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THE STRUCTURE OF *Klebsiella* SEROTYPE 11 CAPSULAR POLYSACCHARIDE*

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ABSTRACT

Using periodate oxidation, methylation analysis, the characterization of oligosaccharides obtained by partial acid hydrolysis, p m r spectroscopy, and analytical ultracentrifugation, the structure of the (mildly alkali-treated) *Klebsiella* serotype 11 capsular polysaccharide has been elucidated. The tetrasaccharide repeating-unit comprises the sequence →3)-β-D-Glc*p*-(1→3)-β-D-GlcUA*p*-(1→3)-α-D-Gal*p*-(1→ with a 4,6-*O*-(1-carboxyethylidene)-α-D-galactosyl residue linked to O-4 of the glucuronic acid residue The structural basis for some serological cross-reactions of the *Klebsiella* K11 antigen is discussed, and it is shown that rabbit antisera against the *Klebsiella* K11 test-strain predominantly contain K agglutinins specific for branch-terminal 4,6-*O*-(1-carboxyethylidene)-D-galactose

INTRODUCTION

About 80 *Klebsiella* K serotypes have been recognized[1,2] Of the capsular polysaccharides carrying the determinants for these different specificities[3], 15 have been subjected to a detailed structural analysis, and 12 of these structures have been published[4–19] Another 24 *Klebsiella* capsular polysaccharides are under investigation[20–22]

Although substituted to a varying degree by pyruvate acetal, *O*-acetyl, *O*-formyl, and, in one case[14], 3-deoxy-L-pentulosonic acid residues, these glycans appear to differ by only rather limited variations of the polysaccharide skeleton Most contain either D-glucuronic or, rarely, D-galacturonic acid, as well as 2–4 of the following aldohexoses D-glucose, D-galactose, D-mannose, L-rhamnose, and L-fucose[3]. Because of this basic similarity, *Klebsiella* capsular glycans of known structure are suitable for a variety of comparative studies, *e g*, by p m r spectroscopy[4,7,19], on the substrate specificity of bacteriophage-borne enzymes[23–27],

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on the structural basis for the serological cross-reactions amongst different *Klebsiella* K antigens[1,28] and between them and surface antigens of other bacteria[29–34], and, probably, on polysaccharide conformation

For these reasons, we have analysed the structure of another *Klebsiella* capsular glycan, the serotype-11 polysaccharide This was of special interest because it is the substrate of the bacteriophage-borne glycanase described in the following paper[27], and because it has been reported[1 28 34] to exhibit serological cross-reactions with some other bacterial exopolysaccharides of known structure[12,35,36]

MATERIAL AND METHODS

Media and PBS — $D_{1\ 5}$ agar[37] was used for plates, and Merck standard I broth for fluid cultures Phosphate-buffered physiological saline (PBS) of pH 7 was prepared by dissolving 8 5 g of NaCl, 1 76 g of $Na_2HPO_4\ 12H_2O$, and 0 1 g of KH_2PO_4 in 1 litre of water, with addition of 0 5 g of NaN_3 for sterility

Bacterial strains — For the isolation of serotype-11 capsular polysaccharide, *Klebsiella* 390 (03·K11) was used It was isolated by Kauffmann[29] from urine, serves as a serological test-strain for the K11 antigen[1 3 38], and is the host of *Klebsiella* bacteriophage No 11 (see following paper[27]) Since the *Klebsiella* 03 and *Escherichia coli* 09 antigens are serologically identical[29], *E coli* Bi316/42[39] (09 K9(L) H12) was employed for the preparation of O sera For determination of serological cross-reactions with the *Klebsiella* K11 antigen, *E coli* Bi161/42[39 40] (09 K29(A) H⁻) and E69[41,42] (09 K30(A) H12) were also used All strains were kindly supplied by Dr Ida Ørskov, WHO International *Escherichia* Center, Statens Seruminstitut, Copenhagen

Isolation and serological identification of Klebsiella *serotype 11 capsular polysaccharide* — *Klebsiella* 390 was grown on $D_{1\ 5}$ agar (48 h at 37°, and then 48 h at room temperature), washed off the plates with PBS containing 2% (w/v) of phenol, precipitated with ethanol, and acetone-dried[43] The dead and dry organisms were extracted with phenol–water, the water phase was ultracentrifuged twice (4 h at 105,000 *g*) to sediment cell-wall lipopolysaccharide, and the acidic serotype-11 polysaccharide was isolated from the supernatant by fractional (0 25→0 06M NaCl) precipitation[43 44] with Cetavlon (cetyltrimethylammonium bromide) To obtain the sodium salt of the polymer, the Cetavlon salt was repeatedly dissolved in M aqueous NaCl and precipitated with ethanol[43] The final product was dialysed against distilled water and lyophilized In this manner, 37 g of dry bacteria, and thence 2 6 g (7%) of type-11 polysaccharide were obtained from 100 large (14-cm diameter) $D_{1\ 5}$ agar-plates

Native type-11 polysaccharide was mildly alkali-treated as follows A 0 25% (w/v) solution of the native material in 0 25M aqueous NaOH was kept at 56° for 2 h The mixture was then cooled with ice, neutralized with M HCl, dialysed, and lyophilized

