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Klebsiella SEROTYPE-13 CAPSULAR POLYSACCHARIDE: PRIMARY STRUCTURE AND DEPOLYMERIZATION BY A BACTERIOPHAGE-BORNE GLYCANASE

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ABSTRACT

Periodate oxidation and Smith degradation, methylation analysis including uronic acid degradation, partial hydrolysis with acid, bacteriophage degradation, and p.m.r. spectroscopy have been used to elucidate the primary structure of the *Klebsiella* serotype-13 capsular polysaccharide. The polymer consists of pentasaccharide repeating-units comprising a 4)-β-D-Man*p*-(1→4)-α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→chain with a 3,4-*O*-(1-carboxyethylidene)-β-D-Gal*p*-(1→4)-α-D-GlcA*p*-(1→ branch at position 3 of the mannose. It is shown that there is a glycanase activity associated with particles of *Klebsiella* bacteriophage No. 13, which catalyses hydrolysis of chain β-D-Glc*p*-(1→4)-β-D-Man*p* linkages in the type-13 polysaccharide. The chemical basis of some serological cross-reactions of the *Klebsiella* K13 antigen is discussed.

INTRODUCTION

The eighty-one different *Klebsiella* capsular polysaccharides[1] basically consist of repeating units comprising various combinations of the seven most common hexoses and hexuronic acids[2–5]. As previously discussed[3], this set of glycans thus lends itself for comparative studies, *e.g.*, of the substrate specificity of bacteriophage-borne glycanases[6,7], of serological determinants[8,9], or of polysaccharide conformation[10,11].

We now report on the elucidation of the primary structure of *Klebsiella* serotype-13 capsular polysaccharide, as well as its depolymerization by a glycanase activity associated with particles of *Klebsiella* bacteriophage No 13. This system was chosen because the viral enzyme also catalyses the hydrolysis of several other *Klebsiella* capsular polysaccharides, a fact which led to some understanding of its substrate specificity[12].

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MATERIALS AND METHODS

Media and PBS. — Generally, Merck standard I broth was used for liquid and for solid media. For the isolation of capsular polysaccharide, however, *Klebsiella* 1470 was grown on $D_{1.5}$ agar[13], and the lysates of phage 13 were prepared in a casein–yeast extract–D-glucose (CYG) broth[13] consisting of 15 g of tryptic digest of casein (CAT; Brunnengräber, Lübeck, GFR), 5 g of yeast extract (Difco), 10 g of D-glucose, 3 g of NaCl, 8 g of $Na_2HPO_4 \cdot 12H_2O$, and 0.2 g of $MgSO_4 \cdot 7H_2O$ in 1 litre of water. The composition of the phosphate-buffered, physiological saline (PBS) has been given previously[3].

Bacteria and bacteriophage. — *Klebsiella* 1470 (R?:K13), originally isolated from urine by Kauffmann[14], and serving as a serological test-strain for the *Klebsiella* K13 antigen[8,14,15], was kindly supplied by Dr. Ida Ørskov, WHO International *Escherichia* Centre, Statens Seruminstitut, Copenhagen, Denmark.

Klebsiella bacteriophage No. 13, which forms plaques with acapsular haloes[16] on *Klebsiella* 1470, was obtained from Dr. Anna Przondo-Hessek, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland. The phage was isolated from sewage[17], and originally designated "Kl 17"; it is a small virus[18] of morphology similar (also Bradley group C[19]) to that of *Klebsiella* bacteriophage No. 11[16,20,21].

Isolation, homogeneity control, and equivalent weight of Klebsiella *type-13 capsular polysaccharide.* — *Klebsiella* 1470 was grown on $D_{1.5}$ agar, harvested, dried, and extracted by the phenol–water–Cetavlon (cetyltrimethylammonium bromide) procedure[22], as described[3] for *Klebsiella* 390. To remove residual RNA, the product was treated with RNAse from bovine pancreas[23] (EC 3.1.4.22; Boehringer No. 15331). 100 large (14-cm diameter) agar plates yielded 32 g of dry bacteria and, from these, 3.4 g (10.7%) of type-13 polysaccharide. For mild alkali-treatment[3], the material was kept for 4 h at 56° in 0.25M NaOH.

