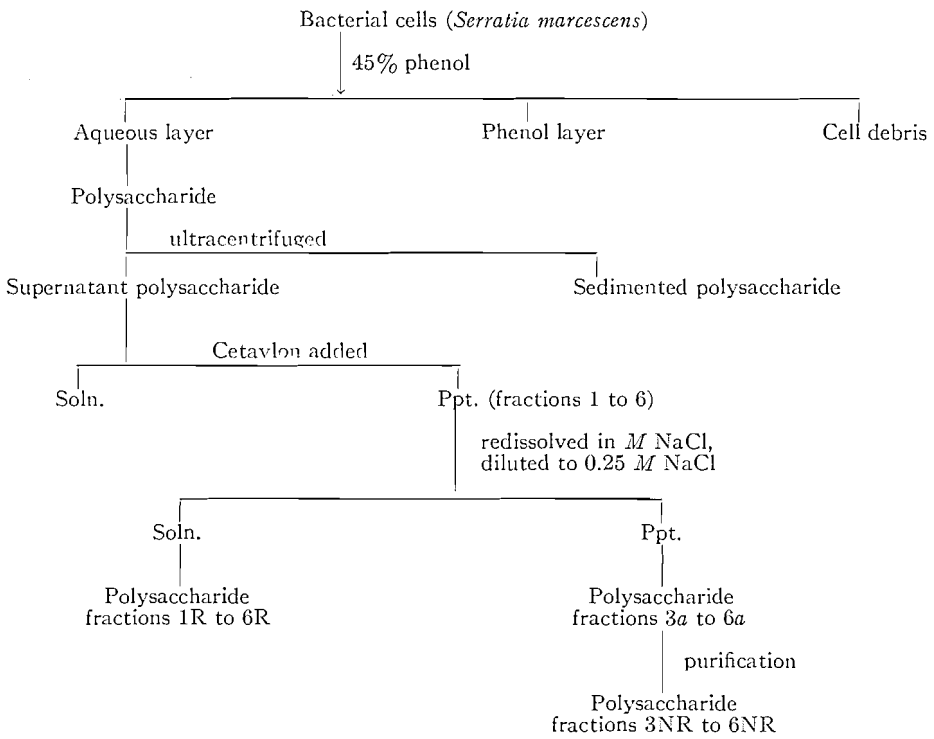


FIG. 1. Preparation of polysaccharides. Fractions 3R, 4R, and 5R were combined for methylation study; fraction 6NR was studied by methylation methods.

hydride to produce the methyl glycoside of a neutral disaccharide. Hydrolysis of the glycoside with acid produced D-glucose and D-mannose in equal proportions. The sugars were characterized as their *p*-nitroanilides. Similar experiments with component C revealed that it was an aldotriouronic acid in which a mannuronic acid residue was combined with two glucose units.

Fractions 6NR and combined fractions 3R, 4R, and 5R (see Fig. 1) were methylated separately with dimethyl sulphate and alkali and the methylation was completed by methyl iodide and silver oxide. The methylated polysaccharides were fractionated and the fractions having the highest methoxyl values were examined.

Methanolysis of 6NR methylated polysaccharide produced a mixture of methylated glycosides, a portion of which was separated by gas–liquid chromatography (11, 12) into three components. One of them crystallized and was shown to be methyl 2,4,6-tri-*O*-methyl-β-D-glucoside, m.p. 68–69° (13).

The methyl glycosides were hydrolyzed with acid to give a mixture of methylated sugars and methylated uronic acids. The uronic acids were converted to their barium salts and separated from the methyl glycosides by selective solvent extraction. The mixture of neutral methylated sugars was resolved by cellulose-column chromatography and the components were weighed and then identified as follows: (i) 2,3,4,6-tetra-*O*-methyl-D-glucose, characterized as *N*-phenyl 2,3,4,6-tetra-*O*-methyl-D-glucosylamine, m.p. 134–135°, $[\alpha]_D$ +240° in chloroform (14); (ii) 2,4,6-tri-*O*-methyl-D-glucose, m.p. and mixed m.p. 123–125° (15), identified as anilide, m.p. 162–165° (16); (iii) 2,4,6-tri-*O*-methyl-D-mannose, m.p. 63–65°, $[\alpha]_D$ +17.6° in water (17); the identity of the compound

