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THE USE OF BACTERIOPHAGE DEPOLYMERIZATION IN THE STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE FROM *Klebsiella* SEROTYPE K3*

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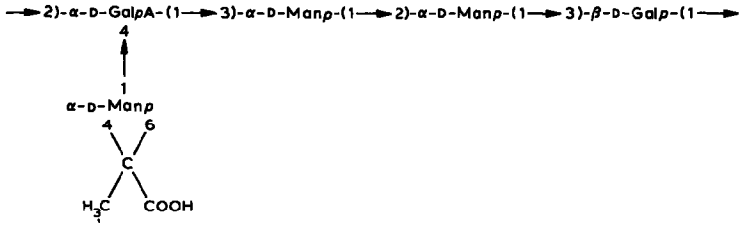
ABSTRACT

The structure of the repeating unit of the capsular polysaccharide from *Klebsiella* serotype K3 has been established from the results of n.m.r. (^1H and ^{13}C) spectroscopy and methylation analysis of (a) **P1**, the pyruvic acetal-bearing pentasaccharide obtained on depolymerization of the polysaccharide with a bacteriophage-borne endogalactosidase, (b) reduced deacetalated **P1**, and (c) the native polysaccharide. The data permit the assignment of the following structure to the repeating unit:



INTRODUCTION

The traditional method for determining the structure of a polysaccharide has been to perform experiments on the polymer, and to examine derived oligosaccharides only when examination was necessary for establishing the sequence of the monosaccharide units. The fact that bacterial polysaccharides have regular structures and that there exist bacterial viruses (bacteriophages) containing an

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endoglycanase specific for the host capsular polysaccharide makes possible an alternative approach. The depolymerase action of the viral enzyme yields an oligosaccharide, corresponding to the repeating unit (or a simple multiple thereof), the study of which provides most of the structural information desired, leaving only a few complementary experiments to be performed on the native polymer.

This approach is described here for the determination of the structure of the capsular polysaccharide (K antigen) of *Klebsiella* serotype K3. This is one of only seven polysaccharides from more than 80 *Klebsiella* strains[1] in which the uronic acid present is D-galacturonic acid[2,3]. Two of these polysaccharides [*Klebsiella* serotypes K34 (ref. 4) and K48] belong to the same chemotype and contain L-rhamnose and D-glucose as the neutral sugars. The polysaccharide of *Klebsiella* K3 belongs to a chemotype that includes K49, K56 (ref. 5), and K68 (ref. 6). These strains have D-mannose and D-galactose as the neutral sugars. The capsules of *Klebsiella* K3 and K68 (ref. 6) also contain 1-carboxyethylidene substituents.

RESULTS AND DISCUSSION

Preparation of oligosaccharide **P1**. — The capsular polysaccharide from *Klebsiella* K3 was depolymerized[7,8] by the homologous bacteriophage $\phi 3$ to give oligosaccharide **P1** in 82% yield. Analysis of **P1**, with and without reduction of the uronic acid, showed the mannose:galactose ratios to be 1.52:1.00 and 2.54:1.00,

TABLE I

METHYLATION ANALYSES OF K3 POLYSACCHARIDE AND DERIVED PRODUCTS

Methylated sugar[a] *(as alditol acetate)*	*Molar ratios*[b]								
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*
2,3,4,6-Man				0.10	0.10	0.15	1.10	0.81	
3,4,6-Man	1.00	0.85	1.00	1.00	0.92	0.90	1.05	0.96	0.90
2,4,6-Man	0.94	1.00	0.70	0.67	1.00	0.95	0.95	1.00	0.90
2,4,6-Gal + 2,5,6-Gal	1.04	0.80		1.11	0.95	1.00	1.00	1.16	1.00
2,3,6-Gal								0.29	
3,4,6-Gal								0.21	
3,6-Gal						0.50		0.70	
2,3-Man	0.98	0.85	0.95	0.60	0.81	0.50		0.17	0.30
2,3-Gal		0.60							
3-Gal					1.00				
1,2,4,5,6-Gal-ol			0.74						