Agar-gel double diffusion was carried out according to Ouchterlony[43,45], using 0 5% (w/v) solutions of type-11 polysaccharide (native or alkali-treated) in

PBS and undiluted *Klebsiella* 03 K11 (K agglutination titre = 1 1280) and *E coli* 09 (O titre = 1 5120) rabbit antisera obtained by standard procedures[38] with *Klebsiella* 390 (formalin-killed) and *E coli* Bi316/42 (boiled) as antigens

Homogeneity controls and physical parameters — The sedimentation analyses were carried out in a Spinco model E analytical ultracentrifuge using the An-H-Ti rotor and Schlieren optics For the determination of the sedimentation coefficient ($s^0_{20,w}$) of alkali-treated type-11 polymer, solutions containing 0 3% (w/v) and less in PBS were sedimented, and the values obtained were extrapolated to infinite dilution and standard conditions[46]

For the determination of the weight-average molecular weight ($\overline{M}_w$), solutions of the alkali-treated glycan (0 25% w/v or less of the sodium salt in PBS) were equilibrated [17 h at 8,000 r p m (4650 *g*), or 24 h at 10,000 r p m (7270 *g*)], and the apparent molecular weights were calculated from the partial specific volume and the sedimentation equilibrium according to Yphantis[47], and then extrapolated to infinite dilution The density of solvent and solutions, and thence the partial specific volume (V_p) were determined by the method of Stabinger *et al* [48] using the equipment manufactured by Parr (Graz, Austria)

The limiting-viscosity number, $[\eta] = \lim_{c \to 0} \frac{\eta - \eta_0}{\eta_0 \times c}$ (ml/g) of native and alkali-treated type-11 polysaccharide (sodium salts) was determined at 37° (±0 1°), using an Ostwald viscosimeter[49] and omitting the extrapolation of the shear gradient (G) to zero[50] With PBS as a solvent, these polyelectrolytes did not show an increase in the viscosity number at low concentrations[50]

Derivatives of type-11 polysaccharide — For autohydrolysis, a 1% aqueous solution of alkali-treated material was passed over a Dowex-50(H^+) column The solution then had pH 2 9 It was heated to 100° for 2 h, cooled, neutralized with dilute NaOH, dialysed against distilled water, and lyophilized Yield 80% of a material having 55% of the original pyruvate content For reduction of the carboxyl groups, the acidic form of alkali-treated polymer (60 mg) was esterified with diazomethane and reduced with sodium borohydride, essentially as described by Aspinall[51] for Type VIII *Pneumococcus* polysaccharide Yield 76% of a material in which 92% of the glucuronic acid, but only 23% of the pyruvate carboxyl-groups were reduced to primary hydroxyls (see Table I)

Constituent analyses of type-11 polysaccharide, its derivatives, and degradation products — The qualitative, constituent analysis of the native glycan was carried out as follows A 1% solution in 0 5M H_2SO_4 was hydrolyzed for 8 h at 100°, and the hydrolysate was neutralized with $Ba(OH)_2$ Glucose, galactose, and mannose were identified by descending p c , using Whatman No 1 paper, 1-butanol–pyridine–water (6 4 3), and the staining method of Trevelyan *et al* [52] D-Glucose and D-galactose were also identified enzymically (see below) Using high-voltage paper electrophoresis[53] (45 V/cm for 2–3 h) on Schleicher & Schull (Dassel, Germany) paper No 2043a, in pyridine–glacial acetic acid–water (10 4 86, pH 5 3), and staining

as above, a hexuronic acid and pyruvate were recognized For further identification, larger amounts were separated on No 2043b paper and the two compounds were isolated Following the details given by Jann *et al* [43], the hexuronic acid was sequentially refluxed with methanolic HCl, reduced with $NaBH_4$, and hydrolysed The hexose thus obtained was identified as D-glucose by p c and enzymically (see below) The pyruvate was identified as its 2,4-dinitrophenylhydrazone[54], m p 216°. After hydroxylaminolysis[55] of native type-11 polysaccharide under the conditions given by Jann *et al* [43], acetyl hydroxamate could be identified by p c , using water-saturated 1-butanol as an irrigant, and $FeCl_3$ in the same solvent for detection

The quantitative constituent analyses were performed in the following manner On hydrolysis of type-11 polysaccharide with 0 5M H_2SO_4 at 100° [neutralization with dry Amberlite IR-410(HCO_3^-) resin], the maximum of free glucose was attained after 4 h, and the maximum of galactose after 15 h, 4 h in 0 5M HCl were sufficient to liberate all of the pyruvate After addition of xylose as an internal standard, the hydrolysates thus obtained (from the native polymer, its derivatives, or its degradation products, see below) were reduced with $NaBH_4$, acetylated, and the alditol acetates were analysed by g l c [56], using a Varian Aerograph (model 1502, equipped with a flame-ionization detector and a digital integrator, model 477), glass columns (80 × 0 125 in) filled with 3% (w/w) ECNSS-M on Chromosorb G (80–100 mesh), a starting temperature of 165° and a temperature increment of 0 5°/min, and nitrogen (30 ml/min) as a carrier gas In the hydrolysates, D-glucose was also determined enzymically with fungal D-glucose oxidase (EC 1 1 3 4)[57 58], using the Boehringer (Mannheim, Germany) No 15982 test combination, and D-galactose with D-galactose dehydrogenase (EC 1 1 1 48) from *Pseudomonas fluorescens*[59] (Boehringer, No 15095) LDH (lactate dehydrogenase, EC 1 1 1 27) from hog muscle (Boehringer, No 15373) served for the estimation[58,60] of pyruvate Both *O*-acetyl and hexuronic acid were determined in unhydrolysed material by the technique of Ludowieg and Dorfman[61], and the carbazole–sulfuric acid method[62], respectively

