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STRUCTURAL INVESTIGATION OF *Klebsiella* SEROTYPE K7 POLYSACCHARIDE

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ABSTRACT

Methylation analysis of and partial hydrolysis studies on the *Klebsiella* K7 capsular polysaccharide and its carboxyl-reduced derivative indicated the recurrence of D-glucopyranuronic acid, D-mannopyranose, and D-glucopyranose residues, linearly linked in a specific manner, in the molecular structure. D-Galactopyranose and pyruvic acid residues are linked to the main chain on the D-mannose residues (at O-3) and the D-glucose residues (at O-4 and O-6), respectively; the simplest interpretation of this evidence is that nine sugar residues and pyruvic acid constitute a repeating unit. The sequence →3)-β-D-GlcA*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-D-Glc*p*→ was demonstrated by the isolation from the polysaccharide of an aldotetraouronic acid of this structure.

INTRODUCTION

A number of molecular structures have been reported recently for the capsular polysaccharides of different *Klebsiella* K-types, a survey of which has been published[1]. Preliminary reference has been made[2,3] to the structural features of the acidic polysaccharide from *Klebsiella* K7, which has $[\alpha]_D$ +40°, equiv. wt. ~500 by titration, and $\bar{M}_w$ 1.2 Mdaltons by gel-permeation chromatography[4]. Proton magnetic resonance (p.m.r.) spectroscopy indicated the presence of one pyruvic acid acetal grouping to every seven or eight sugar residues[5].

Graded hydrolysis with acid, monitored by paper chromatography of the hydrolyzates, showed that galactose and pyruvic acid (detected by the *o*-phenylenediamine spray[6]) were the first residues to be released from the polysaccharide structure, followed by glucose and, more slowly, mannose. Glucuronic acid and four other acidic products of higher molecular weight were then detected, including an aldobiouronic acid chromatographically indistinguishable from 2-O-(β-D-glucopyranosyluronic acid)-D-mannose.

Monitoring of the course of mild hydrolysis with acid by gel-permeation chromatography[7,8] of samples removed at intervals showed an initial, rapid decrease in $\bar{M}_w$, with the subsequent development of several peaks in the gel chromatogram, mostly at molecular weights that were multiples of 1,300 daltons. As hydrolysis progressed, the peaks at mol. wt. 1,300 became increasingly prominent until, after treatment for 48 h, the product corresponding to this peak was the only constituent of the hydrolyzate other than material at the di- and mono-saccharide levels (see Table I and Figs. 1 and 2). A sample of this product, considered to constitute a major portion of the chemical repeating-unit in the polysaccharide molecule, was isolated by preparative, gel-permeation chromatography, and found to contain mannose, glucose, and acidic material, but only a trace of galactose. Methylation analysis of this oligosaccharide established that each of the glucose and glucuronic acid residues present was substituted at O-3, and the mannose residues at O-2.

Further hydrolysis in 0.05M sulfuric acid resulted in breakdown into lower oligosaccharides; chromatography on Bio-Gel P-10 demonstrated the presence of four such components (mol. wts. 960, 790, 660, and 520 daltons) during the initial stages of this hydrolysis, the higher members disappearing after hydrolysis had been continued for 23 h (see Table II and Fig. 3).

From a partial hydrolysis of the polysaccharide with acid were isolated an aldobiouronic acid [2-*O*-(β-D-glucopyranosyluronic acid)-D-mannose], an aldotriouronic acid [*O*-(β-D-glucopyranosyluronic acid)-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-D-mannose], and an aldotetraouronic acid [*O*-(β-D-glucopyranosyluronic acid)-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-α-D-mannopyranosyl-(1→3)-D-glucose], each of

TABLE I

PARTIAL HYDROLYSIS OF *Klebsiella* K7 CAPSULAR POLYSACCHARIDE IN 5mM SULFURIC ACID

Time (*h*)	$M_w{}^a$ (*daltons*)	$[\alpha]_D{}^b$ (*degrees*)	*Degree of*[c] *scission* (*Y*)	$10^6 k^d$ (*sec*$^{-1}$)
0	1,200,000	+40		
				13
1	740,000	+42	0.05	
				13
3	270,000	+50	0.16	
				12
6	6,400	+44	0.26	
				8.5
9	3,200	+35	0.32	
				3.9
24	1,700	+31	0.45	
				1.3
48	1,300	+31	0.51	

[a] Weight-average molecular weight, from gel chromatography of material fractionated by gel. [b] Concentration = 0.48%. [c] Calculated from reducing power relative to polysaccharide hydrolyzed in M sulfuric acid for 18 h at 100°, denoted by Y. [d] Average values of first-order, hydrolysis rate-constant over the time intervals indicated, using appropriate values of Y.