For determination of the equivalent weight, an aqueous solution of alkali-treated polysaccharide was passed over a column of Dowex 50(H^+) resin, and lyophilized; after addition of excess of 0.1M NaOH, a 0.1% solution of the acidic form was back-titrated with 0.1M HCl to pH 7. Aliquots of the solution of the acidic form were also dried at 70° over granular P_2O_5 (40 min, when constant weight was attained), and the equivalent weight was calculated on the basis of the dry weight as obtained with an electrobalance[24].

Constituent analysis. — The qualitative analyses were carried out on 0.25M H_2SO_4 hydrolysates (48 h at 100°). Glucose, galactose, and mannose, as well as glucuronic acid and pyruvate, were identified by p.c. with *A*, ethyl acetate–pyridine–water (4:1:1); or *B*, ethyl acetate–glacial acetic acid–formic acid (95%)–water (18:3:1:4)[25]. The acidic components were also identified by high-voltage paper electrophoresis[26] at pH 5.3; for further details, see Thurow *et al.*[3].

For quantitative constituent analyses, optimal hydrolysis conditions were first established: solutions of type-13 polysaccharide in 0.25M H_2SO_4 were heated

(100°) for different periods of time (1 to 68 h), neutralized with aqueous NaOH, and analysed enzymically; D-glucose (maximum amount obtained after 4 h of hydrolysis), D-galactose (4 h), and pyruvate (3 h) were determined with fungal D-glucose oxidase[27], D-galactose dehydrogenase from *Pseudomonas fluorescens*[28], and lactate dehydrogenase from hog heart[29], respectively[3]. For the estimation of D-mannose (19 h), the sequential action of hexokinase (EC 2.7.1.1; Boehringer No. 15527), D-mannose phosphate isomerase (EC 5.3.1.8; Boehringer No. 15104), D-glucose phosphate isomerase (EC 5.3.1.9; Boehringer No. 15433), and D-glucose 6-phosphate dehydrogenase from yeast (EC 1.1.1.49; Boehringer No. 15527) was employed, with the experimental details given by Gawehn[30]. The other methods used for quantitative constituent analyses[31–33] have been recorded previously[3].

Periodate oxidation and Smith degradation. The periodate consumption of alkali-treated type-13 polysaccharide, and of its repeating-unit pentasaccharide, was determined by the method of Avigad[34], using a two-step procedure[35,36]. When the first consumption of periodate had ceased (after ~24 h), excess of oxidant was reduced by addition of ethylene glycol, and the reaction mixture was dialysed against distilled water. The polyaldehyde was then reduced with $NaBH_4$. After decomposition of excess of reductant with dilute acetic acid, and extensive dialysis against acetate buffer (pH 4), an additional periodate-consumption of the polyalcohol was titrated. For the K13 pentasaccharide, the dialysis steps were replaced by gel filtration (Sephadex G10) with a volatile buffer[6], and lyophilization.

For Smith degradation[37], the product of the second oxidation was reduced, dialysed, and lyophilized; yield, 90%. Using samples of this polyalcohol, and employing paper electrophoresis for the detection of charged oligosaccharides and erythronic acid, and the enzymic determination of free D-glucose for the estimation of unspecific hydrolysis, several hydrolysis conditions[3,25,38,39] were compared; 26 mg of the material were then kept for 16 h at 37° in 125mM H_2SO_4 (optimum conditions). After neutralization and evaporation, the product was desalted (gel filtration with a volatile buffer) and lyophilized. The resulting material was then fractionated on a column of Amberlite IR-410(CO_3^{2-}) resin; elution with water (and evaporation) yielded a neutral fraction, and elution with 10% aqueous formic acid an acidic fraction.

Methylation analysis and uronic acid degradation. — Methylations were performed by the method of Hakomori[40], following the experimental details of Hellerqvist *et al.*[41]. A sample of permethylated type-13 polysaccharide was carboxyl-reduced with calcium borodeuteride as follows[38,42]. A mixture of powdered $CaCl_2$ (1 mmol), $NaBD_4$ (2 mmol), and tetrahydrofuran (50 ml) was stirred overnight at room temperature and then left to settle. The clear supernatant (2–3 ml) was added to 2–5 mg of methylated polymer and again stirred overnight. After addition of water and dilute acetic acid (until pH 6), the reduced–dideuterated product was recovered by dialysis and lyophilization. The acidic oligosaccharides were reduced–trideuterated prior to methylation; at 0°, diazomethane in ether was added to the materials [acidic forms, obtained by treatment with Dowex 50(H^+) resin] in methanol, until the yellow

colour remained. After another 30 min at 0°, and 1 h at room temperature, the solutions were evaporated. The methyl esters were then reduced with calcium borodeuteride as described above [but replacing the final dialysis by decationization with Dowex 50(H^+), and removal of the boric acid with methanol], and methylated.

