Carbohydrate Research, 152 (1986) 205–215
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURE OF THE *Klebsiella* TYPE 10 CAPSULAR POLYSACCHARIDE

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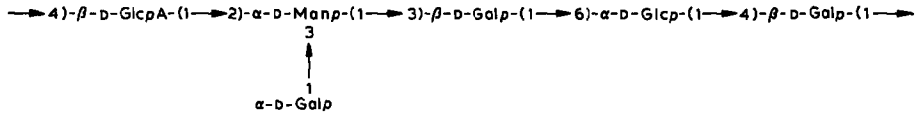
(Received April 23rd, 1985; accepted for publication, December 30th, 1985)

ABSTRACT

The capsular polysaccharide from *Klebsiella* Type 10 was found to contain D-galactose, D-glucose, D-mannose, and D-glucuronic acid in the ratios 3:1:1:1. Acid hydrolysis of the polysaccharide gave one aldobiouronic acid, one aldotriouronic acid, one aldotetraouronic acid, and two neutral disaccharides the structures of which were established. The native and carboxyl-reduced polysaccharide have been subjected as appropriate to methylation analysis and Smith degradation. Degradation of the methylated polysaccharide with base established the identity of the sugar unit preceding the glucosyluronic acid residue. The anomeric configurations of the sugar residues were determined by oxidation of the acetylated native and carboxyl-reduced polysaccharides with chromium trioxide. Based on these studies, the hexasaccharide structure **1** has been assigned to the repeating unit of the K-10 polysaccharide.



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INTRODUCTION

There are 81 serologically recognised strains in the genus *Klebsiella*[1,2], which have been differentiated on the basis of their capsular polysaccharides. These polysaccharides are specific antigens. *Klebsiella* type 10 is one of approximately twenty strains belonging to the same chemotype. We have reported on one member[3] of this chemotype and now report on the structure of the repeating unit of the K-10 polysaccharide.

RESULTS AND DISCUSSION

Klebsiella K-10 bacteria were grown in an agar medium, and the capsular

0008-6215/86/$ 03.50

polysaccharide was isolated by precipitation with Cetavlon[4] and purified on Sephadex G-100. The major peak contained the polysaccharide having $[\alpha]_D^{25}$ +65.5° (water). There was only one small peak of impurity which appeared after the major peak. The polysaccharide was homogeneous in high-voltage electrophoresis in borate buffer. Hydrolysis (0.5M sulfuric acid, 20 h, 100°) of the polysaccharide gave (p.c.) mannose, galactose, glucose, and glucuronic acid together with some slower-moving components. G.l.c. of the derived alditol acetates confirmed the identities of the mannose, galactose, and glucose.

Application of the carbazole method[5] indicated the presence of 17.5% of uronic acid. Carboxyl-reduction of the polysaccharide with 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide metho-*p*-toluenesulfonate[6] followed by hydrolysis and conversion of the products into alditol acetates gave (g.l.c.) derivatives of mannose, galactose, and glucose in the ratios 1:3.1:1.9 (Table I).

Hydrolysis of the K-10 polysaccharide with 0.5M sulfuric acid at 100° for periods up to 20 h showed that the percentage of mannose in the hydrolysate increased with time (Table II).

When the K-10 polysaccharide was hydrolysed with M trifluoroacetic acid for 1.5 h, p.c. of the hydrolysate revealed aldobiouronic acid, aldotriouronic acid, and aldotetraouronic acid in addition to glucose, galactose, and mannose. The acidic and neutral components were isolated by using a column of Dowex 1-X4 (OAc^-)

TABLE I

RESULTS OF ACID HYDROLYSIS

Sugar (as alditol acetate)	*Mole %*[a]						
	A	*B*	*C*	*D*	*E*	*F*	*G*
Galactose	64.5	34.4	52.5	37.0	49.2	48.3	24.2
Glucose	22.1	21.1	—	36.6	50.8	51.7	—
Mannose	13.3	11.1	47.3	26.4	—	—	26.7
Glycerol	—	—	—	—	—	—	49.1
myo-Inositol	—	33.3	—	—	—	—	—

[a]A, K-10; B, carboxyl-reduced K-10; C, aldotriouronic acid; D, aldotetraouronic acid; E, neutral disaccharide N_1; F, neutral disaccharide N_2; G, periodate-oxidised K-10.