[a] 2,3,4,6-Man = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol, etc. [b] I, Methylated **P1**; II, methylated and $LiAlH_4$-reduced **P1**; III, methylated **P1** alditol; IV, original capsular polysaccharide; V, original polysaccharide methylated then reduced with $LiAlH_4$; VI, carbodiimide-reduced polysaccharide, methylated; VII, methylated partially hydrolyzed polysaccharide (**PH**); VIII, methylated, $LiAlH_4$-reduced, partially hydrolyzed, remethylated polysaccharide; IX, degraded polymer obtained after β-elimination.

respectively. Oligosaccharide **P1** is thus composed of mannose, galactose, and galacturonic acid in the ratio 3:1:1, and analysis of **P1** alditol shows galactose to be the reducing sugar.

Methylation of **P1**. — Analyses were conducted on the neutral sugars of methylated **P1**, methylated **P1** reduced with lithium aluminum hydride, and methylated **P1** alditol (Table I, columns I, II and III). These results show **P1** to be a linear pentasaccharide with a 4,6-*O*-(1-carboxyethylidene)mannose unit and a 3-linked galactose residue as the nonreducing and reducing termini, respectively. One may, therefore, write the partial structure pyr$\prec$Man$\overset{1}{\text{—}}$(GalA,Man,Man)$\overset{3}{\text{—}}$Gal. Methylation analysis and a β-elimination experiment on the native polysaccharide (see later) indicated that (a) galacturonic acid is the branch point in the polymer and, (b) the acetalated mannose residue is the sole lateral substituent, linked to galacturonic acid at O-4. Thus, pyr$\prec$Man$\overset{1\ 4}{\text{——}}$GalA—(Man,Man)$\overset{3}{\text{—}}$Gal where the interior mannose units are 2- and 3-linked, in a sequence yet to be determined. This was achieved by a study of the n.m.r. spectra of **P1**.

^{1}H-N.m.r. spectra of **P1** *and deacetalated* **P1** *alditol.* — The numerical data from the ^{1}H- and ^{13}C-n.m.r. spectra of **P1** and acetalated **P1** alditol are collected in Tables II and III, and traces of the anomeric regions are shown in Figs. 1 and 2. The data for **P1** (Table II and Fig. 1a), collected at both ambient temperature and 95°, confirm the pentasaccharide nature of the oligomer. The spectrum (not illustrated) recorded at ambient temperature shows two fractional resonances, at δ 5.29 (0.4 H) and 4.63 (0.6 H), which represent the α- and β-pyranose configurations of the terminal galactose residue. The spectrum (Fig. 1a) recorded at the elevated temperature shows, in addition, a small fractional signal at δ 5.33 which may be assigned to H-1 of the terminal galactose residue in the β-furanose form. The fractional signal representing H-1 of the α-galactofuranose tautomer probably coincides with the signal at δ 5.29. The fractional signals at δ 5.28, 5.27, 5.19, and 5.17 in the spectrum recorded at 95° are attributable to the mannose unit adjacent to the reducing end; the four signals comprise two sets of twins[9] resulting from the presence of the terminal galactose in both ring forms in both α- and β-configurations. It is noteworthy that the signals at δ 5.17 and 5.19 do not appear in the spectrum of the oligosaccharide recorded at ambient temperature. Increased amounts of galactofuranose at higher temperatures have been observed in other cases, *e.g.* in the n.m.r. spectra of the oligosaccharide obtained[10] from *Klebsiella* K26 by the action of phage ϕ26.