The permethylated type-13 polysaccharide (13 mg) was subjected to uronic acid degradation[43], as detailed by Curvall *et* al.[44]. The product, when purified by passage over a column of Sephadex LH20 with chloroform, appeared in the void volume; yield, 4.5 mg). A part of the material was trideuteriomethylated[44].

The methylated products were sequentially hydrolysed with formic and sulfuric acids[41], and the monomers were analysed by descending p.c. of the aldoses[25,45] (irrigant *C*, butanone saturated with 1% aqueous ammonia; spray, 1.5%(w/v) of *p*-anisidine in 5% aqueous trichloroacetic acid[46]), and by g.l.c.–m.s. of the alditol acetates[47,48]. The retention times of the alditol acetates[47] were determined by using a Varian Aerograph instrument and ECNSS-M columns (for further details, see Ref. 3). Mass spectrometry was performed with a Finnigan combined g.l.c. (model 9500)/m.s.(model 3200E-003) instrument coupled to a model 600 Interactive Control and Graphic Output System. Columns of ECNSS-M were employed (starting temperature, 155°; temperature increment, 2°/min), but with helium (25 ml/min) as carrier gas. Electron-impact ionization was used; the ionization potential was 72 eV, and the ionization current 380 μA.

For g.l.c.–m.s. of the permethylated disaccharide alditol[49] obtained from K13 aldobiouronic acid, an ECNSS-M column was also used, but at a starting temperature of 180°.

Partial hydrolysis with acid. — Optimal hydrolysis conditions were worked out by heating (100°) solutions of type-13 polysaccharide in 0.5M H_2SO_4 for 1–5 h, neutralizing, and separating the reaction products by paper electrophoresis at pH 5.3 (*cf.* Ref. 3).

Aldotetrauronic acids (M_{GLCA} 0.42; optimal yield after about 2 h of hydrolysis), aldotriouronic acids (0.52; 2 h), aldobiouronic acid (0.69; 3–4 h; yield, 9%), and (1-carboxyethylidene)galactose (0.91; 2 h; 3%) were then isolated by preparative paper electrophoresis, and checked for homogeneity by p.c. with solvent *D*, 1-butanol–glacial acetic acid–water[25] (2:1:1); R_{GLCA} 0.24, 0.44, 0.79, and 0.95, respectively.

Bacteriophage degradation. — *Klebsiella* bacteriophage No. 13 was propagated on *Klebsiella* 1470, and the virus particles were concentrated and purified, as described previously[6] for bacteriophage No. 11, but with the following minor alterations: (*a*) the lysates were prepared in CYT broth instead of P medium, as phage 13 does not multiply well in the latter; (*b*) the cultures were infected at an absorbance of 1.05, corresponding to $\sim 7 \times 10^8$ colony-forming organisms per ml; (*c*) lysates with titres of $\sim 6 \times 10^9$ PFU (plaque-forming units) were obtained; (*d*) in isopycnic centrifugation, phage 13 banded at 1.46 g/ml; (*e*) the final yields were 40–45% of the PFU in the crude lysates.

Also, the bacteriophage-degradation of (alkali-treated) type-13 polysaccharide was performed essentially as that[6] of the type-11 glycan by phage 11: solutions,

containing 2 mg of glycan, and $\sim 2 \times 10^{10}$ PFU of pure virus/ml of PBS, were incubated at 37° for 48 h. The mixtures were then lyophilized, desalted by passage over Sephadex G10 with a volatile buffer, and lyophilized again. The crude K13 oligosaccharides were finally separated by adsorption to a column of DEAE Sephadex A25 from a 0.05M Tris/HCl buffer (pH 7.2), and elution with a linear (0–0.5M) gradient of NaCl. Three oligosaccharide fractions appeared at ~0.33, 0.44, and 0.48M NaCl; they were desalted as described above, and the first two were checked for homogeneity by paper electrophoresis at pH 5.3 [M_{GLCA} 0.66 (*P1*) and 0.71 (*P2*)], and by p.c. [solvent *D*; R_{GLCA} 0.6 (*P1*) and 0.22 (*P2*)]; yields: 27.5 and 20.5%, respectively.