TABLE II

RESULTS OF GRADED HYDROLYSIS

Time of hydrolysis (h)	*Galactose*	*Glucose*	*Mannose*	myo-*Inositol*
2	11.33	3.7	0.358	10
7	12.6	4.36	1.36	10
14	12.94	4.39	2.29	10
20	12.70	4.36	2.62	10

resin. The acidic components included aldobiouronic acid, aldotriouronic acid, aldotetraouronic acid, and glucuronic acid, which were isolated by preparative p.c. The glucuronic acid thus obtained had $[\alpha]_D^{23}$ +28° (water), indicating it to be D. Preparative p.c. of the neutral fraction gave two disaccharides, galactose, glucose, and mannose. The monosaccharides had specific rotations which indicated them to be D.

Since hydrolysis of the aldobiouronic acid with 2M trifluoroacetic acid for 20 h gave mannose as the only neutral sugar, the structure was probably GlcA→Man. Methylation[7] of the aldobiouronic acid followed by hydrolysis gave 3,4,6-tri-*O*-methylmannose. Reduction of the methylated aldobiouronic acid with lithium aluminium hydride followed by hydrolysis and conversion of the products into the alditol acetates gave derivatives of 3,4,6-tri-*O*-methylmannose and 2,3,4-tri-*O*-methylglucose (Table III). These findings, together with the fact that the aldobiouronic acid had $[\alpha]_D^{25}$ +10° (water), confirmed the structure to be β-D-Glc*p*A-(1→2)-D-Man*p*.

Hydrolysis of the aldotriouronic acid with 2M trifluoroacetic acid for 20 h gave mannose and galactose, the proportion of the latter being greater (Table I). Thus, the aldotriouronic acid contained galactose, mannose, and glucuronic acid. Borohydride reduction of the aldotriouronic acid followed by hydrolysis and acetylation gave (g.l.c.) the alditol acetate of galactose only, indicating the reducing end to be galactose. Methylation[7] of the aldotriouronic acid and hydrolysis of the product with 2M trifluoroacetic acid gave 2,4,6-tri-*O*-methylgalactose and 3,4,6-tri-*O*-methylmannose in the ratio 1:1.06 (Table III). Carboxyl-reduction of the methylated aldotriouronic acid followed by hydrolysis and conversion of the products into the alditol acetates gave (g.l.c.) derivatives of 2,4,6-tri-*O*-methylgalactose, 2,3,4-tri-*O*-methylglucose, and 3,4,6-tri-*O*-methylmannose in the ratios ~1:1:1 (Table III). Thus, the aldotriouronic acid was Glc*p*A-(1→2)-Man*p*-(1→3)-Gal*p*.

Acid hydrolysis of the aldotetraouronic acid gave (g.l.c. of the alditol acetates) galactose, glucose, and mannose in the ratios 1.4:1.4:1.0 (Table I). The reducing end of this oligomer, as determined by the procedure described above, was glucose. Methylation analysis of the aldotetraouronic acid gave 3,4,6-tri-*O*-methylmannose, 2,4,6-tri-*O*-methylgalactose, and 2,3,4-tri-*O*-methylglucose in the ratios 1:1.09:1.1 (Table III). Methylation analysis of the methylated aldotetraouronic acid, after reduction with lithium aluminium hydride, gave 3,4,6-tri-*O*-methylmannose, 2,4,6-tri-*O*-methylgalactose, and 2,3,4-tri-*O*-methylglucose in the ratios 1:1.1:2.1 (Table III). Thus, the aldotetraouronic acid was

Glc*p*A-(1→2)-Man*p*-(1→3)-Gal*p*-(1→6)-Glc*p*.

