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STRUCTURE OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* SEROTYPE K40

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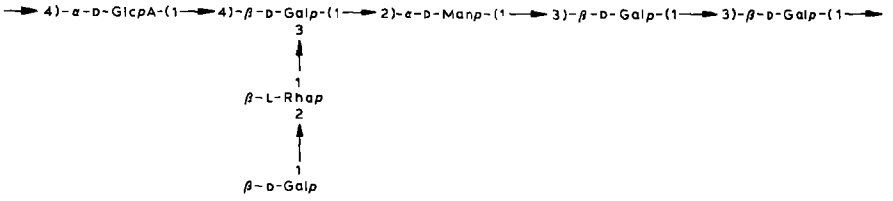
ABSTRACT

The capsular polysaccharide from *Klebsiella* Serotype K40 contains D-galactose, D-mannose, L-rhamnose, and D-glucuronic acid in the ratios of 4:1:1:1. Methylation analysis of the native and carboxyl-reduced polysaccharide provided information about the glycosidic linkages in the repeating unit. Degradation of the permethylated polymer with base established the identity of the sugar unit preceding the glycosyluronic acid residue. The modes of linkages of different sugar residues were further confirmed by Smith degradation and partial hydrolysis of the K40 polysaccharide. The anomeric configurations of the different sugar residues were determined by oxidation of the peracetylated native and carboxyl-reduced polysaccharide with chromium trioxide. Based on all of these results, the heptasaccharide structure **1** was assigned to the repeating unit of the K40 polysaccharide.



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INTRODUCTION

The genus *Klebsiella* was classified by Ørskov[1] into approximately 80 serotypes, based on their antigenic capsular polysaccharides. Nimmich[2] qualitatively analyzed the polysaccharide from each strain. *Klebsiella aerogenes* polysaccharide (K40) was reported[2] to contain galactose, mannose, rhamnose, and glucuronic acid. As part of our continuing investigation of the relationship between the chemical structure and the immunological activity, we now report elucidation of the structure of the repeating unit of the K40 polysaccharide.

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RESULTS AND DISCUSSION

Klebsiella K40 bacteria were grown[3] on an agar medium, and the capsular polysaccharide was isolated by precipitation with ethanol. It was purified first by treatment with Cetavlon, and then by passing it through a column of Sephadex G-100. The homogeneity of the polysaccharide was established by ultracentrifugal analysis and by high-voltage electrophoresis.

Paper chromatography of an acid hydrolyzate of the polysaccharide gave spots corresponding to rhamnose, mannose, galactose, and glucuronic acid, together with some slower-moving fractions. G.l.c. analysis of the alditol acetates from the sugars in the hydrolyzate gave peaks of rhamnose, mannose, and galactose in the ratios of 0.8:1:4.6 (see Table I). The proportion of uronic acid was estimated spectrophotometrically by the carbazole method[4], and was found to be 15.1%. The polysaccharide was reduced with sodium borohydride through its carbodiimide derivative[5]. G.l.c. of the carboxyl-reduced polysaccharide revealed rhamnose, mannose, galactose, and glucose in the ratios of ~1:1:4:1 (see Table I).

The ^{1}H-n.m.r. spectrum of a sample in D_2O showed anomeric-proton signals at δ 4.98 (1 H), 5.04 (1 H), 5.18 (1 H), 5.20 (1 H), 5.24 (2 H), and 5.30 (1 H) as singlets. There was also a three-proton doublet, at δ 1.30, corresponding to one –CH–CH_3 group. The whole spectrum accounted for 66 protons on the same scale, while a seven-sugar repeating unit requires 68 protons. The ^{13}C-n.m.r. spectrum indicated peaks for anomeric carbon atoms at δ 98.8 (1 C), 102.0 (2 C), 103.2 (2 C), and 105.2 (2 C). A peak at δ 19.3 indicated one C–CH_3 carbon atom. The whole spectrum accounted for 43 carbon atoms, while a seven-sugar repeating unit requires 42 carbon atoms.