was confirmed by ionophoresis and by its characteristic crystalline aniline derivative, m.p. and mixed m.p. 132°, $[\alpha]_D$ +7.8° in methanol (18), (iv) 4,6-di-*O*-methyl-D-glucose, m.p. and mixed m.p. 157–158°, $[\alpha]_D$ +62° in water (15); (v) 2,6-di-*O*-methyl-D-glucose, $[\alpha]_D$ +58° (19); and (vi) 2,3- or 2,4-di-*O*-methyl-D-glucose; the lack of sufficient material prevented complete identification of this component. The identification of 2,6-di-*O*-methyl-D-glucose, which was obtained as a sirup, is based upon the following evidence. Demethylation gave glucose and its mobility on paper chromatogram and paper electrophoretogram was the same as that of an authentic specimen of 2,6-di-*O*-methyl-D-glucose. It gave a negative color reaction with alkaline triphenyltetrazolium chloride (20, 21), indicating that C_2-hydroxyl of the sugar was substituted. The sugar was therefore 2,3-, 2,4-, or 2,6-di-*O*-methyl-D-glucose. Periodate oxidation of the sugar on paper followed by benzidine spray (22) showed that oxidation had taken place. Since, of the three possibilities, only 2,6-di-*O*-methyl-glucose is oxidizable, its identity is established.

Three methyl ether derivatives of acidic components were isolated from the hydrolyzate of the methylated polysaccharide. They were shown to be 2,3,4-tri-*O*-methyl-D-mannuronic acid (I), 3-*O*-(2,3,4-tri-*O*-methyl-D-mannuronosyl)-2,4,6-tri-*O*-methyl-D-glucose (II), and *O*-2,3,4-tri-*O*-methyl-D-mannuronosyl-(1 → 3)-*O*-2,4,6-tri-*O*-methyl-D-glucosyl-(1 → 3)-2,4,6-tri-*O*-methyl-D-glucose (III) on the basis of the following experimental evidence. The components I, II, and III were each transformed into their methyl ester methyl glycoside derivatives, which were reduced with lithium aluminum hydride and hydrolyzed. Component I gave only 2,3,4-tri-*O*-methyl-D-mannose, $[\alpha]_D$ +1° in water (23), which was identified by paper and gas chromatography and by preparation of the crystalline derivative 2,3,4-tri-*O*-methyl-D-mannonophenylhydrazide, m.p. 166° (24), via 2,3,4-tri-*O*-methyl-D-mannono-δ-lactone. Component II afforded a mixture of 2,3,4-tri-*O*-methyl-D-mannose and 2,4,6-tri-*O*-methyl-D-glucose (in approximately equal proportions), the methylated sugars being identified by the methods described earlier. Component III also yielded 2,3,4-tri-*O*-methyl-D-mannose and 2,4,6-tri-*O*-methyl-D-glucose but the proportion of the two sugars in this case was approximately 1:2.

Two methylated polysaccharides (S_0,S_I) which were isolated by fractionation of the methylated combined polysaccharides 3R, 4R, and 5R (Fig. 1) were also analyzed in the manner described for methylated 6NR polysaccharide. Both methylated fractions gave upon hydrolysis 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-mannose, and di-*O*-methyl sugars, besides the methylated aldobiouronic acid 3-*O*-(2,3,4-tri-*O*-methyl-D-mannuronosyl)-2,4,6-tri-*O*-methyl-D-glucose. There was also evidence for the presence of small amounts of methylated glucuronic acid, indicating thereby that some D-glucuronic acid residues were present in the polysaccharide in addition to the main acid component, D-mannuronic acid. The ratios of the methylated sugars produced upon hydrolysis of the methyl ethers of different polysaccharide fractions are given in Table I.

TABLE I

Mole ratios of methylated sugars

Methylated sugar	Fraction 6NR	Fraction S_0	Fraction S_I
2,3,4,6-Tetra-*O*-methyl-D-glucose	4.5	1.0	1.0
2,4,6-Tri-*O*-methyl-D-glucose	21.0	20.0	10.0
2,4,6-Tri-*O*-methyl-D-mannose	11.0	9.0	3.0
4,6-Di-*O*-methyl-D-glucose	2.5	1.0	1.0
2,6-Di-*O*-methyl-D-glucose	2.0		
Di-*O*-methyl-D-glucose (?)	1.0	—	—

6NR and 4R polysaccharides were oxidized with sodium metaperiodate, when 0.52 and 0.50 mole of periodate were consumed and 0.122 and 0.125 mole of formic acid were produced respectively per anhydrohexose unit.