Periodate oxidation — The periodate consumption of type-11 polysaccharide (native or alkali-treated) was determined by the method of Avigad[63] The polyaldehyde (100 mg) obtained upon complete oxidation of alkali-treated polymer (1% plus 11 5mM aqueous $NaIO_4$, kept for 24 h at room temperature in the dark) was reduced with $NaBH_4$ (40 mg in 0 01M NaOH, 18 h at 4°), and 50 mg of the polyalcohol thus produced were subjected to mild, acid hydrolysis (0 125M H_2SO_4, 24 h, 37°) (Smith degradation[64]) Neutralization, dialysis against distilled water, and lyophilization yielded 36 mg (72%, from the polyalcohol) of product Amongst the alditol acetates obtained from the polyalcohol, threitol acetate was identified by g l c , using threitol, erythritol, and glycerol acetate as standards

Methylation analysis — Alkali-treated type-11 polysaccharide was esterified with diazomethane and reduced with $NaBD_4$ in D_2O as described above Both the reduced and the partially depyruvylated (autohydrolysed, see above) polymer (10–20-mg portions) were methylated by the method of Hakomori[65] following the experimental details given by Hellerqvist *et al.*[66] The dry products were taken up in

chloroform (5 ml), and the solution was washed with water and passed over a Sephadex LH-20 column[67] (50 cm × 1 75 cm^2, elution at 6 ml/h with ethanol–chloroform, 2 1) The carbohydrate-containing fractions appearing in the void volume were located with phenol–sulfuric acid[68], combined, and concentrated to dryness The completeness of methylation was checked by subjecting a solution in dry chloroform (~10%) to i r spectroscopy with a Perkin–Elmer model 137 instrument (no absorption at 3400–3600 cm^{-1}) The methylated products were hydrolysed (3 h with 90% formic acid, and then 14 h with 0 125M H_2SO_4 at 100°)[66], reduced with $NaBH_4$, acetylated, and subjected to g l c –m s [69–72] The determination of retention times was performed with a Varian Aerograph instrument and ECNSS-M columns (see above) at a constant temperature of 170°, using 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl- (T = 1 00) and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol (T = 5 39) as internal standards The mass spectrometry was carried out with a Perkin–Elmer Model-270B combined g l c –m s instrument equipped with a Honeywell visicorder (model 3508), ECNSS-M columns were used (starting temperature 165°, temperature increment. 1°/min). Helium was used as a carrier gas The ionization potential was 70 eV, the ionization current 80 μA, and the temperature of the ion-source was 180°

Oligosaccharide analysis — Type-11 polysaccharide was partially hydrolysed with 0 5M H_2SO_4 at 100° (4 h to obtain the aldobiouronic acid *H3*. 1 h for the other oligosaccharides, see Fig 1) After neutralization with $Ba(OH)_2$, the acidic oligosaccharides were separated by paper electrophoresis in pyridine–glacial acetic acid–water (see above), and then sub-fractionated by p c with 1-butanol–glacial acetic acid–water (5 2 1) as irrigant, using Schleicher & Schull No 2043b or Whatman 3MM paper, respectively The oligosaccharides thus purified were eluted from the paper (for yields, see Fig 1) and analysed as follows

The qualitative and quantitative constituent analyses, before or after reduction with $NaBH_4$ or periodate oxidation, were carried out as described above Portions (1–3 mg) of the free oligosaccharides were methylated directly (without loss of reducing-end sugar equivalents), essentially as described above, taking care, however, to expose them for not more than 60 min to the methylsulphinyl sodium reagent (before addition of methyl iodide)[66] The dry, methylated methyl glycosides obtained were purified by passage over a column (30 cm × 0 8 cm^2) of Merckogel OR PVA 500 (Merck), with elution by 3 ml of methanol/h[67]

The following procedures were used for the hydrolysis of oligosaccharides by exo-glycosidases Incubations with α-D-glucosidase from yeast (maltase, EC 3 2 1 20, Boehringer, No 15018) were carried out for 7 h in a pH 7 phosphate buffer[73], and those with β-D-glucosidase from sweet almonds (EC 3 2 1 21, Boehringer, No 15399) for 2 h in a pH 5 sodium acetate buffer[58,74] The D-glucose liberated was then determined with D-glucose oxidase (see above), using maltose and cellobiose for controlling anomeric specificity and completeness of hydrolysis In the same manner, α-D-galactosidase from green coffee-beans (EC 3 2 1 22, Boehringer, No 15236), and β-D-galactosidase from *E coli* (EC 3 2 1 23, Boehringer, No 15079) were applied under appropriate conditions[75,76], and the galactose liberated was estimated with

galactose dehydrogenase (see above), using lactose and melibiose as controls Incubations with β-D-glucuronidase from *Helix pomatia* (EC 3 2 1 31, Boehringer, No. 15472; free of α-D-glucuronidase activity[42 58]) were carried out in a pH 4 5 acetate buffer[42,58,77], demonstrating the liberation of glucuronic acid by paper electrophoresis (see above)