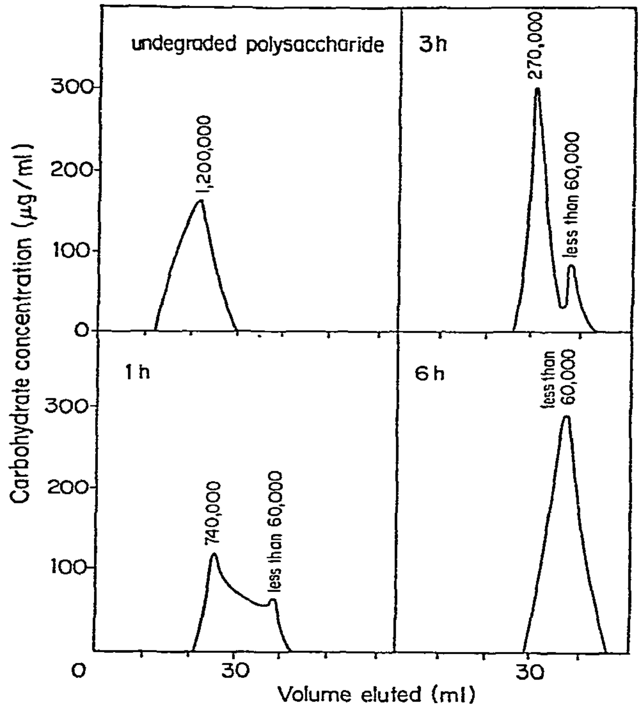

Fig. 1. Agarose elution patterns of *Klebsiella* K7 polysaccharide and hydrolyzates (5mM sulfuric acid at 100°).

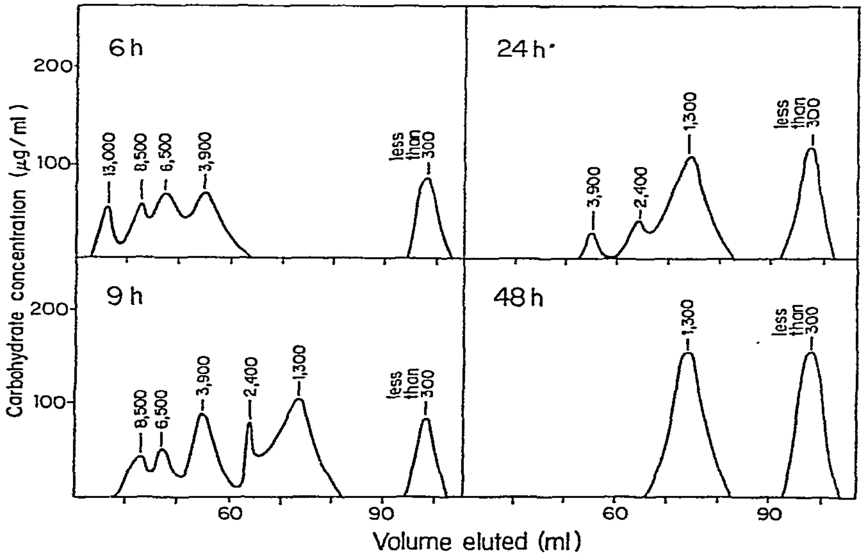

Fig. 2. Bio-Gel P-10 elution patterns of K7 hydrolyzates (6–48 h).

TABLE II

FURTHER HYDROLYSIS OF DEGRADED POLYSACCHARIDE IN 0.05M SULFURIC ACID

Time (*h*)	$[\alpha]_D$[a] (*degrees*)	*Degree of scission* (*Y*)	10^6k (sec^{-1})
0	+31	0.51	
			180
1	+29	0.74	
			30
3	+28	0.78	
			24
6	+27	0.84	
			0.9
23	+25	0.85	

[a] Concentration = 0.44%.