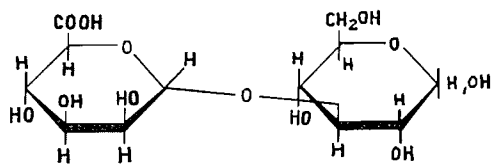
DISCUSSION

Polysaccharide (6NR)

Isolation of D-mannuronolactone from the hydrolyzate of the polysaccharide and 2,3,4-tri-*O*-methyl-D-mannuronic acid from the products of hydrolysis of the methylated polysaccharide established beyond doubt that D-mannuronic acid was a component part of this polymer. As far as the authors are aware, this is the second polysaccharide in which D-mannuronic acid occurred as a building unit of the polymer, the other well-known example being that of alginic acid. D-Mannuronic acid has been suggested by Barker *et al.* to be a constituent of the capsular polysaccharide of *Aerobacter aerogenes*; however, definitive identification of the acid has not been made (25).

Although the methylation data were not sufficient for the formulation of a unique structure for the polysaccharide, the following points of constitutional significance emerged from these studies. The isolation of 2,4,6-tri-*O*-methyl-D-glucose and 2,4,6-tri-*O*-methyl-D-mannose showed that the polysaccharide had a backbone of D-glucose and D-mannose residues linked by 1,3-glycosidic bonds. Some of these glucose residues carried branches at C_2 or C_4 which terminated either in D-glucose or D-mannuronic acid residues, as indicated by the identification of 2,6-di-*O*-methyl, 4,6-di-*O*-methyl, 2,3,4,6-tetra-*O*-methyl ethers of D-glucose and 2,3,4-tri-*O*-methyl-D-mannuronic acid. Di-*O*-methyl sugars may arise from undermethylation and/or demethylation of the polysaccharide, but the isolation of tetra-*O*-methyl-glucose and the fact that no di-*O*-methyl ethers of mannose were obtained showed that the di-*O*-methyl-glucoses have definite structural significance.

Isolation of the aldobiouronic acid (B) from the polysaccharide and the identification of 3-*O*-(2,3,4-tri-*O*-methyl-D-mannuronosyl)-2,4,6-tri-*O*-methyl-D-glucose (II) as one of the methylated fragments from the methylated polysaccharide proved that the D-mannuronic acid was combined to the rest of the polymer through D-glucose residues and by 1,3-glycosidic bonds. It also proved that the D-mannuronic acid was in a terminal position. In the same way, the isolation of an aldotriouronic acid composed of mannuronic acid and two glucose residues and its corresponding methyl ether from the methylated polysaccharide provided evidence that the D-mannuronic acid is joined to one of two continuous D-glucose residues in the molecule.



B

A high positive specific optical rotation of the polysaccharide as well as of its methyl ether indicated that the majority of the intersugar linkages had an α-configuration. The low consumption (0.5 mole) of periodate per anhydrohexose unit indicated that most of the sugar residues in the polysaccharide are immune to periodate oxidation and those which were oxidized were the non-reducing end groups of D-glucose and D-mannuronic

acid. The interpretation of the periodate-oxidation data was complicated by the presence of uronic acid residues, which tend to become oxidized, and also by the possibility that some of the sugar residues might be esterified by the phospholipid moiety of the lipopolysaccharide.

Polysaccharides (Combined 3R, 4R, 5R)

These polysaccharides were built up of similar sugar residues and the sugars were linked in the same manner as in the 6NR polysaccharide. However, they differed from the latter polysaccharide in the degree of branching and in their glucose:mannose ratio.

EXPERIMENTAL

All evaporations were carried out under diminished pressure below 40° unless otherwise stated.