The neutral disaccharide fraction described above was resolved by p.c. into two components (N_1 and N_2) each of which, on hydrolysis, gave galactose and glucose in the ratio 1:1. Methylation analysis of N_1 gave 2,3,4,6-tetra-*O*-methylgalactose and 2,3,4-tri-*O*-methylglucose and of N_2 gave 2,3,4,6-tetra-*O*-methyl-

TABLE III

METHYLATION ANALYSIS OF K-10, CARBOXYL-REDUCED K-10, AND RELATED OLIGOMERS

Methylated sugar (as alditol acetates)	*Retention time (min)*		*Mole %*[a]									
	ECNSS-M	*OV-225*	*I*	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*
2,3,4,6-Tetra-*O*-methyl-D-galactose	1.24	1.18	20.0	16.5	16.4	20.6	22.5	—	—	—	—	—
2,4,6-Tri-*O*-methyl-D-galactose	2.23	2.02	20.4	16.3	16.3	22.4	23.4	—	35.1	34.1	26.0	47.1
2,3,6-Tri-*O*-methyl-D-galactose	2.41	2.21	20.2	16.8	16.7	13.85	4.5	—	—	—	—	—
2,3,4-Tri-*O*-methyl-D-glucose	2.46	2.21	20.2	16.8	16.7	20.6	22.5	46.5	31.8	34.5	50.8	—
2,3,6-Tri-*O*-methyl-D-glucose	2.48	2.30	—	16.6	—	—	—	—	—	—	—	—
4,6-Di-*O*-methyl-D-mannose	3.22	2.90	19.04	16.8	17.7	22.4	24.7	—	—	—	—	—
2,3-Di-*O*-methyl-D-glucose	5.34	4.46	—	—	16.00	—	—	—	—	—	—	—
3,4,6-Tri-*O*-methyl-D-mannose	1.92	1.80	—	—	—	—	—	52.3	32.9	31.2	23.5	—
2,3,4,6-Tetra-*O*-methyl-D-mannose	1.00	0.99	—	—	—	—	—	—	—	—	—	52.3

[a] I, methylated K-10; II, methylated carboxyl-reduced K-10; III, methylated K-10 after reduction with lithium aluminium hydride; IV, methylated K-10 after degradation with sodium methoxide for 0.5 h; V, methylated K-10 after degradation with sodium methoxide for 2 h; VI, methylated aldobiouronic acid after reduction with lithium aluminium hydride; VII, methylated aldotriouronic acid after lithium aluminium hydride reduction; VIII, methylated aldotetraouronic acid; IX, methylated aldotetraouronic acid after reduction with lithium aluminium hydride; X, methylated, periodate-oxidised K-10.

glucose and 2,3,6-tri-*O*-methylgalactose. The disaccharide N_1 had $[\alpha]_D^{23}$ +56°→+40° (water, 24 h), and N_2 had $[\alpha]_D^{23}$ +170°. N_1 and N_2 were therefore β-D-Gal*p*-(1→6)-Glc*p* and α-D-Glc*p*-(1→4)-Gal*p*, respectively.

The polysaccharide K-10 was methylated first by the Hakomori procedure[8] and then by the Kuhn procedure; the product had no i.r. absorption for hydroxyl groups. Hydrolysis of the methylated polysaccharide with 2M trifluoroacetic acid and conversion of the products into the alditol acetates gave (g.l.c.) derivatives of 2,3,4,6-tetra-*O*-methylgalactose, 2,4,6-tri-*O*-methylgalactose, 2,3,6-tri-*O*-methylgalactose, 2,3,4-tri-*O*-methylglucose, and 4,6-di-*O*-methylmannose in the ratios 1.04:1.07:1.06:1.06:0.8 (Table III). Reduction of the methylated polysaccharide, followed by hydrolysis with 2M trifluoroacetic acid for 20 h, and conversion of the products into alditol acetates gave (g.l.c.) derivatives of 2,3,4,6-tetra-*O*-methylgalactose, 2,4,6-tri-*O*-methylgalactose, 2,3,6-tri-*O*-methylgalactose, 2,3,4-tri-*O*-methylglucose, 4,6-di-*O*-methylmannose, and 2,3-di-*O*-methylglucose in approximately equimolecular ratios (Table III). The 2,3-di-*O*-methylglucose must have originated from the D-glucuronic acid residue since it was not formed on methylation analysis of the K-10 polysaccharide.