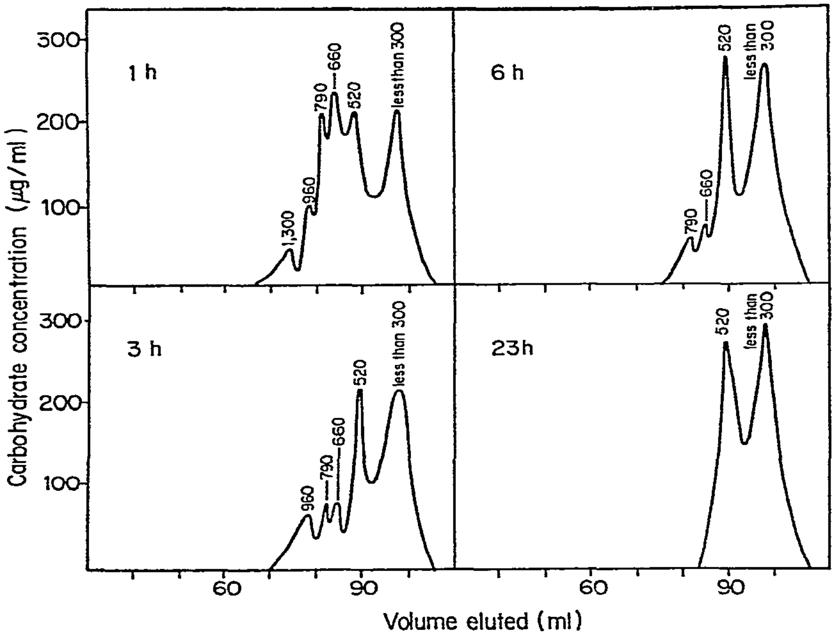

Fig. 3. Bio-Gel P-10 elution patterns of K7 hydrolyzates (0.05M sulfuric acid for 1 to 23 h at 100°).

which was homogeneous by the criteria of paper and gel-permeation chromatography. The structures of the compounds were proved by standard techniques, including partial-hydrolysis studies and methylation analysis, and the use of p.m.r. spectroscopy and measurement of optical rotatory power to determine configuration of anomeric linkages. (That each constituent sugar was of the D configuration was demonstrated by the procedures outlined later).

The ratios of sugars found by quantitative analysis (by use of the Technicon AutoAnalyzer system, borate buffers at pH 7, and type S Chromo-Beads) of a hydrolyzate of the polysaccharide (so prepared as to obtain maximal bond-cleavage without decomposition of the sugars released[9]) were 3:2:4 for mannose, galactose, and glucose, respectively. Analysis[1,10], by gas–liquid chromatography (g.l.c.), of the mixture of derived alditol acetates supported these figures. However, by gel-permeation chromatography on Bio-Gel P-2, and assaying of fractions by the phenol–sulfuric acid method[11], an appreciable proportion (28 mol%) of aldobiouronic acid surviving in the hydrolyzate analyzed was detected; this necessitated modification of the sugar ratios to 3.3:1:2 for mannose:galactose:glucose in order to allow for the mannose remaining bound to glucuronic acid. The individual sugars were identified as crystalline derivatives after manipulation[1] of their methyl ethers derived from the methylated polysaccharide, as outlined next.

Methylated K7 polysaccharide was hydrolyzed, and a portion of the hydrolyzate was analyzed by paper chromatography and by g.l.c. of the trimethylsilylated, derived alditols (with use of characteristic retention-times and response factors determined for the constituent, methylated neutral sugars[12]). The approximate molar proportions found were: 2,3,4,6-tetra-*O*-methylgalactose (1.0), 2,4,6-tri-*O*-methylglucose plus 3,4,6-tri-*O*-methylmannose (2.1), 4,6-di-*O*-methylmannose (0.8), and 2-*O*-methylglucose (1.0). Then, on a larger scale, methylated neutral sugars were separated from methylated acidic sugars by means of ion-exchangers.

Part of the methylated, neutral-sugar mixture from hydrolyzed, methylated K7 polysaccharide was reduced, the alditols acetylated, and the acetates separated into the component methylated alditol acetates[13] by preparative g.l.c. Neither of the stationary phases used (*a* or *b*; see ref. 1) was able to resolve the tri-*O*-methylhexitol mixture. Integrated peak areas (obtained by using an Infotronic electronic integrator) corresponded to values consistent with the figures already given for the analysis using trimethylsilyl ethers. The substitution patterns of the methylated alditol acetates were confirmed by mass spectrometry[14]. Portions of each of the four fractions collected from the gas-chromatographic effluent were *O*-demethylated and the product re-acetylated, yielding the hexa-*O*-acetyl derivatives of galactitol, mannitol + glucitol (in the ratio 1:1.7), mannitol, and glucitol, respectively, as shown by m.p., g.l.c. retention-times, and co-chromatography (in g.l.c.) with authentic specimens[15]. As described earlier[2], the group configuration of the galactose tetramethyl ether in the hydrolyzate of methylated K7 polysaccharide was shown to be D by circular dichroism measurement made on the derived, isolated galactitol tetramethyl ether diacetates dissolved in acetonitrile (acetate absorption at λ_{max} 213 nm). The mannose trimethyl ether was converted into its alditol acetate, the product separated, subjected to *O*-demethylation, and isolated as mannitol hexaacetate, which showed a positive c.d. spectrum, as did that of authentic D-mannitol hexaacetate; in the same way, the mannose dimethyl ether and the glucose tri- and mono-methyl ethers were each shown to have the D configuration.