Paper chromatograms were run by the descending method using the following solvent systems (v/v): (A) pyridine – ethyl acetate – water (2:1:2); (B) butanone–water azeotrope containing ammonia; (C) benzene–ethanol–water–ammonia (200:47:14:1); (D) 1-butanol–ethanol–water (40:11:19); and (E) ethyl acetate – acetic acid – formic acid – water (18:3:1:4). *p*-Anisidine hydrochloride was used to detect the sugars and their methyl ethers on paper chromatograms (26). R_g and M_G represent rates of movement of sugar paper chromatographically and electrophoretically in relation to 2,3,4,6-tetra-*O*-methyl-D-glucose and D-glucose respectively.

Hydrolysis of the Polysaccharide and Isolation of Uronic Acid Components

Polysaccharide (2.37 g) was heated in *N* sulphuric acid (50 ml) at 70° C for 23 hours and then at 100° C for 8 hours. The hydrolyzate was neutralized with barium hydroxide and barium carbonate, and the barium sulphate was removed by filtration. The barium ions were removed by Amberlite IR-120 and the acidic components were adsorbed on a column of Dowex 1 resin (acetate form). After the neutral sugars were washed off with water, the acids were eluted with *N* formic acid (yield of uronic acids 1.003 g). Chromatography on paper in solvent E showed three spots; one spot (component A) had the same mobility as mannuronolactone, the other two spots (components B and C) had $R_{\text{mannurone}}$ values of 0.22 and 0.36 respectively. The acids were separated on Whatman No. 3 MM paper using solvent E and gave the following yields: A, 82 mg; B, 334 mg; and C, 76 mg.

Attempts to crystallize A, $[\alpha]_D^{25}$ +91° (*c*, 1 in water), were unsuccessful. Reduction of its methyl ester methyl glycoside yielded a sugar which was identified chromatographically (solvent A) as mannose.

Component B, which appeared to be an aldobiouronic acid (acid equivalent: calculated 340, found 356), turned dark rapidly on standing and optical rotation measurements were impossible. Component B (134 mg) was heated for 10 hours at 75° C with 2% methanolic hydrogen chloride (3 ml). The methyl glycoside methyl ester was dissolved in tetrahydrofuran (25 ml) and reduced with lithium aluminum hydride (27). The recovered disaccharide glycoside (130 mg), $[\alpha]_D^{25}$ +34.8° (*c*, 2.3 in methanol), was hydrolyzed with *N* sulphuric acid. Chromatographic examination on paper using solvent A showed glucose and mannose in approximately equal amounts.

The sugars were separated on sheets of paper using solvent A, when mannose (53.6 mg) and glucose (57.9 mg) were obtained. The sugars were converted to their *p*-nitroanilides by heating with *p*-nitroaniline in methanol. The recovered *N*-*p*-nitrophenyl-D-mannopyranosylamine dihydrate had a melting point of 219° and the *N*-*p*-nitrophenyl-D-glucopyranosylamine dihydrate a melting point of 184° C, which are in good agreement with the reported values (13).

Similar examination of the derived neutral glycoside of component C showed that it was composed of mannose and glucose in the proportion of approximately 1:2.

Methylation of Fraction 6NR

The polysaccharide (4.01 g) was methylated three times by dimethyl sulphate and alkali (28) to yield chloroform-soluble (0.76 g) and chloroform-insoluble (5.03 g) fractions. These two fractions were then methylated five times by Purdies reagents (29); the chloroform-insoluble fraction yielded a product (0.85 g) with OCH_3 26.9%, and the chloroform-soluble material gave a product (4.73 g) with OCH_3 32.6%. Further methylation of this latter product did not increase its methoxyl content. The methylated polysaccharide (4.7 g) was dissolved in a mixture of chloroform – ethyl ether (60 ml, 1:5, v/v) and fractionated by addition of petroleum ether (b.p. 30–60°). A total of nine fractions were recovered and their specific rotations and methoxyl values are given in Table II. On the basis of similarity of these values, fractions 1, 2, and 3 were combined for methanolysis studies.

Methanolysis of Methylated Polysaccharides

The combined methylated polysaccharides (2.8 g) were refluxed on a water bath in 8% methanolic hydrogen chloride (80 ml). The course of methanolysis was followed by the changes in specific rotation: $[\alpha]_D$ initial (not observable); +75.5° (2 hours); +88° (6 hours); +90° (12 hours, constant value).