Methylation analysis of the carboxyl-reduced K-10 polysaccharide gave 2,3,4,6-tetra-*O*-methylgalactose, 2,4,6-tri-*O*-methylgalactose, 2,3,6-tri-*O*-methylgalactose, 2,3,4-tri-*O*-methylglucose, 2,3,6-tri-*O*-methylglucose, and 4,6-di-*O*-methylmannose in equimolecular ratios (Table III). The 2,3,6-tri-*O*-methylglucose must have originated from the carboxyl-reduced glucuronic acid residue. As all of the mannose appeared as the 4,6-di-*O*-methyl derivative and one galactose residue as the 2,3,4,6-tetra-*O*-methyl derivative, this D-galactose residue was probably attached to O-3 of D-mannose. Moreover, the proportion of 4,6-di-*O*-methylmannose increased after reduction with lithium aluminium hydride, whereas the proportion of the other trimethyl sugars remained unchanged. Consequently, this is the only sugar moiety which is involved in the aldobiouronic acid.

In sequencing the sugar units, the methylated polysaccharide was degraded with base[9] (sodium methoxide), whereby the sugar unit preceding the glycosyluronic residue was eliminated together with that residue. In this process, the yield of 2,3,6-tri-*O*-methylgalactose diminished by ~80% in 2 h, indicating strongly that this 4-linked galactose residue preceded the glucosyluronic acid residue in the repeating unit of the K-10 polysaccharide.

Periodate oxidation[10] of the K-10 polysaccharide and borohydride reduction of the product gave a polyol, hydrolysis of which yielded mannose, galactose, and glycerol in the ratios 1:1.1:2.02 (Table I). These results corroborated the findings of the methylation analysis of the K-10 polysaccharide. Acid hydrolysis of the polyol at room temperature and methylation analysis of the product gave 2,3,4,6-tetra-*O*-methylmannose and 2,4,6-tri-*O*-methylgalactose in the ratio ~1:1 (Table III). These findings showed that periodate oxidation of the K-10 polysaccharide produced the disaccharide *O*-D-mannopyranosyl-(1→3)-D-galactopyranose.

Since the polysaccharide had $[\alpha]_D^{25}$ +65.5°, it was expected that both α and β

TABLE IV

OXIDATION OF K-10, CARBOXYL-REDUCED K-10, AND THE OLIGOMERS WITH CHROMIUM TRIOXIDE

Material	*Time of oxidation (h)*	*Galactose*	*Glucose*	*Mannose*	myo-*Inositol*
Native K-10	0	7.68	2.7	1.6	10
	1	2.4	1.8	1.5	10
	2	1.9	1.4	1.2	10
Carboxyl-reduced K-10	0	14.9	10.1	4.8	10
	1	4.2	3.5	3.2	10
	2	3.2	2.7	2.6	10
Aldotetraouronic acid	0	19.4	18.5	16.4	10
	2	3.4	16.7	11.5	10
Neutral disaccharide N_1	0	10.5	10.7	—	10
	1	1.9	9.9	—	10
	2	1.3	9.6	—	10
Neutral disaccharide N_2	0	11.0	12.5	—	10
	1	10.4	9.8	—	10
	2	9.8	9.2	—	10

linkages would be present. The low specific rotation (+10°, water) of the aldobiouronic acid indicated it to be β-linked. The native and carboxyl-reduced K-10 polysaccharide were acetylated, and the products were oxidised with chromium trioxide[11,12] which abstracts only H-1*a* and thus modifies only β-linked units. In each of the foregoing experiments, the content of galactose decreased during 1 h (Table IV), indicating that at least one of the three galactosyl residues was α. The amounts of mannose and glucose did not change markedly, indicating that they were probably α. The specific rotations of neutral disaccharides N_1 (+56°) and N_2 (+170°) showed the glucose to be α and the galactose moiety preceding it to be β. Results of CrO_3 oxidation of the aldotetraouronic acid and the neutral disaccharides also support this conclusion. Treatment of the K-10 polysaccharide with α-D-galactosidase cleaved only a portion of the galactosyl residues, indicating that the galactosyl groups in the side chains were probably α.