The K40 polysaccharide was methylated, first by the Hakomori procedure[6] and then by the Kuhn method[7,8]. The permethylated material was purified by passing it through a column of Sephadex LH-20. The absence of hydroxyl absorp-

TABLE I

RESULTS OF ACID HYDROLYSIS

Sugars (as alditol acetates)	*Mole %*[a]							
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
Rhamnose	12.28	11.0	—	—	—	—	—	—
Mannose	15.62	14.1	—	—	31.7	46.6	30.6	—
Galactose	72.1	60.1	75	65	68.3	53.4	69.4	100
Glucose	—	14.8	—	—	—	—	—	—
Erythritol	—	—	—	15	—	—	—	—
Glycerol	—	—	25	20	—	—	—	—

[a]Key: A, Native K40 polysaccharide; B, carboxyl-reduced K40 polysaccharide; C, periodate-oxidized K40 polysaccharide; D, periodate-oxidized product of carboxyl-reduced K40 polysaccharide; E, aldotetraouronic acid; F, aldotriouronic acid; G, neutral trisaccharide; and H, neutral disaccharide.

TABLE II

METHYLATION ANALYSIS OF K40 CAPSULAR POLYSACCHARIDE AND DERIVED POLY- AND OLIGO-SACCHARIDES

Methylated sugars[a] *(as alditol acetates)*	T[b]	T[c]	*Mole %*[d]											
			Ia	*Ib*	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*	*XI*
3,4-Rha	0.87	0.92	12.5	10.5	9.9	—	16.5	16.8	—	—	—	—	—	—
2,3,4,6-Gal	1.19	1.25	16.5	15.2	14.8	68	18.7	19.4	—	—	—	—	—	54.4
3,4,6-Man	1.82	1.95	18.2	15.7	16.1	—	21.5	22.7	30.6	39.8	—	40.1	—	—
2,4,6-Gal	2.03	2.28	35.1	29.6	30.2	32	22.7	19.1	22.7	—	—	53.6	60.8	45.6
2,3,6-Glc	2.32	2.50	—	—	14.8	—	—	—	—	—	—	—	—	—
2,6-Gal	3.14	3.65	17.9	14.1	14.2	—	20.6	22.0	—	—	—	—	—	—
2,3-Glc	4.50	5.39	—	14.9	—	—	—	—	—	—	—	—	—	—
2,3,4-Glc	2.22	2.49	—	—	—	—	—	—	21.3	27.4	46.3	—	—	—
2,3,6-Gal	2.22	2.42	—	—	—	—	—	—	25.4	32.8	53.7	26.3	—	—
2,3,4,6-Man	0.99	1.00	—	—	—	—	—	—	—	—	—	—	39.2	—

[a]Key: 3,4-Rha = 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-L-rhamnitol, *etc.* [b,c]Retention time relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on a column of OV-225 and ECNSS-M, respectively. [d]Ia, Original capsular polysaccharide; Ib, LAH-reduced, methylated polysaccharide; II, carboxyl-reduced polysaccharide; III, periodate-oxidized product of carboxyl-reduced polysaccharide; IV and V, methylated polysaccharide after degradation with sodium methoxide for 0.5 h and 2 h, respectively; VI, VII, and VIII, methylated aldotetraouronic acid, aldotriouronic acid, and aldobiouronic acid, respectively, after LAH reduction; IX, aldotetraouronic acid; X and XI, neutral trisaccharide and neutral disaccharide.

tion in the i.r. spectrum of the product indicated complete methylation. A small part of the permethylated polysaccharide was hydrolyzed with 2M trifluoroacetic acid for 20 h, and the alditol acetates were prepared from the hydrolyzate. Analysis by g.l.c. showed peaks for 3,4-di-*O*-methylrhamnose, 2,3,4,6-tetra-*O*-methylgalactose, 3,4,6-tri-*O*-methylmannose, 2,4,6-tri-*O*-methylgalactose, and 2,6-di-*O*-methylgalactose in the ratios of 0.75:1:1.1:2.1:1.1 (see Table II). The other part of the permethylated polysaccharide was reduced[9] with lithium aluminum hydride. The product was hydrolyzed with 2M trifluoroacetic acid for 20 h, and its alditol acetates were prepared. Analysis by g.l.c. showed peaks corresponding to 3,4-di-*O*-methylrhamnose, 2,3,4,6-tetra-*O*-methylgalactose, 3,4,6-tri-*O*-methylmannose, 2,4,6-tri-*O*-methylgalactose, 2,6-di-*O*-methylgalactose, and 2,3-di-*O*-methylglucose in the ratios of 0.74:1.07:1.1:2.1:1:1.05 (see Table II). 2,3-Di-*O*-methylglucose must have come from D-glucuronic acid residues when the permethylated K40 polysaccharide was reduced with lithium aluminum hydride. The original polysaccharide contained no glucose at all.