The correctness of the above interpretation is confirmed by the spectrum of the deacetalated oligosaccharide alditol (Table II and Fig. 1b). Here the galactose unit has been reduced to galactitol, causing the replacement of the mannose signals by a singlet at δ 5.25, having an integral of unity. The fractional mannose signals discussed above are attributed to the 2-linked mannose for the following reasons. The ^{1}H-n.m.r. spectrum of the linear aldotetrauronic acid with the structure

TABLE II

^{1}H-N.M.R. DATA (400 MHz) FOR OLIGOSACCHARIDES **P1** AND REDUCED, DEACETALATED **P1**, AND K3 POLYSACCHARIDE

Sample	*Chemical shift*[a] *(p.p.m.)*		$J_{1,2}$[b] *(Hz)*	*Number of protons*		*Assignment*[c]
	ambient	*95°*		*ambient*	*95°*	
Oligosaccharide **P1**	5.36	5.40	3.0	1.0	1.0	3-Gal*p*A-α-
		5.33	3.5		0.06	3-Gal*f*-β-OH
	5.29	5.29	3.0	0.4	0.42	3-Gal*p*-α-OH 3-Gal*f*-α-OH
	5.31	5.28	n.o.	0.4	0.31	2-Man*p*(α1-3)Gal*p*-α-OH
	5.30	5.27	n.o.	0.6	0.50	2-Man*p*(α1-3)Gal*p*-β-OH
		5.19	n.o.		0.06	2-Man*p*(α1-3)Gal*f*-β-OH
		5.17	n.o.		0.13	2-Man*p*(α1-3)Gal*f*-α-OH
	5.00	5.04	n.o.	1.0	1.0	3-Man*p*-α-
		4.94	n.o.		0.2	Man*p*-α-
	4.91	4.90	n.o.	1.0	0.8	Man*p*-α- 4,6 pyr
	4.65	4.73	n.o.	1.0	1.0	H-5 of Gal*p*A
	4.63	4.64	8.0	0.6	0.52	3-Gal*p*-β-OH
		4.48	n.o.		0.20	H-2 of Man*p*-α-
	4.41	4.45	n.o.	1.0	0.80	H-2 of Man*p*-α- 4,6 pyr
	1.51	1.56	n.o.	3.0	2.5	CH_3-C- COOH
Deacetalated **P1** alditol		5.39	4		1.0	3-Gal*p*A-α-
		5.25	n.o.		1.0	2-Man*p*-α-
		5.07	n.o.		1.0	3-Man*p*-α-
		4.94	n.o.		0.9	Man*p*-α-
		4.90	n.o.		0.1	Man*p*-α- 4,6 pyr
		4.78	n.o.		1.0	H-5 of Gal*p*A-α-
		4.50	2		0.9	H-2 of Man*p*-α-
		4.46	2		0.1	H-2 of Man*p*-α- 4,6 pyr
		1.58			0.3	CH_3-C- COOH

TABLE II *(continued)*

Sample	*Chemical shift*[a] *(p.p.m.)*		$J_{1,2}$[b] *(Hz)*	*Number of protons*		*Assignment*[c]
	ambient	*95°*		*ambient*	*95°*	
Polysaccharide K3		5.59	~2		1.0	Gal*p*A-*α*-
		5.37	n.o.		1.0	2-Man*p*-*α*-
		5.07	n.o.		1.0	3-Man*p*-*α*-
		5.00	n.o.		1.0	Man*p*-*α*- 4 6 \ / pyr
		4.76	7		1.0	3-Gal*p*-*β*-
		4.49	n.o.		2.0	H-2 of Man*p*-*α*- 4 6 \ / pyr H-5 of Gal*p*A-*α*-
		1.54			3.0	CH_3-C- | COOH

[a]Chemical shift downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), measured from internal acetone at 2.23 p.p.m. [b]n.o., not observed. [c]The numerical prefix indicates the position in which the sugar is substituted; the *α* or *β*, the configuration at the glycosidic bond, or the anomer in the case of a reducing-end residue. Thus 3-Gal-*α* refers to the anomeric proton of a 3-linked galactosyl residue in the *α*-anomeric configuration. The absence of a numerical prefix indicates a nonreducing terminal group.