For size determination and identification of the reducing-end sugar, 19 μmol of $NaBH_4/NaB^3H_4$ (specific activity, 133 mCi/mmol) in 300 μl of 0.01M aqueous NaOH were added to 1.5–4.5 μmol of oligosaccharide *P1* or *P2*, and 2.5 μmol of xylose (internal standard) in 300 μl of water. The mixture was kept at room temperature for 6 h. Excess of reductant was then decomposed with dilute acetic acid, the cations were removed with Dowex 50(H^+) resin, and the boric acid was removed with methanol. The products were hydrolysed and analysed by g.l.c. of the alditol acetates as usual[31], but using the B channel of the model 1520B Varian Aerograph, and 1:10 splitting. The single per-*O*-acetylalditols were collected, taken up in chloroform, and counted with a Packard Tri-Carb (model 2450) liquid scintillation counter. The hydrolysates were also analysed for radioactive gulonic acid after paper electrophoresis, using a Packard radiogram scanner.

P.m.r. spectroscopy. — Solutions (0.5–4%) of the poly- and oligo-saccharides in absolute D_2O (Aldrich) were run at 70°, using a Bruker HFX-90 instrument (90 MHz) linked to a Fabritek CAT (model 1074), and acetone (δ 2.17) or the sodium salt of 3-trimethylsilylpropanesulfonic acid-d_4 (δ 0.0) as internal standards. The methylated polysaccharide obtained by uronic acid degradation was measured at 25° in deuteriochloroform.

RESULTS

Isolation, homogeneity, physical parameters, and composition of Klebsiella *serotype-13 capsular polysaccharide.* — As isolated by the phenol–water–Cetavlon (cetyltrimethylammonium bromide) procedure[3,22,23] from *Klebsiella* 1470 (the serological test-strain for the *Klebsiella* K13 antigen[8,14,15]), type-13 polysaccharide sedimented uniformly in the analytical ultracentrifuge (with $s^{0.35\,\%}_{20,\,PBS} = 1.9 \times 10^{-13}$ sec) after mild alkali-treatment (*cf.* Ref. 3). The alkali-treated acidic polysaccharide, which was used for all further investigations, had $[\alpha]^{20}_{589}$ +45° (*c* 0.3, water) and an equivalent weight of 405; its composition is given in Table I (column II).

Periodate oxidation and Smith degradation. — Type-13 polysaccharide consumed periodate only at pH ≪ 4.5. Under these conditions (no loss of viscosity in the absence of periodate), ~1 mol of periodate per pentasaccharide repeating-unit was taken up within 10 h, and another 0.5 mol after reduction with sodium borohydride[35,36]. The constituent analysis of the final polyalcohol, obtained after a second reduction,

is shown in Table I (column IV). The type-13 repeating-unit pentasaccharide *P1* (obtained by bacteriophage degradation, see below) also exhibited a two-step uptake of periodate: an initial 3 mol, and another 1 mol after borohydride reduction of the first oxidation product (see Table I, columns III and V for constituent analyses).

Type-13 polysaccharide, twice oxidized and twice reduced, was subjected to Smith hydrolysis[37,39], and the products were separated into neutral and acidic fractions. The neutral fraction contained nearly equimolar amounts of glucose, mannose, and erythritol (Glc:Gal:Man:erythritol = 0.96:0.14:1.00:0.80), and the acidic fraction contained galactose as the dominant neutral component (0.11:1.00:0.15: 0.10).