TABLE II

Fractionation of methylated polysaccharide from chloroform solution by petroleum ether

Fraction	Weight (g)	$[\alpha]_D^{27}$ in $CHCl_3$	OCH_3 value (%)
1	0.1535	+118°	36.1
2	1.5274	+100°	36.4
3	1.1200	+108°	38.4
4	0.6547	+ 64.5°	32.5
5	0.3929	+ 64°	35.3
6	0.1554	+ 4.56°	20.6
7	1.4791	+ 3.5°	23.0
8	0.1338	+ 55.5°	32.7
9	0.0430	+ 5.5°	—

A small portion of the mixture of methyl glycosides, after neutralization (Ag_2CO_3), was examined by gas–liquid chromatography (11, 12) using Apiezon M at 150° C and a flow rate of 75 ml of argon/min. Three distinct peaks were observed and the components of the mixture were collected separately. One of the components from the column crystallized as needles, which were recrystallized from ethyl – petroleum ether. The crystals had m.p. 67.5–69° and showed no depression when mixed with an authentic sample of methyl 2,4,6-tri-*O*-methyl-β-D-glucoside.

Acid Hydrolysis

A portion (65 mg) of the mixture of methyl glycosides was reserved and the remainder was hydrolyzed with hydrochloric acid (60 ml, 0.8 *N*) on a steam bath for 8 hours. The solution became dark upon hydrolysis and had a final $[\alpha]_D$ of +50° (*c*, 5). The hydrolyzate was neutralized (Ag_2CO_3), filtered, and silver ions were removed by H_2S. Evaporation of the solution afforded a light brown, clear syrup (2.67 g) which upon chromatography using solvent B gave spots with the following M_G values: 0.24; 0.59; 0.66; 1.00. From the chromatographic analysis the following sugars were tentatively identified: (i) 2,3,4,6-tetra-*O*-methyl-D-glucose; (ii) 2,4,6-tri-*O*-methyl-D-glucose; (iii) 2,4,6-tri-*O*-methyl-D-mannose; (iv and v) two di-*O*-methyl-hexoses; and (vi) uronic acid containing material which remained on the starting line.

The mixture of methylated sugars and uronic acids was passed over IR-120 and the acids were converted subsequently to their barium salts by heating with barium hydroxide at 65°. The neutral sugars were removed by continuous extraction with ethyl acetate for 4 days. The extract was evaporated to a sirup (1.734 g) which partly crystallized. The aqueous solution left after ethyl acetate extraction was acidified with *N* sulphuric acid and the extraction was continued with ethyl acetate for 48 hours. The extract was dried (Na_2SO_4) and evaporated to give a mixture of methylated uronic acid as a sirup (0.615 g). Examination of the acids by chromatography (solvent E) showed that there were three main components having R_g values 1.0, 0.8, and 0.55 respectively. They were separated from each other by sheet-filter-paper chromatography using solvent E and were designated fractions I, II, and III respectively.

Separation of the Mixture of Neutral Methylated Sugars on a Cellulose Column

The mixture (1.484 g) was put on a cellulose column and the column was developed with 1-butanone–water azeotrope. Ten-milliliter fractions were collected every 20 minutes. The appropriate fractions were combined and identified as described below.

Identification of Methylated Sugars

2,3,4,6-Tetra-O-methyl-D-glucose

The sirupy product (253 mg), $[\alpha]_D$ +83° in water (*c*, 1), was dissolved in 50% aqueous methanol and the insoluble material removed by filtration. Chromatographic examination in three different solvent systems (B, C, and D) showed that the sugar moved as a single spot and had the same mobility as 2,3,4,6-tetra-*O*-methyl-D-glucose. Examination of the methyl glycoside of the sugar by gas–liquid partition chromatography (11, 12) gave the same retention time as methyl 2,3,4,6-tetra-*O*-methyl-α,β-D-glucoside. Demethylation with boron trichloride (30) showed that glucose was the only parent sugar. The methylated sugar was identified as *N*-phenyl 2,3,4,6-tetra-*O*-methyl-D-glucosylamine, m.p. and mixed m.p. 134–135°; $[\alpha]_D$ +240° ±3° in chloroform (*c*, 0.3) (14).

The tri-*O*-methyl fraction, which was a mixture, was resolved into two components by chromatography on Whatman No. 3MM paper using solvent C. The two methylated sugars were identified as follows.