The carboxyl-reduced K40 polysaccharide was similarly methylated, and the alditol acetates were prepared. Analysis by g.l.c. showed peaks corresponding to 3,4-di-*O*-methylrhamnose, 2,3,4,6-tetra-*O*-methylgalactose, 3,4,6-tri-*O*-methylmannose, 2,4,6-tri-*O*-methylgalactose, 2,3,6-tri-*O*-methylglucose, and 2,6-di-*O*-methylgalactose in the ratios of 0.66:1:1.08:2.04:1:0.95 (see Table II). Here also, the 2,3,6-tri-*O*-methylglucose must have come from the carboxyl-reduced glucuronic acid residue. The presence of 2,3,4,6-tetra-*O*-methylgalactose and 2,6-di-*O*-methylgalactose indicated that one of the galactose residues is present as a nonreducing end, and this terminal galactose is attached to another galactose residue of the main chain, either directly or through another sugar residue.

In order to obtain further information regarding the sequence of the sugar units, the permethylated polysaccharide was subjected to degradation with base[8]. It was observed that the amount of 2,4,6-tri-*O*-methylgalactose diminished with time. This sugar unit was degraded to the extent of ~45.6% in 2 h, which indicated that one of the two O-3-linked galactose residues preceded the glycosyluronic acid residue in the repeating unit of the K40 polysaccharide.

On oxidation of the polysaccharide with sodium periodate[10], followed by treatment of the product with sodium borohydride, a polyol was obtained. Total hydrolysis of a portion of the polyol gave glycerol and galactose in the ratio of 1:3 (see Table I). In a similar experiment, the carboxyl-reduced K40 polysaccharide gave glycerol, erythritol, and galactose in the ratios of 1.3:1.0:4.3 (see Table I). In either case, not a trace of rhamnose was found. Absence of glucose in the case of the carboxyl-reduced polymer indicated that the glucuronic acid in the original polysaccharide is (1→4)-linked. These results supported the results of the methylation analysis.

The rest of the polyol from the oxidized native polymer was hydrolyzed at room temperature, and the product was methylated[8]. Analysis, by g.l.c., of the alditol acetates showed the presence of 2,3,4,6-tetra-*O*-methylgalactose and 2,4,6-

tri-*O*-methylgalactose in the molar ratios of ~2.12:1 (see Table II). It was, therefore, quite clear that periodate oxidation followed by hydrolysis of the product with acid at room temperature resulted in the formation of galactose and a disaccharide, namely, 3-*O*-galactopyranosylgalactose.

The native K40 polysaccharide was hydrolyzed with M trifluoroacetic acid for 2 h. Neutral and acidic fractions were separated by passing the hydrolyzate through a column of Dowex-1 X-4 (OAc^-) resin. On paper-chromatographic analysis in solvent system *A*, the acid part gave spots corresponding to an aldobiouronic acid, an aldotriouronic acid, and an aldotetraouronic acid, in addition to glucuronic acid, which were separated by preparative paper-chromatography. The glucuronic acid thus obtained had $[\alpha]_D^{24}$ +29.5°, corresponding to D-glucuronic acid. From the neutral fractions, one disaccharide and one trisaccharide were isolated, in addition to rhamnose, mannose, and galactose, by means of preparative paper-chromatography in solvent system *B*. The monosaccharides thus isolated had specific rotations corresponding to those of D-galactose, D-mannose, and L-rhamnose, respectively.