GlcA$\underset{\alpha}{\overset{1\ 3}{---}}Man\underset{\alpha}{\overset{1\ 2}{---}}Man\underset{\alpha}{\overset{1\ 3}{---}}$Gal isolated from a partial hydrolyzate of *Klebsiella* K47 shows[11] the chemical shifts of H-1 of the 2- and 3-linked mannoses at δ 5.29 and 5.07, respectively. A similar oligosaccharide alditol from *Klebsiella* K24 shows[12] H-1 of the 2- and 3-linked *α*-mannosyl residues resonating at δ 5.27 and 5.05, respectively. The assignments in both studies[11,12] were established from spectral data derived from the appropriate aldobiouronic and aldotriouronic acids. In the spectra of **P1** and **P1** alditol recorded at 95° the signals at δ 5.04 and 5.07 can now be assigned to the 3-linked *α*-mannosyl residue, which must be joined to the 2-linked mannose unit. The remaining mannose signal at δ 4.90 (0.8 H) in the spectrum of **P1** recorded at 95° can thus be attributed to the terminal 4,6-acetalated mannosyl residue attached to O-4 of the galacturonic acid.

In recording the spectrum of **P1** at elevated temperature approximately 20% of the 1-carboxyethylidene group was lost, as judged by the integral at δ 1.51. The partial removal of the acetal is accompanied by the appearance of a fractional signal at δ 4.94 (0.2 H), consistent with the presence of two oligosaccharides[9], one without and one with the 1-carboxyethylidene group, in the ratio 1:4. In the spectrum of

TABLE III

^{13}C-N.M.R. DATA (100 MHz) FOR OLIGOSACCHARIDES **P1** AND DEACETALATED **P1** ALDITOL, AND K3 POLYSACCHARIDE

Sample	*Chemical shift*[a] *(p.p.m.)*	*Assignment*[b]
Oligosaccharide **P1**	103.06	3-Man*p*-α- and
	102.98	4-Gal*p*A-α-
	101.39	Man*p*-α-
		4 6
		\ /
		pyr
	97.19	3-Gal-β-OH
	95.45	2-Man*p*(α1-3)Gal-β-OH
	95.17	2-Man*p*(α1-3)Gal-α-OH
	93.09	3-Gal-α-OH
	25.53	CH_3 of pyruvate
Deacetalated **P1** alditol	102.76	3-Man*p*-α-
	102.53	4-Gal*p*A-α-
	101.34	Man*p*-α-
	100.32	2-Man*p*-α-
Polysaccharide K3	105.41	3-Gal*p*-β-
	103.28	3-Man*p*-α-
	102.86	2,4-Gal*p*A-α-
	100.76	Man*p*-α-
		4 6
		\ /
		pyr
	95.89	3-Man*p*-α-
	25.89	CH_3 or pyruvate

[a]Chemical shift downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), measured from internal acetone at 31.07 p.p.m. [b]As footnote [c], Table II.

deacetalated **P1** alditol the ratio of the signals at δ 4.94 and 4.90 is now 0.9:0.1, in keeping with the presence of 10% of residual pyruvic acetal. The only other signals to be similarly affected by the 1-carboxyethylidene group are the resonances at δ 4.45, 4.48 and δ 4.46, 4.50 in the spectra of the **P1** and **P1** alditol, respectively. These may be assigned with confidence to H-2 of the terminal acetalated and deacetalated mannosyl residues, respectively. The signals at δ 5.40 ($J_{1,2}$ 2 Hz) and δ 5.39 ($J_{1,2}$ 2 Hz) in the spectra of **P1** and deacetalated **P1** alditol can then be assigned to H-1 of the α-galactopyranosyluronic acid residue. The remaining signal (δ 4.73 and 4.78, respectively) in the anomeric region of each spectrum is clearly not due to an anomeric proton, but most likely represents H-5 of the 4-linked α-galacturonic acid residue.