From the above data, it was concluded that the K-10 polysaccharide has the hexasaccharide repeating-unit **1**.

EXPERIMENTAL

Materials and methods. — All the acid hydrolyses were performed at 100° in sealed ampoules. P.c. was performed on Whatman No. 1 paper with *A*, 9:2:2 ethyl acetate–acetic acid–water; *B*, 8:2:1 ethyl acetate–pyridine–water; and *C*, 4:1:5 (upper layer) 1-butanol–acetic acid–water; and detection with alkaline silver

nitrate[11]. All solvents were distilled before use, and all evaporations were conducted at 50°, unless otherwise stated. All aqueous solutions were lyophilised by using an Eyela Model FD-1 freeze-dryer. G.l.c. was performed on a Hewlett–Packard Model 5730 A instrument fitted with a flame-ionisation detector, a Model 3380A electronic integrator, and glass columns (1.83 m × 6 mm) packed with *A*, 3% of ECNSS-M on Gas Chrom Q (100–120 mesh); and *B*, 3% of OV-225 on Gas Chrom Q (100–120 mesh). The chromatography was performed at 180° on the alditol acetates[3] of unsubstituted sugars and at 170° on methylated sugars. Retention times were measured with respect to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol. Optical rotations were measured with a Perkin–Elmer Model 241MC spectropolarimeter. Colorimetric estimations were conducted by using a Hitachi Model 100-60 spectrophotometer.

Isolation and purification[4] of Klebsiella *K-10 polysaccharide.* — A culture of *Klebsiella* K-10 was grown on a 3% sucrose–yeast extract–agar medium composed of NaCl (4.5 g), K_2HPO_4 (2.25 g), $MgSO_4 \cdot 7\ H_2O$ (0.56 g), $CaCO_3$ (0.45 g), sucrose (67.5 g), bacto yeast extract (4.5 g), and agar (33.75 g) in water (2.25 L). The cells were harvested after 3 days and a very viscous slime (400 mL) was collected. Aqueous phenol (1%, 10 mL) was added, the mixture was centrifuged in batches for 2 h at 15,000 r.p.m. and 7°, and the clear supernatant liquids were decanted, combined (~350 mL), and poured into ethanol (1800 mL). The crude polysaccharide was collected and to a solution in water (400 mL) was added aqueous 10% Cetavlon until precipitation was complete. The precipitate was collected by centrifugation (20 min, 8000 r.p.m.) and a solution in 4M NaCl (400 mL) was poured into ethanol (2 L). The purified polysaccharide was collected by centrifugation (20 min, 8000 r.p.m.), and an aqueous solution was dialysed against distilled water for 4 days and then freeze-dried to yield the K-10 polysaccharide (625 mg). The polysaccharide was purified in batches (125 mg) by elution from a column (70 × 1.6 cm) of Sephadex G-100 with pyridine–acetic acid–water (4:10:1000). Fractions (64, 5 mL) were monitored with a Waters Associates Differential Refractometer Model 403 fitted with a recorder. Fractions 20–44 contained the polysaccharide, which emerged as a broad peak, and they were combined and lyophilised to yield purified K-10 polysaccharide (95 mg), $[\alpha]_{589.5}^{25}$ +65.5° (*c* 1, water). The homogeneity of the polysaccharide was established by high-voltage electrophoresis in borate buffer, using a Shandon Model L-24 Apparatus.