The bulk of the methylated, neutral-sugar mixture was separated into its components by cellulose-column and thick-paper chromatography. 2,3,4,6-Tetra-*O*-methyl-D-galactose (affording a crystalline alditol, the trimethylsilyl derivative of which was identical in g.l.c. with an authentic specimen of this compound), crystalline 3,4,6-tri-*O*-methyl-D-mannose, 2,4,6-tri-*O*-methyl-D-glucose, and 2-*O*-methyl-D-glucose were thus isolated, together with syrupy 4,6-di-*O*-methyl-D-mannose whose identification was supported by p.m.r. spectroscopy[16,17] and by periodate oxidation–paper chromatography[18].

The methylated acidic sugars were converted into a mixture of the methyl ester methyl glycosides; these were ester-reduced with lithium aluminum hydride[19] and the products hydrolyzed. Paper chromatography of the hydrolyzate showed the presence of 3,4,6-tri-*O*-methylmannose (+), 2,4,6-tri-*O*-methylglucose (trace), 4,6-di-*O*-methylmannose (+ +), 2,4-di-*O*-methylglucose (+ + + +), and 2-*O*-methylglucose (+). A portion of the hydrolyzate was next reduced, the product acetylated, and the mixture of acetates analyzed by g.l.c. as for the mixture of methylated neutral sugars. The retention times of the five alditol derivatives, and the mass spectrum of the 2,4-di-*O*-methylglucitol tetraacetate, confirmed their characterization. Furthermore, *O*-demethylation and subsequent treatment proved that glucose was the parent sugar of the 2,4-di-*O*-methylglucitol tetraacetate, and c.d. measurement of the hexitol hexaacetate showed the glucose to be of the D configuration. It appears certain that the origin of the 2,4-di-*O*-methyl-D-glucose found as the major component in the hydrolyzate of the reduced, acidic fraction must be the glucuronic acid present in the original *Klebsiella* K7 polysaccharide; this acid must, accordingly, also be of the D configuration. An estimate by g.l.c.[13] of the ratios of the five methylated sugars derived from the acidic fraction, so expressed as to correspond as nearly as possible (on the basis of yields) with the ratios of methylated neutral sugars given earlier, is: 4:2:12:20:3.

Carboxyl-reduction of *Klebsiella* K7 polysaccharide by the methyl ester propionate–$LiBH_4$ method[20] gave a product having $[\alpha]_D$ +35° in which up to 80% of the acidic groups were converted into primary alcohol groups; acid hydrolysis afforded mannose, galactose, and glucose in the approximate ratios of 2:1:3. Methylation of the reduced polysaccharide and hydrolysis of the product gave 2,3,4,6-tetra-*O*-methylgalactose (1), 3,4,6-tri- (0.75) and 4,6-di-*O*-methylmannose (1.25), 2,4,6-tri- (2) and 2-*O*-mono-methylglucose (0.7), and a minor amount of (probably) 2,6-di-*O*-methylglucose, as determined by appropriate manipulation and g.l.c. of the derived alditol acetates. Corresponding reduction of K7 polysaccharide with $LiBD_4$ was more successful, acid hydrolysis yielding mannose, galactose, and glucose, and no detectable acidic sugars. From the derived alditol acetates, deuterated glucitol hexaacetate was isolated by g.l.c.; its mass spectrum, in comparison with that of authentic D-glucitol hexaacetate[21], showed enhanced primary, secondary, and tertiary peaks corresponding to deuterium-labelled fragments, thus proving conclusively the presence, in substantial proportion, of glucuronic acid in the original, unreduced polysaccharide.