2,4,6-Tri-O-methyl-D-glucose

This sugar gave a bright red color with *p*-anisidine spray and yielded only glucose on demethylation. Its R_g (solvent C) was 0.47, which is the same as that of an authentic sample of 2,4,6-tri-*O*-methyl-D-glucose. The sirup crystallized and, after recrystallization from ethyl ether, had m.p. and mixed m.p. 123–125° (15).

Refluxing with aniline in ethanolic solution yielded *N*-phenyl 2,3,6-tri-*O*-methyl-D-glucosylamine, m.p. 162–165° (16). Examination of the methyl glycoside of the sugar by gas–liquid partition chromatography showed that it had the same retention time as that of authentic methyl 2,4,6-tri-*O*-methyl-α,β-D-glucoside.

2,4,6-Tri-O-methyl-D-mannose

This sugar had R_g 0.61 (solvent C) and M_G 0.0 in borate buffer, values which are in agreement with those of an authentic sample of 2,4,6-tri-*O*-methyl-D-mannose. On demethylation with boron trichloride it gave only mannose. Upon seeding with 2,4,6-tri-*O*-methyl-D-mannose, the sirup crystallized and, after recrystallization from ether–hexane, had m.p. and mixed m.p. 63–65° and showed $[\alpha]_D$ +17.6° in water (*c*, 1). These values are in good agreement with those reported for this sugar (17). The anilide, after recrystallization from ethyl ether, had m.p. and mixed m.p. 132° and $[\alpha]_D$ +7.8° in methanol (*c*, 0.35). The reported values for *N*-phenyl 2,4,6-tri-*O*-methyl-D-mannosylamine are m.p. 134° and $[\alpha]_D$ +8° (18).

The di-*O*-methyl sugar fraction did not give satisfactory separation of its components on paper using solvents B, C, and D. However, it was separable by electrophoresis on paper strips in 0.05 *M* borate buffer at 800 volts for 2 hours. Two main components (X, Y) and a minor one (Z) were separated. The methylated sugars were recovered from the paper by elution with water. Sodium ions were removed by Amberlite IR-120 and boric acid was removed by repeated distillation with methanol. The M_G values of the fractions were: X, 0.17; Y, 0.071; Z, 0.00 and the colors produced with the *p*-anisidine spray were: X, brown; Y, pink; and Z, bright red. Identifications of the sugars were made as follows.

4,6-Di-O-methyl-D-glucose

The rate of movement of the sugar on paper chromatogram (solvents B and C) was the same as that of 4,6-di-*O*-methyl-D-glucose and the brown color given by the *p*-anisidine spray was the same as that of an authentic specimen. Demethylation of this component produced only glucose. The sugar crystallized as fine needles from ethyl acetate solution, and after recrystallization from the same solvent, the crystals had m.p. 157–158° and showed $[\alpha]_D$ +62° in water (*c*, 1.9). The reported melting point and $[\alpha]_D$ for 4,6-di-*O*-methyl-D-glucose are 156–158° (19) and +62.4° (15) respectively.

2,6-Di-O-methyl-D-glucose

The sugar gave glucose on demethylation. Its mobility on paper in solvents B and C and by electrophoresis in borate buffer were the same as that of an authentic sample of 2,6-di-*O*-methyl-D-glucose. The specific rotation, +58° in water (*c*, 1), agreed closely with the reported value of +58.3° (19). The sugar gave a negative reaction with triphenyltetrazolium chloride (20, 21) but was detectable on the paper with periodate–benzidine spray (22).

Di-O-methyl Sugar (Component Z)

Its M_G value in borate buffer was 0.0, and upon demethylation, the sugar yielded glucose. It gave a negative triphenyltetrazolium test, showing thereby that C_2 was substituted. It was not oxidized by periodate. From these results the component appeared to be either 2,3- or 2,4-di-*O*-methyl-D-glucose. Scarcity of material precluded further investigation.