The paper-electrophoretic analysis of partially hydrolysed, tritium-labelled oligosaccharide *H5 1* was done as follows At 4°, 20 μl of 0 01M NaOH, containing 500 μCi of $NaBH_4/NaBT_4$ (specific activity 480 μCi/μmole) were added to solutions of oligosaccharides *H3*, *H5 1*, and *H5 3* (~1 mg of each, in 0 01M NaOH), and the mixtures were kept at 4° overnight After decomposition of excess reducing agent with dilute acetic acid, boric acid was removed by evaporation with methanol, and a portion of the reduced *H5.1* was partially hydrolysed (0 5M H_2SO_4, 20 min, 100°) All radioactive products were subjected to paper electrophoresis in pyridine–glacial acetic acid–water (see above), and the electropherogram strips were analysed with a Packard radiogram scanner

P m r spectroscopy — The p m r spectra of native *Klebsiella* type 11 polysaccharide, and of the polymer obtained after Smith degradation (see above) in absolute D_2O (2–3%) were run at 90 to 95°, using a Varian HR 220 instrument at 220 MHz, with the sodium salt of 3-trimethylsilylpropanesulfonic acid as an internal standard

Serological cross-reactions. — The standard procedures described by Kauffmann[38] were used for the immunization of rabbits, as well as for bacterial slide and tube agglutinations

RESULTS

Isolation, serological identification, homogeneity, and physical properties of Klebsiella *serotype 11 capsular polysaccharide* — As expected[42 43] for a material carrying the serological *Klebsiella* K11 determinants, type-11 polysaccharide, as isolated from *Klebsiella* 390 (03 K11) by the phenol–water–Cetavlon technique[43 44], yielded a precipitation reaction in Ouchterlony agar gel double-diffusion[45] against a *Klebsiella* 390 OK serum—one precipitation line with alkali-treated, two (hardly separated) with native material—but none against an *E coli* 09 serum (although the *Klebsiella* 03 and *E coli* 09 antigens are serologically identical[29]) In the analytical ultracentrifuge, native type-11 polysaccharide (0 4% in PBS) showed a double peak with $s^c_{25,\,solv} = 2\,8 \times 10^{-13}$ and $3\,0 \times 10^{-13}$ sec After mild alkali-treatment, however, homogeneous sedimentation ($s^c_{25\ solv} = 2.9 \times 10^{-13}$ sec) was observed For the alkali-treated product, a sedimentation coefficient ($s^0_{20\ w}$) of 4.55×10^{-13} sec, a partial specific volume (V_p) of 0 6129 ml/g, a molecular weight ($\overline{M}_w$) of 298,000, $[\alpha]_D^{25}$ +106° (*c* 1 0, water), and a limiting viscosity number ([η], at 37° in PBS) of 290 ml/g (against 620 ml/g for the native material) were determined.

Constituents — Qualitative analysis of type-11 polysaccharide showed the presence of D-glucuronic acid, D-glucose, D-galactose, acetate, pyruvate, and trace

amounts of mannose, in agreement with the results reported by Nimmich[3] The quantitative composition of the material is given in Table I; it approaches molar ratios of Gal Glc GlcUA pyruvate acetate of 2 1 1 1.0 5, if the trace amounts of mannose are considered to be due to a small contamination with *Klebsiella* 03 cell-wall lipopolysaccharide, which is known to contain large proportions of this sugar[20 78] The analysis of type-11 polysaccharide after esterification with diazomethane and subsequent reduction with sodium borohydride is also included in Table I, 92% of the glucuronic acid, but only 23% of the pyruvic acid carboxyl-groups were reduced by this procedure (possibly due to more successful competition of hydrolysis during reduction of the latter)

Periodate oxidation — Both the native and the alkali-treated polymer consumed 0 8 mole of periodate per mole of glucose Periodate-oxidized material was quantitatively analysed for loss of original constituents before and after reduction with sodium borohydride and "Smith hydrolysis"[64] (Table I) The results show that approximately one mole of galactose per mole of glucose was destroyed by periodate The material obtained after "Smith hydrolysis", which was essentially non-dialysable (72% yield from the polyalcohol), had also lost most of the pyruvate By g l c of the alditol acetates obtained from oxidized and then reduced type-11 polysaccharide, threose could be identified as one of the products formed by periodate treatment.