Nimmich's identification[22] of the component sugars of *Klebsiella* K7 (*aerogenes*

4140) polysaccharide as galactose, glucose, mannose, and glucuronic acid has been confirmed, and it is now established that each is of the D configuration. The p.m.r. data obtained support the paper-chromatographic evidence for the presence of pyruvic acid acetal residues, one per (approximately) each eight sugar residues; the acetal system, furthermore, survives the processes of carboxyl-group reduction and methylation. The evidence obtained from analysis of hydrolyzates, and from methylation studies on the methylated polysaccharide, the methylated reduced polysaccharide, and the permethylated derivatives of the oligosaccharides produced on hydrolysis, shows that the major component residues of the polysaccharide are, in the approximate proportions indicated, D-Gal*p*→ (1); →2)-D-Man*p*→ (3); $\rightarrow 2)\text{-D-}\underset{\substack{3\\ \uparrow}}{\text{Man}p}\rightarrow$ (1); →3)-D-Glc*p*→ (1); $\rightarrow 3)\text{-D-}\underset{\substack{4\ \ 6\\ \uparrow\ \uparrow}}{\text{Glc}p}\rightarrow$ (1); and →3)-D-GlcA*p*→ (2).

The molar proportions shown are rounded off to the nearest whole number, and the acid is scaled up to two units; in addition to those shown, certain of the acid groups may be 3- and 4-linked branch-points. Paper chromatography of partial hydrolyzates of the polysaccharide and its reduced derivatives supported the presence of galactose residues as end-groups, and structural analysis of the only oligosaccharides at and below the tetrasaccharide level that were isolated from hydrolyzates afforded clear proof that the sequence of sugars →3)-β-D-GlcA*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-D-Glc*p*→ is repeated in a linear chain. For the pendant D-galactopyranose groups, the position of linkage that is best supported by all of the evidence is through O-3 of one in every two of the D-mannose residues that are themselves bound to glucuronic acid; this postulate is based upon the high proportion of 4,6-di-*O*-methylmannose found in the acid fraction of the hydrolyzate of methylated K7 polysaccharide. Pyruvic acid is placed as an acetal grouping engaging O-4 and O-6 of alternate, 3-linked D-glucopyranose residues in the chain. The possible stabilizing effect of this sequence upon contiguous glycosidic linkages has been noted[23], and it may account for the very high proportion of material of molecular weight 1,300 accumulating on partial hydrolysis of the polysaccharide.

From the work of this and other laboratories, there is evidence for a relatively simple pattern of sugars within repeating units in the molecular structures of the capsular polysaccharides from a considerable number of the 80 known *Klebsiella* K-types[1]. Despite the few component sugars present, no two structures have yet been found to be identical. Of the structural features indicated for the K7 polysaccharide, the mode of linkage of glucuronic acid to mannose differs from that present[24] in K24 and K26, but it is probably the same as that reported[25] for *Aerobacter aerogenes* (K-type unknown) polysaccharide; in the structure of the latter, however, the acidic group is pendant, and is not part of the linear chain. The mannose residues in K7 polysaccharide constitute both 2-linked chain units and branch points, linked at O-2 and O-3 as in many of the other related polysaccharides. Galactose has not been reported elsewhere as a terminal group, except in the sense that it is the nonreducing sugar, pyruvic acid being linked to it as an acetal, in K11 (ref. 26) and K21 (ref. 1) polysaccharides. O-3-Linked glucose residues occur in several other *Klebsiella* K-type polysaccharides, but the K7 polysaccharide is the first reported to exhibit the attachment of pyruvic acid to certain of these residues.

EXPERIMENTAL

General methods. — The chromatographic and spectroscopic methods used were as detailed in earlier papers[1,27,28]. The g.l.c. analysis of trimethylsilylated methyl ethers and derived alditols was conducted with a Beckman GC-4 chromatograph with flame-ionization detector, and glass columns (1.80 m × 6.25 mm o.d.) packed with 3% (w/w) of SE-52 on Chromosorb W (80–100 mesh), at a flow rate of helium carrier gas of 60 ml. min^{-1}, isothermally at 140° (ref. 12); that of methyl glycosides, with a Beckman GC-2A instrument, copper column (1 m × 6.25 mm o.d.) packed with 14% of poly(ethylene glycol succinate) on Chromosorb W (80–100 mesh), helium carrier gas, isothermally at 155° (ref. 29). Molecular-weight distributions were found from elution patterns obtained by gel-permeation chromatography on calibrated columns of Sagavac 6F (6% agarose), Bio-Gel P-10, or Bio Gel P-2, with M sodium chloride as the eluant[7,8], carbohydrate assay by the phenol–sulfuric acid method[11]. Preparative, gel-permeation chromatography was performed with a column (93 × 3.6 cm) packed with Bio-Gel P-10, with water as the eluant. The reducing power of hydrolyzates was determined by Nelson's method[30]. Optical rotations were determined for aqueous solutions, unless stated otherwise.