Identification of Methylated Uronic Acid Components

Fractions I, II, and III (10 mg each) were converted to their methyl ester methyl glycoside derivatives by heating with 2.5% methanolic hydrogen chloride (2 ml) at 100° in a sealed tube for 15 hours. Reduction with lithium aluminum hydride (50 mg) in tetrahydrofuran (40 ml) yielded sugars, which, after recovery by extraction with chloroform, were hydrolyzed with *N* sulphuric acid for 15 hours. The hydrolyzates were neutralized ($BaCO_3$) and chromatographed in solvent C. Fraction I yielded one main component, R_g 0.61; fractions II and III gave two components each, R_g 0.61 and 0.47 respectively. Chromatography of the sugars on paper using solvents B and C indicated that the sugar having R_g 0.61 (solvent C) and R_g 0.71 (solvent B) was 2,3,4-tri-*O*-methyl-D-mannose and the other sugar (R_g 0.47 (solvent C) and R_g 0.60 (solvent B)) was 2,4,6-tri-*O*-methyl-D-glucose.

Thus, fraction I yielded only 2,3,4-tri-*O*-methyl-D-mannose and fractions II and III yielded a mixture of this sugar and 2,4,6-tri-*O*-methyl-D-glucose. The mixtures were resolved by sheet-filter-paper chromatography and the sugars identified in the following manner.

2,4,6-Tri-O-methyl-D-glucose

The sugar crystallized and, after recrystallization from ethyl ether, had m.p. and mixed m.p. 123–125° (15). It gave an anilide, m.p. 162–165° (16). The identification was further confirmed by examination of its methyl glycosides by gas–liquid chromatography as described earlier.

2,3,4-Tri-O-methyl-D-mannose

The sirup, $[\alpha]_D$ +1° in water (*c*, 1), when examined on paper chromatograms in solvents B and C showed the same rate of movement as an authentic sample of 2,3,4-tri-*O*-methyl-D-mannose. Gas–liquid partition chromatography of the methyl glycoside of the sugar gave the same retention time as an authentic specimen of methyl 2,3,4-tri-*O*-methyl-α-D-mannoside.

The sugar (107 mg) was dissolved in water (2 ml), and barium carbonate (90 mg) and bromine (0.5 ml) were added. The oxidation was allowed to take place in the dark for 48 hours, after which time the excess of bromine was expelled by aeration. The reaction mixture was filtered, the filtrate acidified and extracted with chloroform. Evaporation of the chloroform extract gave 2,3,4-tri-*O*-methyl-D-mannonic acid, which

was distilled, b.p. (bath temp.) 110–120° (0.005 mm), to afford 2,3,4-tri-*O*-methyl-D-mannono-δ-lactone. Treatment of methanolic solution of the lactone (63 mg) with phenylhydrazine (33 mg) for 3 hours at 100° yielded crystalline 2,3,4-tri-*O*-methyl-D-mannonophenylhydrazide, which on recrystallization from ethyl acetate had m.p. 166° (reported value 166°) (26).

Methylation of Combined Fractions 3R, 4R, and 5R

The combined fractions (2.0 g) were methylated three times with dimethyl sulphate and alkali, using each time 50 ml of dimethyl sulphate and 150 ml of 45% potassium hydroxide. The first methylation was done in an atmosphere of nitrogen; acetone was added in subsequent methylations to keep the methylated product in solution. The rest of the procedure was the same as that described for 6NR. The chloroform-soluble material was methylated five times with methyl iodide and silver oxide, acetone being added in the first methylation to dissolve the partially methylated product. The methylated polysaccharide was recovered as a light brown friable glass (1.2 g; OMe 41.55%). The methylated polysaccharide was fractionated from chloroform – ethyl ether solution with petroleum ether and the analytical data on the fractions are given in Table III.

TABLE III

Analytical data of fractions of methylated polysaccharide

Fraction*	Weight (g)	$[\alpha]_D^{27}$ in $CHCl_3$	OCH_3 (%)
P I	0.1582	+106°	43.56
P II	Negligible	—	—
P III	0.0860	+109°	35.76
P IV	0.2536	+110°	38.8
S I	0.5704	+ 62°	41.34
S II	0.1000	+ 67°	25.95
S III	0.0396	+ 10°	2.4
S IV	0.0522	+ 11.5°	7.6

*P I, P II, P III, and P IV were combined to give S_0.

Examination of Methylated Fractions S_0 and S_I (See Table III)

Methanolysis and Hydrolysis

Fraction S_0.—This had $[\alpha]_D$ +114° (*c*, 1.18 in methanol) and was heated for 12 hours in 8% methanolic hydrogen chloride (20 ml). A portion of the methyl glycosides was hydrolyzed by heating with 0.5 *N* hydrochloric acid for 10 hours at 100° (final $[\alpha]_D$ +69°).