Acid hydrolysis of the K-10 polysaccharide. — The polysaccharide (2 mg) was hydrolysed with 0.5M sulfuric acid for 20 h at 100°. The hydrolysate was neutralised ($BaCO_3$) and filtered through Celite. One part was treated with Dowex 50W-X8 (H^+) resin, filtered, concentrated to small volume, and subjected to p.c. To the other part was added an equal volume of aqueous 40% sodium borohydride, and the alditol acetates were prepared in the usual way[3] and analysed by g.l.c. The results are given in Table I.

Carboxyl-reduction of the K-10 polysaccharide. — A solution of the K-10 polysaccharide (11 mg) in water (12 mL) was stirred with 1-cyclohexyl-3-(2-

morpholinoethyl)carbodi-imide metho-*p*-toluenesulfonate (300 mg), and cyclohexanol (4 drops) was added as an antifoaming agent. The pH was kept at 4.75 by the addition of 0.01M hydrochloric acid. After 2 h, 2M sodium borohydride (5 mL) was added during 1 h and the pH was kept at 7.00 by simultaneous addition of 4M hydrochloric acid. The solution was then dialysed for 24 h against distilled water and freeze-dried. The procedure was repeated on the same material, to ensure complete carboxyl-reduction. The yield of product was 9 mg.

Determination of the sugar components. — The uronic acid content of the native K-10 polysaccharide, determined by the carbazole method, was 17.5%. The carboxyl-reduced polysaccharide (2 mg) and *myo*-inositol (0.8 mg) were treated with 0.5M sulfuric acid for 20 h, and the products were converted into the alditol acetates. G.l.c. revealed mannose, galactose, and glucose in the ratios 1:3.1:1.9 (Table I).

Graded hydrolysis. — A solution of the polysaccharide (9.2 mg) and *myo*-inositol (2.5 mg) in 0.5M sulfuric acid (10 mL) was divided into four parts which were hydrolysed in ampoules for 2, 7, 14, and 20 h. Alditol acetates were prepared from the products in the usual way and analysed by g.l.c. The results are summarised in Table II.

Isolation of aldobio-, aldotrio-, and aldotetrao-uronic acids and neutral disaccharides. — The polysaccharide (77 mg) was hydrolysed with M trifluoroacetic acid (10 mL) for 1.5 h. The acid was evaporated and the residue was eluted from a column (20 × 1.2 cm) of Dowex 1-X4 (AcO^-) resin with water to give the neutral products (32 mg), and with aqueous 30% acetic acid to give the acidic products (40 mg). Preparative p.c. (solvent *A*) of the acidic fraction gave glucuronic acid (11 mg), $[\alpha]_D^{25}$ +28° (*c* 0.4, water); an aldobiouronic acid (4 mg), $R_{Lactose}$ 1.02, $[\alpha]_D^{25}$ +10° (*c* 0.1, water); an aldotriouronic acid (7.3 mg), $R_{Lactose}$ 0.70, $[\alpha]_D^{25}$ +27.5° (*c* 0.28, water); and an aldotetraouronic acid (3.7 mg), $R_{Lactose}$ 0.40, $[\alpha]_D^{25}$ +35° (*c* 0.12, water). Preparative p.c. of the neutral fraction (solvent *C*) gave two disaccharides N_1 and N_2 together with glucose, galactose, and mannose. N_1 ($R_{Lactose}$ 0.77) had $[\alpha]_D^{23}$ +56° (*c* 0.12, water) (lit.[13] for 6-*O*-β-D-galactopyranosyl-D-glucose, $[\alpha]_D$ +54°); and N_2 ($R_{Lactose}$ 1.04) had $[\alpha]_D^{23}$ +170° (*c* 0.1, water) (lit.[14] for 4-*O*-α-D-glucopyranosyl-D-galactose, $[\alpha]_D$ +140°). The monosaccharides had specific rotations corresponding to those of D-galactose, D-glucose, and D-mannose.

The aldobio-, aldotrio-, and aldotetrao-uronic acids (~1 mg) were each hydrolysed with 2M trifluoroacetic acid for 20 h. P.c. (solvent *B*) of the products and g.l.c. of their alditol acetates gave the results shown in Table I. Each oligomer (~0.5 mg) was also reduced with sodium borohydride, and the products were hydrolysed and then acetylated. The reducing ends of the oligomers were thus converted into alditol acetates which were identified by g.l.c.