The chemical shift of H-1 of the terminal 4,6-*O*-(1-carboxyethylidene)-D-mannopyranosyl unit at δ 4.90 does not permit an unambiguous assignment of the α- or β-configuration to this residue. The value is, however, in agreement with that

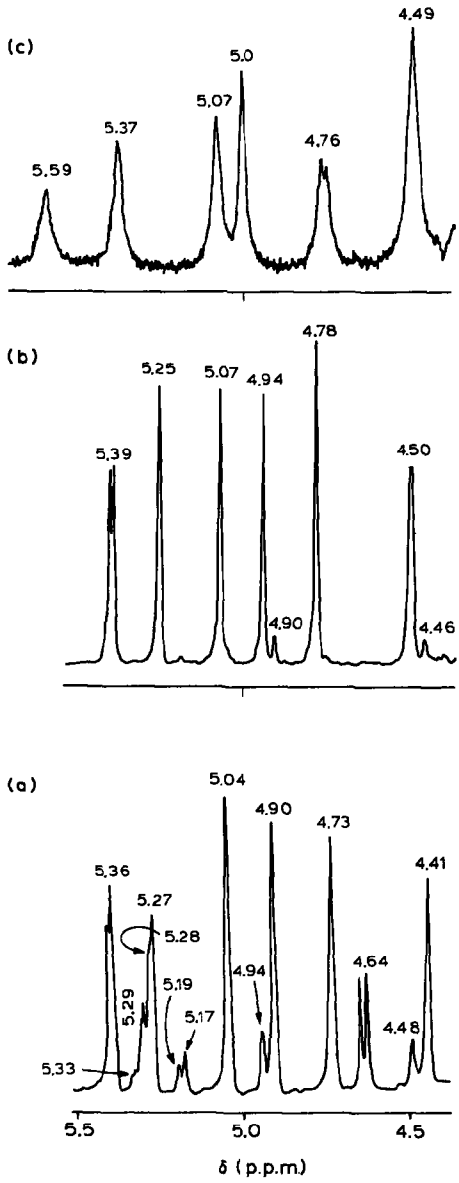

Fig. 1. ^{1}H-N.m.r. spectra (400 MHz) at 95° of (a) oligosaccharide **P1**, (b) deacetalated **P1** alditol, (c) K3 native polysaccharide. Anomeric region (δ 4.5–5.5) only.

found for a similar substituent in K68, where an α-configuration was assigned[6], and the signal is at lower field than the corresponding one from *Klebsiella* K6 polysaccharide, where a β-linkage (in-chain) was deduced to be present[13].

The remaining information required to fully describe the structure of polysaccharide K3 is the anomeric configuration of the galactose, which is linked to O-2

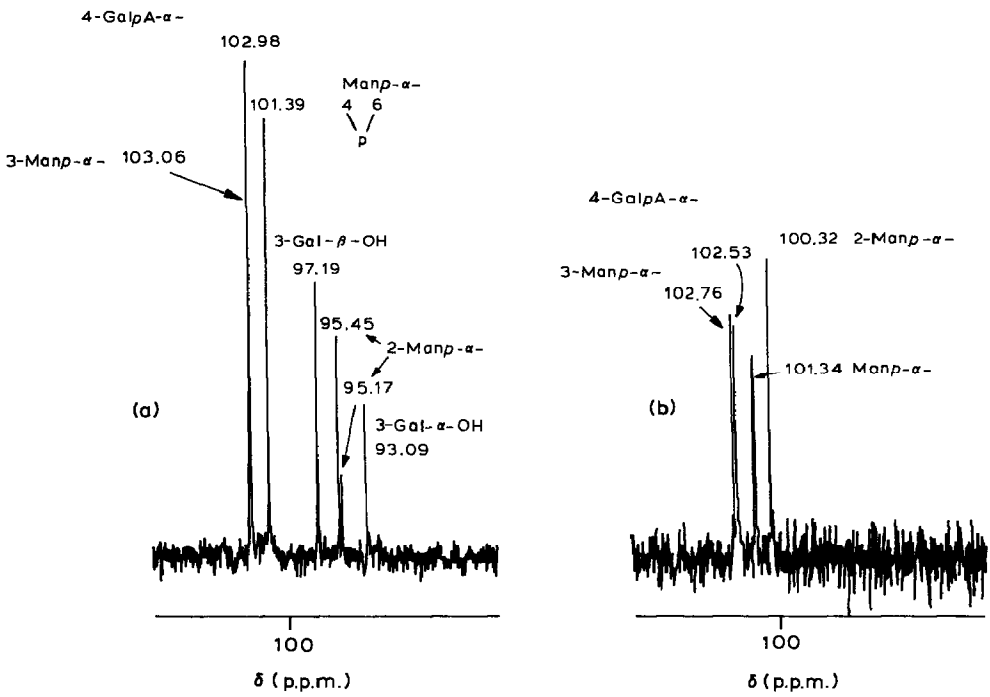

Fig. 2. ^{13}C-N.m.r. spectra of (a) oligosaccharide **P1**, and (b) deacetalated **P1** alditol. Anomeric region only.