TABLE I

QUANTITATIVE COMPOSITION (PERCENT ANHYDRO RESIDUES AND THEIR MOLAR RATIOS BASED ON GLUCOSE) OF *Klebsiella* SEROTYPE 11 CAPSULAR POLYSACCHARIDE AND ITS DERIVATIVES

	I[a]	*II*	*III*	*IV*	*V*	*VI*
D-Glucose	17 4 ±0 5(1 00)	17 9(1 00)	34 2(2 00)	17 3(1 00)	17 2(1 00)	24 4(1 00)
D-Galactose	31 8 ±1 2(1 83)	32 4(1 81)	33 0(1 93)	19 4(1 12)	18 1(1 05)	24 9(1 02)
D-Glucuronic acid	20 6 ±0 8(1 09)	20 5(1 06)	1.7(0 09)	20 0(1 07)	20 7(1 11)	26 8(1 02)
Pyruvate[b]	6 9 ±0 3(0 93)	6 8(0 89)	5 3(0 72)	6 7(0 90)	6 9(0 94)	1 3(0 13)
Acetyl[b]	2 1 ±0 2(0 45)	0	0	n d	0	0
Total[c]	96 5					

[a]I, Native, II, alkali-treated, III, alkali-treated, esterified with diazomethane, and reduced with sodium borohydride, IV, native, oxidized with periodate, V, alkali-treated, and oxidized with periodate, VI, alkali-treated, oxidized with periodate, reduced with sodium borohydride, subjected to "Smith hydrolysis", and dialysed. [b]Calculated as CH_3–CO–COO^- and CH_3COOH minus H_2O [c]Including 2 5% mannose, 10 0% water (loss of weight after 24 h at 50° *in vacuo* over phosphorus pentaoxide), 0 2% nucleic acid (estimated from the absorption at 260 nm with yeast RNA as a standard), and 5 0% sodium (calculated on the basis of the glucuronic and pyruvic acid values)

Methylation analysis — Alkali-treated type-11 polysaccharide, esterified with diazomethane and then reduced with sodium borodeuteride in deuterium oxide, as well as partially autohydrolysed polymer (selective removal of 45% of the pyruvate substituents) were permethylated, and the methylated sugars obtained upon hydrolysis were analysed by g l c–m s (Table II) The results showed that the glycan contained

approximately equal amounts of 3-substituted glucose and galactose, 4,6-substituted galactose, and 3,4-substituted glucuronic acid residues This latter inference followed from the appearance of fragment *m/e* 47 (instead of *m/e* 45) in the mass spectrum of 2,6-Glc The results with the partially autohydrolysed polymer confirmed the substitution pattern of the glucuronic acid residues (disappearance of 2,6-Glc) and indicated that the pyruvate was linked to positions 4 and 6 of a branch, terminal galactose (partial disappearance of 2,3-Gal, and appearance of 2,3,4,6-Gal) The occurrence of 2,6-Gal in this chromatogram is discussed below

TABLE II

IDENTIFICATION AND RATIOS OF *O*-ACETYL-*O*-METHYLALDITOLS OBTAINED FROM PERMETHYLATED *Klebsiella* SEROTYPE 11 POLYSACCHARIDE DERIVATIVES AND OLIGOSACCHARIDES

Alditol derivative[a]	T[b]		*Primary fragments found* (m/e)						*I*[c]	*II*	*III*	*IV*
	Lit	*Found*	*45*	*117*	*161*	*205*	*233*	*261*	*Ratio of peak integrals*			
2,3,4,6-Gal	1 25	1 23	+	+	+	+			—	0 30	—	—
2,4,6-Glc	1 95	1 95	+	+	+		+		1 00[d]	1 00[d]	—	—
2,4,6-Gal	2 28	2 37	+	+	+		+		0 95	0 83	1 00[d]	—
2 6-Gal	3 65	3 62	+	+					—	0 18	—	0 28
2,6-Glc	3 83	3 78	+ (47)[e]	+					1 10	—	—	—
2,3-Gal	5 68	5 63		+	+			+	0 95	0 48	—	1 00[d]

[a]2,3,4,6-Gal = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, etc [b]Retention time, relative to that of 2,3,4,6-Glc ($T = 1\ 00$) and 2,3-Glc ($T = 5\ 39$) on an ECNSS-M column at 170°[69–72] [c]I, Alkali-treated type-11 polysaccharide, esterified with diazomethane, and reduced with sodium borodeuteride; II, alkali-treated type-11 polysaccharide, partially autohydrolysed (selective hydrolysis of 45% of the pyruvate residues), III, oligosaccharide *H3*, IV, oligosaccharide *H5 3* [d]Ratio of peak integrals based on these peaks [e]Doubly deuterated fragment found instead of the normal one

Oligosaccharide analysis — *Klebsiella* type-11 capsular polysaccharide was subjected to partial, acid hydrolysis, and the oligosaccharides obtained were separated by preparative paper electrophoresis Subsequent p c of the electrophoretic fractions (Fig 1) yielded sufficient amounts of some acidic oligosaccharides, which were analysed as follows

The aldobiouronic acid *H3* contained the most acid-resistant glycosidic linkage, and comprised glucuronic acid and galactose in the molar ratio 1 0 0 95 All of the galactose could be reduced with sodium borohydride, and β-D-glucuronidase split *H3* into the constituents G l c –m s of the alditol acetates obtained from permethylated *H3* led to the identification of 2,4.6-Gal (Table II) The structure of *H3* is thus β-D-GlcUA*p*-(1→3)-D-Gal.