Methylation analyses. — (*a*) *The aldobiouronic acid.* To a solution of the aldobiouronic acid (2 mg) in *N*,*N*-dimethylformamide (1.5 mL) were added silver oxide (0.8 g) and Drierite (0.5 g). The mixture was stirred for 30 min and then methyl iodide (0.5 mL) was added. Stirring was continued for 35 h, and chloroform

(20 mL) was added whilst the mixture was vigorously stirred. The mixture was filtered through Celite, washed with water, dried (Na_2SO_4), and concentrated. A portion of this product was hydrolysed with 2M trifluoroacetic acid for 20 h and the products were converted into alditol acetates. G.l.c. then revealed the derivative of 3,4,6-tri-*O*-methylmannose.

To a solution of another portion of the methylated aldobiouronic acid in dichloromethane (4 mL) and ethyl ether (2.5 mL) was added lithium aluminium hydride (20 mg). The mixture was boiled under reflux for 6 h and then kept for 16 h at room temperature. The excess of lithium aluminium hydride was decomposed with ethyl acetate and water. The mixture was then neutralised with M phosphoric acid, filtered through a cotton plug, washed with water, dried (Na_2SO_4), and concentrated to dryness. The residue was hydrolysed with 2M trifluoroacetic acid for 20 h. The products were converted into the alditol acetates which were then analysed by g.l.c. The results are given in Table III.

(*b*) *The aldotriouronic acid and aldotetraouronic acid.* The procedure in (*a*) was followed and the results are given in Table III.

Neutral disaccharides. — (*a*) *Hydrolysis.* The neutral disaccharides N_1 and N_2 were each hydrolysed with 2M trifluoroacetic acid for 20 h, and the products were converted into the alditol acetates which were then analysed by g.l.c. The results are summarised in Table I.

(*b*) *Methylation analysis.* The disaccharides N_1 and N_2 were methylated by the procedure described above and the products were then hydrolysed with 2M trifluoroacetic acid for 20 h. The products were converted into the alditol acetates which were then analysed by g.l.c. N_1 gave derivatives of 2,3,4,6-tetra-*O*-methylgalactose and 2,3,4-tri-*O*-methylglucose, and N_2 gave derivatives of 2,3,4,6-tetra-*O*-methylglucose and 2,3,6-tri-*O*-methylgalactose.

Methylation analyses. — (*a*) *The K-10 polysaccharide.* The polysaccharide (20 mg) in methyl sulfoxide (10 mL) was methylated by the Hakomori procedure, using 2M methylsulfinylmethanide (10 mL) and methyl iodide (10 mL). The products, after further methylation with methyl iodide and silver oxide[7], showed no i.r. absorption for hydroxyl. A portion of the methylated K-10 was hydrolysed with 2M trifluoroacetic acid for 20 h, and the products were converted into the alditol acetates which were analysed by g.l.c. The results are summarised in Table III.

Another portion of the methylated K-10 was reduced with lithium aluminium hydride by the method described above. The products were hydrolysed, and the alditol acetates prepared from the products were analysed by g.l.c. The results are summarised in Table III.

(*b*) *Carboxyl-reduced K-10.* Carboxyl-reduced polysaccharide (2 mg) was methylated as in (*a*). The methylated product was then hydrolysed with 2M trifluoroacetic acid for 20 h, and the alditol acetates prepared from the products were analysed by g.l.c. The results are summarised in Table III.

Base-catalysed degradation[15] of the methylated K-10 polysaccharide. — To a solution of the methylated K-10 polysaccharide (7 mg) in 10:1:2 dry methanol–2,2-

dimethoxypropane–dichloromethane (20 mL) was added a trace of toluene-*p*-sulfonic acid, and the solution was boiled under reflux for 30 min. Freshly cut sodium (250 mg) was added to the cooled solution which was then boiled under reflux. Portions were removed after 0.5 and 2 h, and cooled, and the pH was adjusted to 6.0 by the addition of aqueous 50% acetic acid. Water (50 mL) was added and each mixture was partitioned between water and chloroform. Each organic phase was washed with water (2 × 25 mL), dried (Na_2SO_4), and concentrated to dryness. Each residue was hydrolysed, and the products were converted into the alditol acetates which were analysed by g.l.c. The results are given in Table III.