Preparation and properties of Klebsiella *K7 polysaccharide.* — A culture of *Klebsiella* K7, obtained by courtesy of Dr. I. Ørskov (Copenhagen), was grown for 6 days on agar containing sucrose, yeast extract, and mineral salts; it was then scraped from the surface, treated with aqueous phenol, and added to ethanol. Portions of the stringy precipitate, composed of the polysaccharide desired and bacterial debris, were centrifuged, repeatedly if necessary, at concentrations of 0.1 to 0.5% in distilled water, at up to 55,000 *g*. The moderately clear, supernatant solutions were combined and freeze-dried. Precipitation of the acidic K7 polysaccharide from aqueous solution with cetavlon bromide, according to Nimmich's well established procedure[22], and deproteinization (Sevag)[31] gave *Klebsiella* K7 polysaccharide that had $[\alpha]_D$ +40°, equiv. wt. (as acid, by titration) ~500, and the sodium salt of which showed no absorption at 255 nm; i.r. spectrum (KBr disc), 1710 cm^{-1} (w) and 1600 cm^{-1} (s), indicating un-ionized and ionized carboxyl groups. A broad singlet at τ 8.35 in the 100-MHz p.m.r. spectrum (solution in D_2O) indicated pyruvate acetal (7% by weight, according to colorimetric assay[32] of a hydrolyzate). The purified polysaccharide had $\bar{M}_w$ 1.2 Mdaltons, and was monodisperse according to gel-permeation chromatography. Electrophoretograms on cellulose acetate strips at pH 3.0 and 8.8 showed a rate of migration consistent with the acid content of the polysaccharide[1].

Acid hydrolysis of the polysaccharide; molecular-weight distribution analysis. — A sample (1 g) of the polysaccharide in 5mM sulfuric acid (100 ml) was heated for

48 h at 100°, coagulated insoluble material being removed by centrifugation after 1 h. Samples (5 ml) were neutralized at intervals with barium carbonate, centrifuged, and the $[\alpha]_D$ and reducing power of each determined. Molecular-weight distributions of hydrolyzate samples were found from elution patterns obtained on gel-permeation chromatography of aliquots (each containing ~2 mg of carbohydrate), and further information concerning the lower molecular-weight components was sought by paper chromatography. After 48 h of hydrolysis, the acid concentration in the solution was adjusted to 0.05M by addition of 0.5M sulfuric acid, and heating was continued for a further 23 h, samples being removed and examined as described.

Sugar analysis. — A small sample (10 mg) of polysaccharide was hydrolyzed in a sealed ampoule, under nitrogen, in 2M trifluoroacetic acid (2 ml) for 8 h at 100°. A portion of this hydrolyzate was chromatographed on Bio-Gel P-2; the aldobiouronic acid present was well resolved from the monosaccharide, and its molar proportion could be estimated from the elution pattern obtained. The molar ratio of neutral sugars present was determined by analysis (Technicon AutoAnalyzer) of further portions of the hydrolyzate.

Isolation and examination of oligosaccharide of $\bar{M}_w$ 1,300 daltons. — The amorphous material separated by gel-permeation chromatography from lower molecular-weight products of partial acid hydrolysis had $[\alpha]_D$ +23° (*c* 0.82); its p.m.r. spectrum (in D_2O after deuterium exchange) indicated the presence of both α and β anomeric protons in similar proportions. The fully methylated (i.r. evidence) derivative, $[\alpha]_D$ +21° (*c* 5.3, chloroform), gave, upon hydrolysis at 100° (first in 90% formic acid and then, for 5 h, in 2M trifluoroacetic acid), the neutral sugars 2,4,6-tri-*O*-methylglucose and 3,4,6-tri-*O*-methylmannose (paper chromatography, and g.l.c. of derived acetylated alditols), and an acidic fraction which was separated from the neutral one by ion-exchange chromatography. The acidic material was converted into methyl ester methyl glycosides which were reduced with lithium aluminum hydride, and the product hydrolyzed, yielding the neutral sugars 2,4-di-*O*-methylglucose and 2,3,4-tri-*O*-methylglucose (both from the glucuronic acid moiety), and 3,4,6-tri-*O*-methylmannose, all of which were observed by paper chromatography. Reduction, conversion into the acetates, and g.l.c.–mass spectrometry confirmed the presence of these sugars (ratios 1.8:1:0.4), but also indicated the presence of 2-*O*-methylglucose (1.1).