Acid hydrolysis of the aldobiouronic acid with 2M trifluoroacetic acid for 20 h showed, in g.l.c., galactose as the only neutral sugar. Paper chromatography of the hydrolyzate showed galactose and glucuronic acid, together with some unreacted aldobiouronic acid, confirming assignment of the structure of the disaccharide as GlcA→Gal. Methylation[8] of the aldobiouronic acid gave a fully methylated product which, on hydrolysis, gave 2,3,6-tri-*O*-methylgalactose. In another experiment, the permethylated material was reduced with lithium aluminum hydride, and the product was hydrolyzed with acid. Alditol acetates prepared from the resulting mixture gave peaks corresponding to 2,3,6-tri-*O*-methylgalactose and 2,3,4-tri-*O*-methylglucose in the ratio of 1.2:1 (see Table II). These results, together with $[\alpha]_D^{24}$ +54.8°, confirmed that the aldobiouronic acid was α-D-Glc*p*A-(1→4)-D-Gal*p*.

On hydrolysis with 2M trifluoroacetic acid for 20 h, the aldotriouronic acid gave mannose and galactose, in the ratio of 1:1.1 as analyzed by g.l.c. (see Table I). It was evident from these results that the aldotriouronic acid is composed of mannose, galactose, and glucuronic acid. Analysis showed that the trisaccharide contained the mannose residue as its reducing end. Methylation analysis (see Table II), as described for the aldobiouronic acid, revealed the structure of the aldotriouronic acid as Glc*p*A-(1→4)-Gal*p*-(1→2)-Man*p*.

Hydrolysis of the aldotetraouronic acid with acid afforded a mixture that, on g.l.c. analysis, gave peaks of mannose and galactose in the ratio of 1:2.2 (see Table I). Galactose was found to be at the reducing end of this aldotetraouronic acid. Methylation analysis (see Table II) as already described showed that the structure of the aldotetraouronic acid was Glc*p*A-(1→4)-Gal*p*-(1→2)-Man*p*-(1→3)-Gal*p*.

The aldotetraouronic acid has a (1→3)-linked galactose residue as its reducing end. Periodate oxidation showed two (1→3)-linked galactose residues connected together. Again, a (1→3)-linked galactose residue precedes the glucuronic acid, as shown by degradation of the permethylated K40 polysaccharide with sodium methoxide. It is, therefore, most probable that the rhamnosyl residue lies

TABLE III

OXIDATION OF K40 AND CARBOXYL-REDUCED K40 POLYSACCHARIDE WITH CHROMIUM TRIOXIDE

Polysaccharide	*Time of oxidation (h)*	*Rhamnose*	*Mannose*	*Galactose*	*Glucose*	myo-*Inositol*
Native K40	0	2.06	2.64	12.2	—	10.00
	1	0.29	1.34	0.51	—	10.00
	2	0.09	1.2	0.27	—	10.00
Carboxyl-reduced K40	0	1.95	2.5	10.67	2.61	10.00
	1	0.25	1.24	0.45	1.12	10.00
	2	0.08	1.1	0.25	0.96	10.00

in the side chain and links up the galactosyl group with the main chain.

More light on the sequence of the sugar residues was provided by the neutral oligomers. Hydrolysis and methylation analysis proved that the neutral disaccharide was Gal-(1→3)-Gal, and that the trisaccharide was Man-(1→3)-Gal-(1→3)-Gal.

It was necessary to determine the anomeric configurations of the different sugar residues in the polysaccharide. The polysaccharide has $[\alpha]_D^{25}$ +1.6°. It was therefore expected that the polysaccharide has, in its repeating unit, linkages which contribute to rotations in both the positive and negative directions. The high specific rotation of the aldobiouronic acid indicated that the glucuronic acid is α-linked. The native polysaccharide and the carboxyl-reduced polysaccharide were acetylated, and the peracetates were subjected to oxidation with chromium trioxide[11]. In both experiments, the amount of galactose and rhamnose decreased very rapidly during the first hour, and almost vanished in the second hour (see Table III), indicating that the L-rhamnose and all four D-galactose residues in the polysaccharide have β linkages. In the case of native K40, the amount of mannose, and in the case of carboxyl-reduced K40 polysaccharide, the amounts of glucose and mannose did not decrease appreciably with time. These results indicated that the mannosyl and glucosyluronic residues have α-glycosidic linkages.