TABLE I

MOLAR RATIOS OF CONSTITUENTS (AND PERCENTAGE OF "ANHYDRO" RESIDUES) IN *Klebsiella* SEROTYPE-13 CAPSULAR POLYSACCHARIDE AND ITS DERIVATIVES

	I[a]	*II*		*III*	*IV*[b]	*V*[c]	*VI*	*VII*
D-Glucose	1.91 (22.9)	2.15 (28.7)[d],	1.96 (24.8)[e]	2.11; 1.89	1.21	0.09	—	—
D-Galactose	1.00 (12.0)	1.00 (13.3),	1.00 (12.7)	1.00; 1.00	0.78	1.00	—	1.00
D-Mannose	0.94 (11.3)	1.03 (13.7),	0.91 (11.5)	0.73; 0.72	1.00	0.80	0.93	—
D-Glucuronic acid[f]	0.95 (12.2)	0.99 (14.1),	1.03 (14.1)	0.86; 0.77	0.15	0.13	1.00[g]	—
Pyruvate[h]	n.d.	0.77 (4.3),	0.59 (3.6)	0.96; 0.90	0.70	0.76	—	0.77
Acetyl[h]	0.19 (0.6)	—	—					
Total[i]		(93.9)[i]						

[a]I, Native polysaccharide; II, alkali-treated polysaccharide; III, repeating-unit pentasaccharide *P1*; double repeating-unit decasaccharide *P2*; IV, alkali-treated polysaccharide, twice oxidized with periodate and reduced with borohydride; V, pentasaccharide *P1*, twice oxidized with periodate and reduced with borohydride; VI, aldobiouronic acid; VII, pyruvylgalactose. [b]In addition, 0.86 mol. equivalent of erythritol was found, and erythronic acid was detected by paper electrophoresis. [c]In addition, 0.78 and 0.52 mol. equivalent of erythritol and glycerol, respectively, were found, and erythronic acid was detected by paper electrophoresis. [d]Determined enzymically after optimal hydrolysis for each constituent (see Materials and Methods); hexuronic acid with carbazole–sulfuric acid[32]. [e]Hexoses determined by g.l.c. of the alditol acetates[31] (after the same hydrolysis conditions for all constituents). [f]The identification of the hexuronic acid as D-glucuronic acid follows from the analysis of the aldobiouronic acid (see *g*). [g]Determined by g.l.c. of the alditol acetates, as well as with D-glucose oxidase, after esterification, reduction with calcium borodeuteride, and hydrolysis. [h]Calculated as CH_3–CO–COO^- and CH_3CO. [i]Including 15.0% of water (looss of weight after 40 min at 70° *in vacuo* over P_2O_5), 0.2% of protein (determined by the Folin method, with bovine serum albumin as a standard), 0.6% of nucleic acid (estimated from the absorption at 260 nm, with yeast RNA as a standard), and 4.0% of sodium (calculated on the basis of the glucuronic and pyruvic acid values).

Methylation analysis and uronic acid degradation. — Type-13 polysaccharide was methylated according to Hakomori[40,41], and a part of the product was reduced with calcium borodeuteride[38,42]. Upon hydrolysis, the *O*-methylaldoses were identified by p.c.[45], and by g.l.c.–m.s. of the alditol acetates[47,48]. The results are shown in Table II (columns I and II).

Uronic acid degradation[43,44] of the methylated type-13 glycan, $[\alpha]_{589}^{22}$ +44° (*c* 0.1, chloroform), yielded a polymer, $[\alpha]_{589}^{22}$ +82° (*c* 0.1, chloroform), the composi-

TABLE II

IDENTIFICATION AND RATIOS OF *O*-METHYLALDOSES OBTAINED FROM METHYLATED *Klebsiella* SEROTYPE-13 CAPSULAR POLYSACCHARIDE AND ITS DERIVATIVES

O-Methylaldose[a]	R_F *in p.c. of free aldoses*[b]		T[c] *in g.l.c. of alditol acetates*		*Mass spectrum*												
					Primary fragments (m/e)									*Ratio of peak integrals*				
	Lit.[45]	*Found*	*Lit.*[47,48]	*Found*	*45*	*89*	*117*	*133*	*161*	*205*	*233*	*261*	*305*	*I*[d]	*II*	*III*	*IV*	*V*
1,2,4,5,6-Glc	—[e]	—	—	0.49	+[f]	+[f]		+						—	—	—	—	0.3[f,g]
2,3,4,6-Glc[h]	0.78	0.78	1.00	1.00	+		+		+	+				—	0.2	—	0.1	—
2,4,6-Glc	0.48	0.51	1.95	1.89	+		+		+		+			0.9	1.0	0.9	0.9	0.2[g]
2,4,6-Man[h]	0.55	0.56	2.09	2.03	+		+		+		+			—	—	—	—	0.9
2,3,6-Man	0.50	0.48	2.20	2.20	+		+		+[i]		+[i]			—	—	—	0.8[i]	—
2,3,6-Glc	0.56	0.56	2.50	2.42	+[j]		+		+		+[j]			1.0	1.1	0.9	1.0	1.9[j]
2,6-Man	—	0.20	3.35	3.31	+		+						+	1.0	1.0	1.0	0.1	—
2,6-Gal[h]	—	0.23	3.65	3.63	+		+						+	1.1	0.9	0.1	—	1.0
2,3-Glc[h]	0.28	0.27	5.39	5.39			+		+			+[k]		—	0.7[k]	—	—	—