Oligosaccharide *H2 1* contained glucose, glucuronic acid, and galactose in the molar ratios 1 0 1 0 0 8, all of the galactose being reducible by sodium borohydride As determined with D-glucose oxidase, 0 8 mole of D-glucose could be cleaved from *H2 1* by the action of β-D-glucosidase, whereas α-D-glucosidase had no effect Partial,

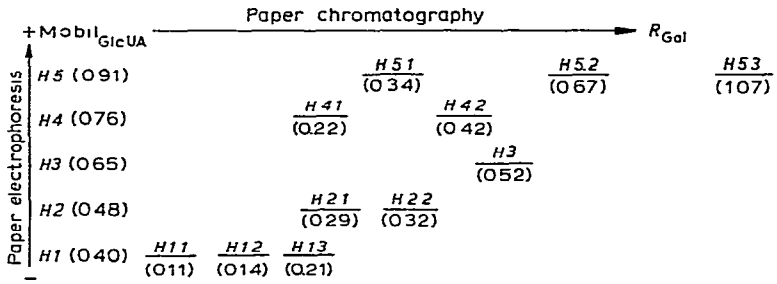
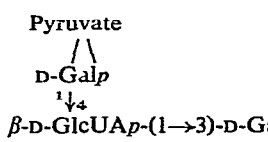


Fig 1 Separation of acidic oligosaccharides obtained from *Klebsiella* serotype 11 capsular polysaccharide by partial, acid hydrolysis Mobilities in relation to glucuronic acid, and p c values in relation to galactose Pyridine–glacial acetic acid–water was used for paper electrophoresis, and 1-butanol–glacial acetic acid–water as an irrigant for p c The yields were *H3*, 13, *H2 1*, 5, *H5 3*, 4, *H4 1*, 2, *H5 1*, 1–2, *H2 2*, below 1% (w/w) from dry serotype-11 polysaccharide The residual acidic oligosaccharides, as well as the neutral ones, were obtained in trace amounts only

acid hydrolysis of *H2 1* gave a fraction having the same electrophoretic mobility as *H3* Comparative determination of glucuronic acid in *H2 1* before and after periodate oxidation showed this constituent to carry no free vicinal hydroxyl groups. These results are in agreement with the following structure for *H2 1*. β-D-Glc*p*-(1→3)-β-D-GlcUA*p*-(1→3)-D-Gal

Oligosaccharide *H5 3* contained galactose and pyruvate in the molar ratio 1 0 0 8 Sodium borohydride reduced all of the galactose Methylation analysis yielded 2,3- and 2,6-Gal in the ratio of ~3.5 1 0 (Table II) *H5 3* thus consisted of a mixture of 4,6- and 3,4-*O*-(1-carboxyethylidene)-D-galactose (see Discussion)

Oligosaccharide *H5 1* contained galactose, glucuronic acid, and pyruvate in the molar ratios 2 0 1 0 0 8, with one equivalent of galactose being reducible by sodium borohydride To establish whether the terminal, reducing galactose residue was substituted by pyruvate (as in *H5 3*) or by glucuronic acid (as in *H3*), *H5 1* was labelled by treatment with $NaBH_4/NaBT_4$, partially hydrolysed, and subjected to paper electrophoresis It was found that reduced *H5.1* thus lost a fragment having the same mobility as reduced *H3* From these results, and from the facts that the glucuronic acid residue was doubly substituted in positions 3 and 4 (see methylation analysis of the reduced polymer), position 3 being occupied by glucose (see oligosaccharide *H2 1*), it was concluded that *H5 1* had the following structure



Oligosaccharide *H2 2* was obtained in very small amounts Qualitative analysis showed that it contained galactose and glucuronic acid On incubation with α-, but not with β-, D-galactosidase, galactose and an oligosaccharide having the same mobility as *H3* were liberated This suggested the following structure

α-D-Gal*p*
$^{1}\downarrow_{4}$
β-D-GlcUA*p*-(1→3)-D-Gal

Oligosaccharide *H4 1* contained glucose, galactose, glucuronic acid, and pyruvate in the molar ratios 1 0 1 85 0 9 1 1 Half of the galactose could be reduced with sodium borohydride *H4 1* thus constituted a tetrasaccharide repeating-unit of the polymer, the structure of which is discussed below

Oligosaccharides *H5 2* and *H4 2*, obtained in very small amounts, contained the same constituents as *H5.1* and *H4 1*, respectively Possibly the former differed from the latter only in the linkage of the pyruvate (see oligosaccharide *H5 3* and Discussion) Qualitative analysis of *H1 1*, *H1 2*, and *H1 3* yielded galactose, glucuronic acid, and glucose in all cases

P m r spectroscopy — The results with native type-11 polysaccharide are recorded in Table III. It can be seen[7] that the polymer contained two equatorial (α-linkages, $\tau<5$, $J_{1\,2}$ 4 Hz) and two axial (β-linkages, $\tau>5$, $J_{1\,2}$ 8–9 Hz) anomeric protons, acetate (τ 7 86), and pyruvate methyl protons (τ 8 57) in the ratios of ~1·1·1.1 1 6 3. The signals of one of the equatorial anomeric protons (at τ 4 5), as well as those of the acetyl and pyruvate methyl protons, were largely diminished or absent in the spectrum of Smith-degraded material