All of these results led to structure **1** for the repeating unit of the polysaccharide from *Klebsiella* type 40.

EXPERIMENTAL

Materials and methods. — Optical rotations were measured with a Perkin–Elmer Model 241 MC spectropolarimeter. Paper chromatography was performed on Whatman No. 1 and No. 3 papers. Solvent systems (v/v) used were (*A*) 9:2:2 ethyl acetate–acetic acid–water, and (*B*) 4:1:5 1-butanol–acetic acid–water (upper layer). The spray reagent used was alkaline silver nitrate. All solvents were distilled before use, and all evaporations were conducted at 50°, unless otherwise stated. All aqueous solutions were lyophilized by using an Eyela model FD-1 freeze-dryer.

Gas-liquid chromatography (g.l.c.) was performed by using a Hewlett–

Packard Model 5730A instrument fitted with a flame ionization detector and a Hewlett–Packard Model 3380A electronic integrator. The columns used were glass (1.83 m × 6 mm) packed with (*1*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) and (*2*) 3% of OV-225 on Gas Chrom Q (100–120 mesh). The chromatography was performed at 180° for neutral sugars, and at 170° for methylated sugars by converting the sugars into their corresponding alditol acetates[8]. Retention times of partially methylated alditol acetates were measured with respect to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol as unity. Colorimetric estimations were performed by using a Hitachi Model 100-60 spectrophotometer. N.m.r. spectra (^1H and ^{13}C) were recorded with a 500-MHz Bruker instrument at 80°, using D_2O as the solvent.

Isolation and purification[3] *of* Klebsiella *K40 polysaccharide.* — A culture of *Klebsiella* K40 obtained from Dr. I. Ørskov was grown, and the polysaccharide was isolated as described previously. The crude polysaccharide (200 mg) was purified by passing it through a column of Sephadex G-100. The column was eluted with 4:10:1000 pyridine–acetic acid–water, and 65 fractions (5 mL each) were collected. Fractions 29–43 contained the polysaccharide, which emerged as a sharp single peak. The fractions containing the polysaccharide were combined, and lyophilized: yield 185 mg; $[\alpha]_D^{24}$ +1.6° (*c* 0.2, water). The homogeneity of the polysaccharide was established by ultracentrifugation, using the sedimentation velocity procedure at 40,000 r.p.m., of a 1% solution of the polysaccharide in 0.1M phosphate buffer, and by high-voltage electrophoresis in borate buffer.

Acid hydrolysis of the polysaccharide. — The polysaccharide (2.5 mg) was dissolved in 2M trifluoroacetic acid (1.5 mL), and the solution was heated in a sealed ampoule for 20 h at 100°. The acid was removed *in vacuo* by codistillation with water. The product was dissolved in water, and the solution was divided into two parts. One part was examined by paper chromatography in solvent systems *A* and *B*. To the rest of the hydrolyzate (~2 mL) was added sodium borohydride (15 mg), and the alditol acetates were prepared in the usual way and analyzed by g.l.c. (column *1*). The results are shown in Table I.

Preparation of carboxyl-reduced K40 polysaccharide. — 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (300 mg) was added to a stirred solution of the polysaccharide (15 mg) in water (15 mL). Cyclohexanol (4 drops) was added as an antifoaming agent, and the pH of the solution was kept at 4.75 by dropwise addition of 0.01M hydrochloric acid. After 2 h, 2M aqueous sodium borohydride (7 mL) was added dropwise during 1 h, and the pH was kept at 7.0 by concurrent addition of 4M hydrochloric acid. The solution was dialyzed against distilled water for 30 h, and then lyophilized. The whole process was repeated on the same material, in order to ensure complete reduction of the carboxyl group of the uronic acid; yield 13.0 mg.

Analysis of sugar components in the K40 polysaccharide. — The carboxyl-reduced polysaccharide (2 mg) was hydrolyzed with 2M trifluoroacetic acid for 20 h at 100°, and its alditol acetates were prepared in the usual way. Examination by g.l.c.

gave the ratios of the sugar components (see Table I). The uronic acid content in the polysaccharide was estimated by the carbazole method[4], with D-glucuronic acid as the standard. The uronic acid content was found to be 15.1%.