[a]1,2,4,5,6-Glc = 1,2,4,5,6-penta-*O*-methyl-D-glucose, etc. [b]Solvent *C*[45]. [c]Retention time relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (*T* 1.00) and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol (*T* 5.39) on ECNSS-M[47,48]. [d]I, type-13 polysaccharide, methylated; II, polysaccharide, methylated, and reduced with calcium borodeuteride; III, polysaccharide, methylated, and subjected to uronic acid degradation[43,44] (polymeric fragment); IV, polysaccharide, methylated, subjected to uronic acid degradation, and trideuteriomethylated; V, type-13 repeating-unit pentasaccharide *PI*, esterified, reduced with calcium borodeuteride, and methylated. [e]Hexitol, not stained. [f]The monodeuterated fragments, *m/e* 46 and 90 were found in addition to the normal ones. [g]The presence of 2,4,6-Glc, and the low yield of 1,2,4,5,6-D-glucitol may result from the formation of some methyl glycoside during esterification, and from the volatility of the penta-*O*-methyl derivative. [h]Standards available. [i]The trideuterated fragments, *m/e* 164 and 236 were found instead of the normal ones. [j]In the case of V (methylated pentasaccharide *PI*), the dideuterated fragments, *m/e* 47 and 235 were found in addition to the normal ones. [k]The dideuterated fragment, *m/e* 263 was found instead of the normal one.

TABLE III

P.M.R. SIGNALS OF ANOMERIC, AND OF PYRUVATE-ACETAL, PROTONS IN *Klebsiella* SEROTYPE-13 CAPSULAR POLYSACCHARIDE AND ITS DERIVATIVES

	δ-Value ($J_{1,2}$, *Hz*)		*Approximate ratio of peak integrals*	*Proton assignment* (cf. *Refs. 25, 51–53*)
Type-13 polysaccharide alkali-treated[a]	5.13 5.07	(2.5)	2.0	α-GlcA*p*-(1→ 4)-α-Glc*p*-(1→
	4.78	(4)	1.0	β-Man*p*-(1→
	4.47	(8)	2.0	3)-β-Glc*p*-(1→ and -β-Gal*p*-(1→
	1.55		3.0	Pyruvate acetal
Aldobiouronic acid[a]	5.25	(3)	1.0	α-GlcA*p*-(1→
	5.12	(2)	0.5	α-Man*p*-(1→
	4.86	(1.5)	0.4	β-Man*p*-(1→
Type-13 repeating-unit pentasaccharide *P1*[a]	5.32 5.26	(4) (3.5)	2.5	α-GlcA*p*-(1→ 4)-α-Glc*p*-(1→ and 3)-α-Glc*p*-(1→
	4.79	(1.5)	0.9	β-Man*p*-(1→
	4.65	(7.5)	0.6	3)-β-Glc*p*-(1→
	4.55	(8)	1.0	β-Gal*p*-(1→
	1.50		3.0	Pyruvate acetal
Type-13 polysaccharide, methylated, and subjected to uronic acid degradation (polymeric fragment)[b]	5.38	(3)	1.2	4)-α-Glc*p*-(1→
	4.66	(5)	1.0	β-Man*p*-(1→
	4.28	(7)	0.9	3)-β-Glc*p*-(1→

[a]Run at 70° in deuterium oxide. [b]Run at 25° in deuteriochloroform.

tion of which (before and after remethylation with trideuteriomethyl iodide[43,44]) is given in Table II (columns III and IV).