TABLE III

P M R DATA ON NATIVE *Klebsiella* SEROTYPE 11 CAPSULAR POLYSACCHARIDE

τ Value[a] (*Coupling constant*, $J_{1\,2}$ *Hz*)	*Approximate ratio of integrals*	*Proton assignment*[7]
4 5 (4)	1	equatorial, anomeric
4 65 (4)	1	
5 08 (8–9)	1	axial, anomeric
5 35 (8–9)	1	
7 86	1 6	CH_3 of *O*-acetyl
8 57	3	CH_3 of pyruvate

[a]Spectra run in deuterium oxide at 90 to 95° with an internal standard

Serological cross-reactions — As the capsular polysaccharides carrying the *E coli* K29[40,79,80] and K30[42] determinants are known to contain the same aldobiouronic acid (*H3*) as *Klebsiella* type 11 glycan (for *E coli* K29, the two polysaccharides even have the trisaccharide *H2 1* in common), the serological K cross-reactions between the corresponding *E coli* test-strains and *Klebsiella* 390 were determined by slide and tube agglutination[1 38] The results are given in Table IV. It can be seen that only exceedingly weak cross-reactions were observed

DISCUSSION

As isolated by the phenol–water–Cetavlon procedure[43 44], the *Klebsiella* type 11 capsular polysaccharide shows a double peak in the analytical ultracentrifuge,

TABLE IV

SEROLOGICAL CROSS-REACTIONS OF *Klebsiella* 390

OK serum against strain	*Agglutination[a] with strain*		
	Klebsiella *390* (*03 K11*)	E coli *Bi161/42* (*09 K29(A) H⁻*)	E coli *E69* (*09 K30(A) H12*)
Klebsiella 390	128	4	<4
	+++	(+)	—
E coli Bi161/42	8	256	<4
	++	+++	—
E coli E69	4	4	256
	++	+	+++

[a]The (reciprocal) titers in tube agglutination (1 2 dilution series), and the results of slide agglutination (+, aggl in undiluted serum, +++, aggl in serum diluted 1 10) are given

after mild alkali-treatment, however, the material sediments uniformly This behaviour is typical for *Enterobacteriaceae* capsular polysaccharides, the single glycan strands of which have repeatedly been postulated to be cross-linked by uronic acid ester-bonds[40 42,43,81] Although, in one instance[40 82], this view could be substantiated by the detection of tritiated hexose on reduction of the native (but not of the alkali-treated) polymer with $NaBH_4/NaBT_4$, alternative explanations for the ultracentrifugational behaviour of these polysaccharides, such as some cleavage by β-elimination, or aggregational and/or conformational alterations caused by the alkali-treatment, have not been rigorously excluded and necessitate further investigation

The constituent analyses of the native type-11 polymer (Table I) show that the material consists of D-galactose, D-glucose, D-glucuronic acid, pyruvate, and *O*-acetyl in the molar ratios 2·1·1 1 0 5, if it is accepted that the small amount of mannose is due to a contamination with cell-wall lipopolysaccharide[20,78] The absence of mannose in pure type-11 polysaccharide was confirmed by the analysis of the oligosaccharides obtained from it by bacteriophage degradation (see following paper[27])

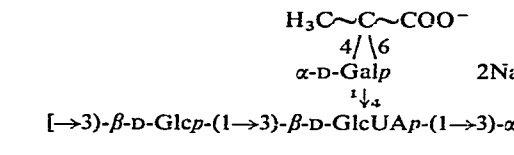
As shown by the results of methylation analysis (Table II, four different methylated monomers in about equimolar amounts) and of p m r spectroscopy (Table III, four different anomeric protons in equal amounts), type-11 polysaccharide consists of tetrasaccharide repeating-units This was directly confirmed by quantitative isolation of the repeating-unit tetrasaccharide and double repeating-unit octasaccharide after phage depolymerization of alkali-treated material (see following paper[27]) In agreement with the results of periodate oxidation (one pair of free, vicinal hydroxyl groups at C-2 and C-3 of a galactose residue), the methylation analyses further show that the tetrasaccharide repeating-unit consists of 3-substituted glucose and galactose, 4,6-substituted galactose, and 3,4-substituted glucuronic acid

Its alkali-stability (Table I) already indicates that the pyruvate is linked as an acetal, as in many similar cases[11,12,30,35,36,79 80] The results of the methylation analyses [Table II, disappearance of 4,6-Gal and concomitant appearance of 2,3,4,6-

Gal upon partial depyruvylation (autohydrolysis)] and of Smith degradation [Table I, concomitant loss of one branch galactose and of pyruvate (the product of Smith degradation is non-dialysable)], as well as the direct isolation and identification of 4,6-*O*-(1-carboxyethylidene)-D-galactose (oligosaccharide *H5 3*), confirm this assumption and show that at least the major part of the pyruvate is linked to positions 4 and 6 of branch, terminal D-galactose residues The detection of some 2,6-Gal upon methylation of partially autohydrolysed material and oligosaccharide *H5 3* (Table II), as well as the incomplete periodate-oxidation of the branch galactose (Table I), seem, however, to indicate that some of the pyruvate acetal residues are linked to positions 3 and 4 of galactoses also[35] But since 2,6-Gal was obtained only from products which had been subjected to acidic conditions before methylation (see Table II and following paper[27]), it is considered more likely that, under acidic conditions (by analogy with similar cases[20,83 84]) some of the pyruvate acetal residues migrate from 4,6 to the (thermodynamically more stable[85]) 3,4 position This view is also strengthened by the finding (possibly due to the increased rigidity of the double-chair structure) that periodate oxidation of 4,6-*O*-(1-carboxyethylidene)hexoside residues generally appears to proceed comparatively slowly[80].