Methylation analysis of native and carboxyl-reduced K40 polysaccharides. — Methylation of the polysaccharides (4–8 mg) was conducted by standard procedures[6–8]. The products showed no hydroxyl band in their i.r. spectra. The permethylated, native K40 polysaccharide (1 mg) was hydrolyzed with 2M trifluoroacetic acid for 20 h, and the alditol acetates were prepared, and analyzed by g.l.c. (see Table II).

Another portion of the permethylated K40 polysaccharide (2 mg) was dissolved in a mixture of dichloromethane (4 mL) and diethyl ether (2.5 mL). To this solution was added an excess of lithium aluminum hydride (25 mg), and the mixture was boiled under reflux for 6 h in a hot-water bath, and cooled. The excess of lithium aluminum hydride was decomposed by adding ethyl acetate and, finally, a few drops of water. The mixture was then made neutral with M phosphoric acid, and filtered through a bed of Celite. The filtrate was washed with water, dried (anhydrous sodium sulfate), and evaporated to dryness. The product was hydrolyzed, and its alditol acetate were analyzed by g.l.c. The results are given in Table II.

Methylation analysis of carboxyl-reduced K40 polysaccharide. — Carboxyl-reduced polysaccharide (4 mg) was methylated[6–8]. The permethylated, carboxyl-reduced K40 polysaccharide was hydrolyzed with acid, and its alditol acetates were analyzed by g.l.c. (see Table II).

Base degradation[9] of permethylated K40 polysaccharide. — Permethylated K40 polysaccharide (3 mg) and *p*-toluenesulfonic acid (a trace) were dissolved in 18:1:2 dry methanol–2,2-dimethoxypropane–dichloromethane (20 mL), and the solution was refluxed for 30 min, and cooled. A piece of freshly cut sodium (200 mg) was then added, and the turbid solution resulting was refluxed. An aliquot was taken after 0.5 h, and for the rest the reaction was allowed to proceed for 2 h. For both of these fractions, after cooling, the pH was adjusted to 6.0 by the addition of 50% acetic acid. Water (50 mL) was added, and the mixtures were partitioned between water and chloroform. The combined organic phases were washed with water (25 mL), and evaporated to dryness. The two samples thus obtained were separately hydrolyzed with acid, and the alditol acetates prepared from the hydrolyzates were analyzed by g.l.c. (see Table II).

Periodate oxidation of the polysaccharide. — To a 0.05% solution of the polysaccharide (40 mL) was added 0.2M sodium periodate solution (10 mL), and the mixture was kept in the dark for 48 h at 5°. Ethylene glycol (2 mL) was added, and the solution was stirred for 2 h before dialysis for 3 days. The solution was concentrated to 5 mL, sodium borohydride (50 mg) was added, and the mixture was kept for 4 h at room temperature. The mixture was de-cationized with Dowex-50W X-8 (H^+) ion-exchange resin, and evaporated to dryness. Boric acid was removed by repeated addition and evaporation of methanol. Alditol acetates prepared from a portion of the polyol thus obtained were analyzed by g.l.c.; the results are summarized in Table I.

The rest of the polyol was hydrolyzed with 0.5M sulfuric acid for 8 h at room temperature. The acid was neutralized with barium carbonate, the suspension was filtered through a bed of Celite, and the filtrate was de-cationized with Dowex-50W X-8 (H^+) ion-exchange resin, and lyophilized. The resulting material was methylated, and the alditol acetates prepared from the product were analyzed by g.l.c. The results are shown in Table II.

Carboxyl-reduced K40 polysaccharide was treated with sodium periodate in a similar way. Its alditol acetates were analyzed by g.l.c., and the results are shown in Table I.

Oxidation of K40 polysaccharide and carboxyl-reduced K40 polysaccharide with chromium trioxide[11]. — A mixture of the polysaccharide (5 mg) and *myo*-inositol (3 mg) was completely acetylated by two treatments with acetic anhydride–pyridine, using a trace of formamide in the first treatment[11].