Partial, acid hydrolysis. — Four major fractions were isolated by paper electrophoresis after partial, acid hydrolysis of type-13 polysaccharide. These comprised aldotetrauronic acids, aldotriouronic acids, aldobiouronic acid, and pyruvylgalactose, and contained Glc:Gal:Man:GlcA:pyruvate in ratios approaching 2:0:1:1:?, 1:0:1:1:?, 0:0:1:1:0, and 0:1:0:0:1, respectively (see Table I, columns VI and VII). The first two fractions were not further investigated, because p.m.r. spectroscopy showed that they were mixtures.

The aldobiouronic acid had $[\alpha]_{589}^{25}$ +42° (*c* 0.1, water), and was not split by β-D-glucuronidase[3,50]. When the material was esterified and deuterated–reduced, and the resulting disaccharide alditol was methylated and analysed by g.l.c.–m.s.[49], it yielded, *inter alia*, the primary fragment with *m/e* 133 (23% of the fragment with *m/e* 88), but no fragment (<6%) with *m/e* 134, indicative of 3-substitution of the alditol moiety[49]. In total, these findings show that the aldobiouronic acid is 3-*O*-(α-D-glucopyranosyluronic acid)-D-mannose.

Bacteriophage degradation. — By analogy with the previously described case of *Klebsiella* bacteriophage No. 11 and type-11 capsular polysaccharide[6], a virus that forms plaques with acapsular haloes[6,7,16] on *Klebsiella* 1470 was employed to degrade type-13 polysaccharide. The alkali-treated glycan was incubated with purified particles of *Klebsiella* bacteriophage No. 13, and the oligosaccharides obtained were separated by ion-exchange chromatography. Two pure type-13 oligomers were isolated: *P1*, $[\alpha]_{589}^{22}$ +108° (*c* 0.3, water); and *P2*, +88° (*c* 0.1). After addition of xylose as an internal standard, samples of *P1* and *P2* were reduced with $NaBH_4/NaB^3H_4$, hydrolysed, and analysed by g.l.c. of the alditol acetates (Table I, column III); additionally, the single fractions were collected, and their radioactivities determined. In this manner, molecular weights of 810 and 1630 were obtained for *P1* (pentasaccharide, type-13 repeating unit) and *P2* (decasaccharide), glucose being the reducing sugar in each compound. See Table I (column V) and Table II (column V) for further analyses of pentasaccharide *P1*.

P.m.r. spectroscopy. — The p.m.r. signals of anomeric and pyruvate acetal protons in the spectra of alkali-treated type-13 polysaccharide and some of its degradation and methylation products are recorded in Table III.

DISCUSSION

Composition of the Klebsiella *type-13 capsular polysaccharide repeating-unit.* Type-13 glycan consists of D-glucose, D-galactose, D-mannose, D-glucuronic acid, and pyruvate in molar ratios approaching 2:1:1:1:1 (Table I, columns I, II, and VI). These data suggest a composition of pentasaccharide repeating-units, in agreement with the equivalent-weight titration, and the products of bacteriophage degradation. In the native (*i.e.* not alkali-treated) polymer, approximately every fifth unit carries an (unlocalized) *O*-acetyl residue (Table I, column I).

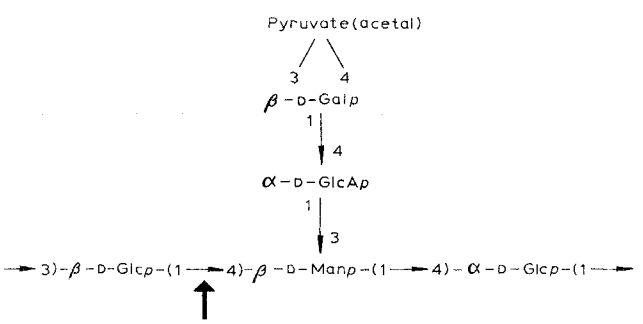
Substitution pattern of the constituents. Methylation analysis of the type-13 polysaccharide before and after carboxyl-reduction (Table II, columns I and II) showed that the repeating unit contains 3- and 4-substituted D-glucoses, 3,4-disubstituted D-galactose and D-mannose, as well as 4-substituted D-glucuronic acid (besides pyruvate). The results of periodate oxidation (Table I, column IV) are in agreement with this conclusion, if a slightly reduced accessibility, or a preferred hemiacetal protection[35,36], of the 4-substituted glucose is assumed. The presence of a small proportion of unsubstituted glucose in the reduced polymer (Table II, column II) is unexplained.