Oxidation of the K-10 polysaccharide. — (*a*) *With periodate.* To an aqueous 0.05% solution of the polysaccharide (100 mL) was added 0.2M sodium periodate (25 mL), and the mixture was kept in the dark for 46 h at 5°. The excess of periodate was decomposed by adding an excess of ethylene glycol, and the mixture was kept for 3 h at room temperature and then dialysed for 2 days against distilled water. Sodium borohydride (100 mg) was added, and the solution was kept for 4 h at room temperature, decationised with Dowex 50W-X8 (H^+) resin, and concentrated to dryness. Boric acid was removed from the residue by repeated addition and evaporation of methanol. Alditol acetates, prepared from a portion of the residue, were analysed by g.l.c. The results are given in Table I.

The rest of the polyol was hydrolysed with 0.5M sulfuric acid for 8 h at room temperature. The hydrolysate was neutralised ($BaCO_3$), filtered through Celite, decationised with Amberlite IR-120 (H^+) resin, and lyophilised. The product was subjected to methylation analysis. The results are given in Table III.

(*b*) *With chromium trioxide.* A solution of the K-10 polysaccharide (1.0 mg) and *myo*-inositol (0.4 mg) in formamide (0.5 mL) was stirred with acetic anhydride (1 mL) and pyridine (1.5 mL) for 20 h at room temperature, then diluted with chloroform (25 mL), and washed with water, dried (Na_2SO_4), and concentrated to dryness. The product was reacetylated with acetic anhydride and pyridine for 20 h at room temperature.

Powdered chromium trioxide (300 mg) was added to a solution of the foregoing product in glacial acetic acid (3 mL). The mixture was stirred at 50°, aliquots were removed at 0, 1, and 2 h, immediately diluted with water, and partitioned between water and chloroform, and the chloroform phases were dried (Na_2SO_4) and concentrated to dryness. The samples were deacetylated with methanolic 0.2M sodium methoxide for 4 h. Solutions were decationised with Amberlite IR-120 (H^+) resin, and the products were converted into the alditol acetates and analysed by g.l.c. The results are given in Table IV.

Oxidation of carboxyl-reduced K-10 polysaccharide with chromium trioxide. — A mixture of carboxyl-reduced K-10 (1.0 mg) and *myo*-inositol (0.25 mg) was acetylated, and the product was oxidised with chromium trioxide as for the native polysaccharide. The results are summarised in Table IV.

Oxidation of aldotetraouronic acid and neutral disaccharides N_1 and N_2 with

chromium trioxide. — Each compound (~1 mg), together with *myo*-inositol (~0.3 mg), was reduced with sodium borohydride, and the products were acetylated and then oxidised with chromium trioxide as described above. The results are given in Table IV.

Enzymic degradation[3] *of the K-10 polysaccharide.* — To a solution of the polysaccharide (10 mg) in 0.2M sodium acetate buffer (pH 5.0; 3 mL) was added an excess (1.0 mL) of α-D-galactosidase (10 mg/mL, from *Coffeae arabicae*). A few drops of toluene were added to prevent bacterial growth, and the mixture was incubated for 50 h at 37°. The enzyme was then deactivated by heating for 30 min at 70°, the mixture was filtered through Celite and dialysed for 1 day against distilled water, and the difusate was collected and concentrated to dryness. The alditol acetate prepared from the material contained (g.l.c., column *A*) only the derivative of galactose.

ACKNOWLEDGMENTS

The authors thank Dr. I. Ørskov (Statens Seruminstitut, Copenhagen) for providing the culture, and Dr. A. K. Guha for growing the bacteria, help in isolation of the polysaccharide, and helpful suggestions.

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