Isolation and characterization of aldo-bio-, -trio-, and -tetrao-uronic acids. — Neutral and acid products of the hydrolysis (0.1M trifluoroacetic acid for 25 h at 100°) of a sample (1.1 g) of the polysaccharide were separated on ion-exchangers, yielding, after suitable application to and elution [solvent: 2:1:1 (v/v) 1-butanol–acetic acid–water] from thick paper sheets, the following fractions.

(i) Aldobiouronic acid, R_{Glc} 0.60, $[\alpha]_D$ −25° (*c* 1.0), whose methylated derivative, on methanolysis and g.l.c., gave methyl glycosides of methyl 2,3,4-tri-*O*-methylglucuronate (removed by saponification) and 3,4,6-tri-*O*-methylmannose in the same proportions as were given upon methanolysis of methylated 2-*O*-(β-D-glucopyranosyluronic acid)-D-mannose. The identity of the 3,4,6-tri-*O*-methylmannose was confirmed by hydrolysis of the methylated aldobiouronic acid, and g.l.c. of the trimethylsilyl derivatives of the anomers of the tri-*O*-methyl sugar.

(ii) Aldotriouronic acid, R_{Glc} 0.37, $[\alpha]_D$ $-5°$ (*c* 2.5), homogeneous in gel-permeation chromatography on Bio-Gel P-10; after reduction with sodium borohydride, hydrolysis, and treatment for 4 h in hot M sodium hydroxide under oxygen, a portion of this yielded mannitol (identified by g.l.c. of its hexaacetate). Hydrolysis of the aldotriouronic acid afforded mannose, glucuronic acid, glucuronolactone, and aldobiouronic acid (i) (paper chromatography); permethylation gave a derivative, $[\alpha]_D$ $-4°$ (*c* 2.0, chloroform), which, on hydrolysis, afforded 3,4,6-tri-*O*-methylmannose (identified by paper chromatography, and by g.l.c. of the trimethylsilyl ethers) as the only neutral sugar. The methylated aldotriouronic acid (which was purified on a short column of Merckogel OR-PVA 500, methanol eluant) gave, on methanolysis, methyl glycosides of methyl 2,3,4-tri-*O*-methylglucuronate and 3,4,6-tri-*O*-methylmannose. The p.m.r. spectrum in D_2O of the borohydride-reduced aldotriouronic acid (after deuterium exchange) showed anomeric signals consistent with those expected from D-glucuronic acid (β) and D-mannose (α) moieties.

(iii) Aldotetraouronic acid, R_{Glc} 0.22, $[\alpha]_D$ $+35°$ (*c* 4.1), homogeneous in Bio-Gel P-10 chromatography. The reducing end-group was glucose, as shown by borohydride reduction of the tetraouronic acid, hydrolysis, oxidative decomposition of the sugars in hot alkali, and conversion of the glucitol that remained into its hexaacetate. Graded, acid hydrolysis of the reduced tetraouronic acid yielded the trio- and bio-uronic acids and mannose (paper chromatography). Methylated aldotetraouronic acid, $[\alpha]_D$ $+15°$ (*c* 2, chloroform), purified on Merckogel OR-PVA 500 as already described, gave, on methanolysis and g.l.c., the methyl glycosides of 2,4,6-tri-*O*-methylglucose, 3,4,6-tri-*O*-methylmannose, and methyl 2,3,4-tri-*O*-methylglucuronate. Its hydrolyzate was shown (by paper chromatography) to contain 2,4,6-tri-*O*-methylglucose, 3,4,6-tri-*O*-methylmannose, and methylated acid, the identity of the neutral sugars being confirmed by g.l.c. of their trimethylsilyl derivatives. The anomeric signals observed in the p.m.r. spectrum (in D_2O) of the reduced aldotetraouronic acid (after deuterium exchange) indicated the presence of a β-linked glucuronic acid moiety, and, separated from each other by 0.05 p.p.m., two broadened signals for the α-D-mannosyl linkages.