Fraction S_I.—This showed $[\alpha]_D$ +83° (*c*, 0.93 in methanol). It was treated the same way as described for S_0. After hydrolysis it had $[\alpha]_D$ +66°.

Chromatographic Examination of Methylated Sugars

The hydrolyzate of S_0 when examined by chromatography using solvents B and D showed the following components: 2,3,4,6-tetra-*O*-methyl-D-glucose; 2,4,6-tri-*O*-methyl-D-glucose; 2,4,6-tri-*O*-methyl-D-mannose; dimethyl sugar fraction; and uronic acid fraction.

Hydrolysis of fraction S_I gave the same components as S_0. The uronosides and glycosides from S_0 were separated by converting the former into their barium salts, from which the neutral glycosides were extracted with chloroform.

Examination of Neutral Sugar Fractions

The neutral glycosides of fraction S_0 were examined by gas–liquid partition chromatography and the retention times were compared with those of known sugar glycosides. By this means, the following sugars were found: 2,3,4,6-tetra-*O*-methyl-D-glucose; 2,4,6-tri-*O*-methyl-D-glucose; 2,4,6-tri-*O*-methyl-D-mannose; and an unresolved dimethyl fraction. Paper electrophoresis of the dimethyl fraction in borate buffer showed the presence of 4,6-di-*O*-methyl-D-glucose, 2,6-di-*O*-methyl-D-glucose, and an unidentified dimethyl spot.

Examination of fraction S_I by the same means as described for fraction S_0 showed that the same sugars were present, although the proportions were different. The ratios of the methylated fragments are given in Table I. Since it was clear from chromatographic evidence that the same sugars were present in all three fractions (6NR, S_0, and S_I) of the methylated polysaccharide and identification had been made of those in fraction 6NR, it was considered unnecessary to identify those in fractions S_0 and S_I further.

Methylated Uronic Acid Components of Fractions S_0 and S_I

The amounts of material were small and the whole quantity of each was treated as in the case of 6NR methylated polysaccharide. Reduction of the methyl ester methyl glycosides with lithium aluminum hydride yielded disaccharides which were subjected to acid hydrolysis. The sugars were separated by paper chromatography using solvent C and were found to be 2,3,4-tri-*O*-methyl-D-mannose and 2,4,6-tri-*O*-methyl-D-glucose. A portion of the sugars in the acid hydrolyzate was treated with methanolic hydrogen chloride

and the methyl glycosides thus formed were examined by gas–liquid chromatography. In addition to the above-mentioned sugars, there was present in the mixture a small amount of the glycosides of 2,3,4-tri-*O*-methyl-D-glucose.

Periodate Oxidation

Samples 4R (0.0854 g) and 6NR (0.0864 g) were each dissolved in water (50 ml), and 0.1 *M* sodium periodate (50 ml) was added. The samples and the blanks were put in brown, 250-ml glass-stoppered flasks and stored in the refrigerator (5° C). From time to time aliquots were taken out and periodate consumption and formic acid production were determined in the following manner.

Periodate Consumption

To 5 ml of aliquot, sodium bicarbonate (1.7 g), sodium arsenite solution (10 ml), and potassium iodide (1 ml) were added. The flasks were stored in the dark for 15–20 minutes and then titrated against 0.0203 *N* iodine using starch as indicator.

Formic Acid Production

To 10 ml of aliquot, seven drops of 2-methyl-1,2-propanediol were added. After 20 minutes, 1 ml of 20% potassium iodide was added and the liberated iodine was titrated against 0.01 *N* sodium thiosulphate.

Since the rate of oxidation was very slow during the first 24 hours, the samples were removed from the refrigerator and stored in a room maintained at 15° C. The results are given in Table IV.

TABLE IV

Periodate oxidation of fractions 4R and 6NR

Sample	Time (hours)	IO_4^- consumption (moles)	HCOOH produced (moles)
4R	5	0.27	0.076
	24	0.32	—
	48	0.29	0.080
	72	0.37	0.096
	196	0.50	0.125
6NR	5	0.17	0.071
	24	0.19	—
	48	0.38	0.086
	72	—	0.095
	196	0.52	0.122

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