The peracetates were dissolved in glacial acetic acid (3 mL), powdered chromium trioxide (300 mg) was added, and the mixture was stirred in a water bath at 50°. Aliquots were removed at 0, 1, and 2 h, and immediately diluted with water. Each mixture was partitioned between water and chloroform, and the respective chloroform extracts were combined, dried (Na_2SO_4), and evaporated to dryness. The materials were deacetylated with 0.2M sodium methoxide for 3 h, de-cationized with Dowex-50W X-8 (H^+) ion-exchange resin, and the alditol acetates prepared in the usual way. The alditol acetates were analyzed by g.l.c. (see Table III).

The carboxyl-reduced K40 polysaccharide (2 mg) was oxidized with chromium trioxide in a similar way, and the alditol acetates prepared from it were analyzed by g.l.c. (see Table III).

Partial hydrolysis of K40 polysaccharide. — The polysaccharide (90 mg) was hydrolyzed with M trifluoroacetic acid for 2 h at 100°. The acid was removed by evaporation. The mixture was then passed through a column of Dowex-1 X-4 (OAc^-) anion-exchange resin. The neutral sugars were obtained by elution of the column with water. The eluate was freeze-dried. The acidic sugars were isolated by eluting the column with 30% acetic acid and then freeze-drying the eluate (yield 20 mg). Preparative paper-chromatography (solvent *A*) of the acid fraction, gave glucuronic acid (5 mg), $[\alpha]_D^{24}$ +29.5° (*c* 0.6, water); one aldobiouronic acid (4.7 mg), $R_{Lactose}$ 1.12, $[\alpha]_D^{24}$ +54.8° (*c* 0.66, water); one aldotriouronic acid (3.1 mg), $R_{Lactose}$ 0.75, $[\alpha]_D^{24}$ +62.3° (*c* 0.43, water); and one aldotetraouronic acid (3.2 mg) $R_{Lactose}$ 0.45, $[\alpha]_D^{24}$ +72° (*c* 1.2, water).

The neutral fraction was resolved by paper chromatography (solvent *B*) into one neutral trisaccharide (3.5 mg), R_{Gal} 0.44, $[\alpha]_D^{24}$ +72.7° (*c* 0.9, water), one disaccharide (2.5 mg), R_{Gal} 0.59, $[\alpha]_D^{24}$ +51.5° (*c* 0.8, water), and three monosaccharides having specific rotations corresponding to those of L-rhamnose, D-mannose, and D-galactose.

Hydrolysis of oligosaccharides. — Aldobio-, aldotrio-, and aldotetrao-uronic acid (~0.5 mg) were hydrolyzed with 2M trifluoroacetic acid for 20 h. Paper-chromatographic (solvent *A*) and g.l.c. analysis of the products as their alditol

acetates provided information about the sugars present in individual oligomers. The results are shown in Table I. In another set of experiments, each oligomer (0.5 mg) was reduced with sodium borohydride, and the reduction product was hydrolyzed with acid, and then acetylated. The reducing ends of the oligomers were thus converted into alditol acetates which were analyzed by g.l.c.

Methylation analysis of acidic oligosaccharides. — The aldobiouronic acid (0.6 mg) was methylated by the Kuhn method. The methylation product was hydrolyzed with 2M trifluoroacetic acid for 20 h, and the alditol acetates were prepared. Analysis by g.l.c. showed 2,3,6-tri-*O*-methylgalactose. Another portion of the methylated aldobiouronic acid was reduced with lithium aluminum hydride in the usual way, and the reduction product was hydrolyzed with 2M trifluoroacetic acid. The alditol acetates prepared from this material were analyzed by g.l.c. (see Table II).

Methylation analyses of aldotriouronic acid and aldotetraouronic acid were carried out similarly. The results are given in Table II.

Hydrolysis of neutral disaccharide and trisaccharide. — The neutral disaccharide and trisaccharide (~0.5 mg) were hydrolyzed with 2M trifluoroacetic acid for 20 h; the alditol acetates were prepared in the usual way, and analyzed by g.l.c. (see Table I).

Methylation analysis of neutral disaccharide and trisaccharide. — The neutral disaccharide and trisaccharide (~1 mg each) were methylated by the Kuhn method, and the methylation products were analyzed in the usual way by g.l.c. The results are summarized in Table II.

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