The sequence of the constituents within the type-11 repeating unit, as well as the anomeric configurations, follow from the structure of the oligosaccharides obtained by partial, acid hydrolysis and from the results of p m r spectroscopy (Table III) The fact that the chain glucose is linked to position 3 and the branch pyruvate-galactose to position 4 of the glucuronic acid was confirmed by the results of phage degradation[27]

In summary, the results presented are in agreement with the following structure for alkali-treated *Klebsiella* type 11 polysaccharide, calculating the d p from the weight-average molecular weight of the sodium salt



The configuration at C-2 of the pyruvate acetal residue is not known The native polymer additionally carries one *O*-acetyl residue on every second repeating-unit, which, from the results of periodate oxidation, and if it is not randomly distributed, cannot be located on the branch galactose

The p m r data allow a partial assignment of the signals of the anomeric protons (see Table III and Results) The signal at τ 4 5 must originate from the equatorial anomeric protons of the branch galactose residues, it is largely diminished (to ~17%) in the spectrum of Smith-degraded polymer, and therefore the signal at τ 4 65 is from the anomeric protons (which are also equatorial) of the chain galactose residues The results presented do not show, however, which of the signals at τ 5 08 and 5 35 originates from the axial anomeric protons of the chain glucose and glucuronic acid residues, respectively

TABLE V

CHEMICAL BASIS OF SEROLOGICAL CROSS-REACTIONS OF THE *Klebsiella* K11 CAPSULAR ANTIGEN

Capsule (slime) antigen	*Oligosaccharide region in common with the* Klebsiella *serotype 11 capsular polysaccharide repeating unit*	*Serological cross-reaction with* Klebsiella *K11*
	Branch	
Enterobacteriaceae		
M (colanic acid)[35 36]	Pyruvate 4,6< D-Gal*p*	+++[a]
Klebsiella K21[12]	Pyruvate 4,6< α-D-Gal*p*-(1→4)-D-GlcUA*p*	+++[a]
	Chain	
Klebsiella K7[22 86]	D-Glc*p*-(1→3)-β-D-GlcUA*p*[b]	−[c d]
Klebsiella K20[10 11]		−[c d]
Klebsiella K52[20]	β-D-GlcUA*p*-(1→3)-α-D-Gal*p*[e]	−[c]
E coli K30[42]		(+)[f]
	Branch and chain	
E coli K29[40,79,80]	Pyruvate 4,6 / X / Y / 1,4 — β-D-Glc*p*-(1→3)-β-D-GlcUA*p*-(1→3)-α-D-Gal*p*[g]	(+)[f]

[a]Strong cross-reactions repeatedly reported[1 28 34] [b]No data available on the anomeric configuration of the glucose residues, which also carry pyruvate acetal residues[22 86] [c]No cross-reactions given by Edwards and Ewing[1] [d]No cross-reactions observed in own control experiments [e]In the case of *Klebsiella* K20, the anomeric configurations have been established[10 11], in the case of *Klebsiella* K52, as yet not totally conclusive observations suggest that they are the same[20], in the case of *E coli* K30, the uronic acid residues were found to be in the β-D configuration, but no data on the anomeric configuration of the galactose residues were given[42] [f]See Table IV [g]The structure of the *E coli* serotype 29 capsular polysaccharide[40 82] has recently been reinvestigated[79 80], it is

Pyruvate
4,6
β-D-Glc*p*
1,2
α-D-Man*p*
1,4
→3)-β-D-Glc*p*-(1→3)-β-D-GlcUA*p*-(1→3)-α-D-Gal*p*-(1→2)-α-D-Man*p*-(1→

In Table V, the serological cross-reactions of the *Klebsiella* K11 with some other *Enterobacteriaceae* K and M antigens of partially homologous structure are compiled It can be seen that the majority of the capsule antibodies (K agglutinins) produced by rabbits upon injection with *Klebsiella* 390 are specific for terminal 4,6-*O*-(1-carboxyethylidene)-D-galactose Only a very small fraction of the K-antibody population is directed against chain determinants (no, or very weak, cross-reactions with *Klebsiella* K7, K20, K52, and *E coli* K29 and K30), or, on the other hand, against pyruvate acetal alone (very weak cross-reaction with *E coli* K29) Obviously, K antibodies totally specific for pyruvate-galactose should be obtained by absorption of a *Klebsiella* 390 OK-serum with *E coli* Bi161/42 (09 K29(A) H⁻) cells

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