Sequence of the constituents. The repeating unit contains two disubstituted hexoses, as well as alkali-stable pyruvate, and is thus branched with a branch-terminal (1-carboxyethylidene)hexose. The latter must be 3,4-*O*-(1-carboxyethylidene)-D-galactose, because a (rather acid-stable[3]) pyruvylgalactose could be isolated by partial hydrolysis with acid, and because the mannose carries a chain glucose at position 4 (*cf.* methylation analysis of polysaccharide, and of depolymerization product *P1*; Table II, columns I, II, and V) and glucuronic acid at position 3 (see analysis of aldobiouronic acid, and results of uronic acid degradation; Table II, columns III and IV).

These latter considerations additionally imply that the D-glucuronic acid also constitutes part of the side chain.

Because uronic acid degradation[43,44] yielded a polymer product containing the 3- and 4-substituted D-glucoses, as well the disubstituted D-mannose (Table II, column III), the main chain must consist of these three constituents, and the branch of 3,4-*O*-(1-carboxyethylidene)-D-galactopyranosyl-(1 → 4)-*O*-D-glucopyranosyluronic acid (**1**). This conclusion is corroborated by the detection of substantial (and approximately equimolar) amounts of glucose and erythritol only in the neutral fraction obtained after Smith degradation.

The sequence of the chain constituents, *O*-D-glucopyranosyl-(1 → 4)-*O*-D-mannopyranosyl-(1 → 4)-*O*-D-glucopyranosyl-(1 → 3)- (**1**) follows from the finding that bacteriophage depolymerization, besides liberating position 4 of the mannose, also liberates 3-substituted reducing glucose (see Table I, columns IV and V; and Table II, columns I, II, and V).



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Anomeric configurations. As seen in Table III, the p.m.r. spectrum of type-13 polysaccharide was consistent with two equatorial anomeric protons (α-linkages), and three axial protons (β-linkages) (*cf.* Ref. 53).

As judged from the spectrum of the aldobiouronic acid (*cf.* Refs. 25,52,53), one of the signals at δ 5.07 and 5.13 results from α-linked D-glucuronic acid; in agreement with this, the aldobiouronic acid was not split by β-D-glucuronidase. One of the signals at δ 4.47 must result from β-linked D-galactose, because it is lost, together with the glucuronide signal, upon uronic acid degradation. The other signal at δ 4.47 is contributed by a β-linked 3-substituted D-glucose, as it is diminished in the spectrum of the repeating-unit pentasaccharide *P1*. The signal at δ 4.78 is assigned to β-linked D-mannose, because of its chemical shift (*cf.* the p.m.r. data of *Klebsiella* K2 oligosaccharides[53]), and because of its coupling constant (*cf.* $J_{1,2}$ of anomeric mannose protons in the K13 aldobiouronic acid, and in the pentasaccharide *P1*). The remaining signals at δ 5.07 and 5.13 must be due to α-linked 4-substituted D-glucose.

Bacteriophage degradation. The above discussion implies that there is a glycanase activity associated with the particles of *Klebsiella* bacteriophage No. 13, which catalyses hydrolysis (and not β-elimination) of 4-*O*-β-D-glucopyranosyl-β-D-mannopyranose linkages in type-13 polysaccharide (arrow, **1**). The results of the labelling with NaB^3H_4 further show that, as in the case of *Klebsiella* phage 11 and type-11 polysaccharide[6], a mixture of type-13 oligomers is formed, comprising one and two repeating-unit fragments.

Serological cross-reactions. Serological cross-reactions between the *Klebsiella* K13 on the one hand, and the *Klebsiella* K2[8] and *Pneumococcus* type-IV[9] capsular polysaccharide antigens on the other hand have been reported. Inspection of these primary structures for homologies reveals that the *Klebsiella* type-2 repeating-unit[53] is identical with that of type-13, except for the absence of the pyruvylgalactose, and that the *Pneumococcus* type-IV glycan also contains terminal (1-carboxyethylidene)-galactose residues[9,54].

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