Methylation analysis. — A portion (0.70 g) of *Klebsiella* K7 polysaccharide, in the acid form, was methylated once by Hakomori's method[33,34] and four times by the method of Purdie and Irvine[35] to give, in good yield, a colorless, amorphous product, $[\alpha]_D$ $+48°$ (*c* 1.2, chloroform), which showed no absorption at 3700–3200 (OH), and strong absorption at 1740 cm^{-1} (methyl ester CO); pyruvate acetal singlet at τ 8.60 ($CHCl_3$ solution). The methylated polysaccharide was hydrolyzed by heating with 90% aqueous formic acid for 2 h and then with 0.5M sulfuric acid in a sealed tube under nitrogen for 6 h in a boiling-water bath. Neutral sugars (0.38 g) were separated from acidic sugars (0.15 g, recovered as methyl ester methyl glycosides) by ion-exchange chromatography on a column of Duolite A4 (OH^-) resin.

The bulk (0.3 g) of the neutral-sugar mixture was applied to a column (65 ×

4 cm) of cellulose, water-jacketed at 28°, and separated into its components by elution with butanone–water azeotrope, followed by aqueous acetone. Where necessary, mixtures were re-separated by use of thick-paper chromatography, and further characterized as follows.

Fraction I (53 mg), $[\alpha]_D$ +100° (*c* 1.3), was chromatographically identical with 2,3,4,6-tetra-*O*-methyl-D-galactose, and, on being reduced, afforded a crystalline alditol on nucleation (in cyclohexane solution) with 2,3,4,6-tetra-*O*-methyl-D-galactitol; the trimethylsilylated alditol was identical in its retention characteristics in g.l.c. with an authentic sample of this derivative.

Fraction II (21 mg), on reduction and trimethylsilylation, gave derivatives of tetra-*O*-methylgalactose and 3,4,6-tri-*O*-methylmannose (see Fraction III), in the ratio 4:1 according to g.l.c.

Fraction III. From the syrup (37 mg), colorless prisms, m.p. 104–105°, of 3,4,6-tri-*O*-methyl-D-mannose were obtained by extracting with ether and nucleating with the authentic sugar.

Fraction IV (4 mg) was converted into the trimethylsilyl derivatives of 3,4,6-tri-*O*-methylmannose and 2,4,6-tri-*O*-methylglucose, as indicated by g.l.c.

Fraction V. The crystalline sugar (50 mg) had m.p. (from ether–methanol) 110–111°, undepressed by admixture with a sample of 2,4,6-tri-*O*-methyl-D-glucose (m.p. 114–121°). The trimethylsilylated, derived alditol co-chromatographed (on g.l.c.) with authentic material.

Fraction VI (36 mg), $[\alpha]_D$ +15° (*c* 2.0), consisted of 4,6-di-*O*-methyl-D-mannose containing a small proportion of 2,4,6-tri-*O*-methylgalactose as judged by g.l.c. of the derived, trimethylsilylated alditols. The p.m.r. spectrum (in D_2O) showed clear evidence of mannose as the parent sugar, and two well-separated signals for the methoxyl groups, which, in turn, were not resolved into α and β components[17]. Periodate oxidation of the syrupy sugar mixture gave the anticipated product[18], as shown by paper chromatography.

Fraction VII. Crystalline 2-*O*-methyl-D-glucose (55 mg), m.p. 159°, was further characterized by (*a*) the production of methoxymalondialdehyde on periodate oxidation[18], and (*b*) its p.m.r. spectrum (in D_2O), which showed widely-spaced (12.5 Hz) methoxyl signals for the anomeric forms[16]. Traces of glucose and acidic sugars were eluted finally from the column.

A sample (30 mg) of the methylated polysaccharide in 2M trifluoroacetic acid was heated for 8 h at 100° in a sealed tube under nitrogen. The products were reduced, trimethylsilylated, and analyzed by g.l.c. (using characteristic retention times and response factors determined for the constituent sugars). The analytical figures agreed well with the recoveries of methylated sugar from the cellulose-column separation.

Reduction of the polysaccharide. — Polysaccharide (as sodium salt, 1 g) was converted into the propionate and, thence, into the propionate methyl ester (1.47 g), one portion of which was reduced in tetrahydrofuran with $LiBH_4$, and the other with $LiBD_4$ (98% Koch–Light Co.)[20].

Methylation of the reduced polysaccharide (0.20 g) by the methods of Hakomori[33,34], Kuhn[36], and Purdie and Irvine[35] (twice) gave the methylated reduced product (0.10 g), $[\alpha]_D$ +39° (*c* 1.4, chloroform), free from ester and hydroxyl groups according to the i.r. spectrum.

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