of the galacturonic acid. Comparison of the ^{1}H spectra of K3 polysaccharide (Fig. 1c) and of **P1** (Fig. 1a) shows that the signal at δ 4.76 ($J_{1,2}$ 7 Hz) disappears in the formation of the oligosaccharide, being replaced by the fractional signals at δ 4.64, 5.29, and 5.23. The signal at δ 4.76 is thus due to the galactose residue, which must therefore be β-linked.

^{13}C-N.m.r. data. — The ^{13}C-spectral data (Table III), although not as informative as the ^{1}H data, nevertheless are in agreement with the proposed structure. The signals at 97.19 and 93.09 p.p.m. in Fig. 2a correspond to the β- and α-anomeric carbons of the terminal galactose unit in **P1**, while the fractional signals at 95.45 and 95.17 p.p.m. are attributable to the anomeric carbon of the 2-linked mannose adjacent to the galactose. The anomeric carbon atom of a mannose unit substituted at O-2 is known[14] to resonate at higher field than the 3-isomer, confirming the placement of the 2-linked mannose as the penultimate residue in **P1**. As expected, the ^{13}C spectrum of the deacetylated **P1** alditol (Fig. 2b) shows only four anomeric carbon signals. A comparison of the data (Table III) for the polysaccharide and **P1** shows the presence, in the former spectrum, of a signal at 105.41 p.p.m. Since this signal is absent from the **P1** spectrum, it may be assigned to the 3-linked β-galactopyranosyl residue. In the ^{13}C spectrum of a partially hydrolyzed sample of polysaccharide (data not shown) the signal at 100.76 p.p.m. is slightly

reduced, consistent with the loss of some mannose during the partial hydrolysis. This signal is consequently attributed to the terminal acetalated mannopyranosyl residue. The remaining two signals in the anomeric region of the polysaccharide spectrum, at 103.28 and 102.86 p.p.m., have been assigned to the anomeric carbons of 3-linked α-mannopyranose and 2,4-linked α-galactopyranosyluronic acid, respectively. The appearance of the 1-carboxyethylidene methyl carbon resonance at 25.89 and 25.53 p.p.m. in the spectra of the polysaccharide and **P1**, respectively, indicates that the acetal carbon has the *S* configuration[15].

Polysaccharide from Klebsiella *K3.* — The polysaccharide had the same composition as **P1**. The n.m.r.-spectral data are recorded in Tables II and III. Comparison of the optical rotation of the polysaccharide ($[\alpha]_D$ +83°) with that of **P1** ($[\alpha]_D$ +126°) is consistent with the assignment of a β-D-galactosidase activity to the phage enzyme. Individual sugars were isolated by paper chromatography from a hydrolyzate of the polysaccharide, and each was shown to have the D configuration by measurement of its optical rotation.

When the polysaccharide was subjected to methylation analyses without and with reduction of the uronic ester group, comparison of the results demonstrated (Table I, columns IV and V) that galacturonic acid was a branch-point residue, substituted at O-2 and O-4.

Partial hydrolysis of K3 polysaccharide gave a small quantity of mannose and a nondialyzable polymeric product (**PH**, $[\alpha]_D$ +97°). Methylation analyses of **PH**, and of a carboxyl-reduced[16] sample of native K3 polysaccharide, confirmed the presence of a lateral 4,6-*O*-(1-carboxyethylidene)mannosyl unit in the original polysaccharide (Table I, columns VI and VII).

In an attempt to establish the nature and the position of the side chain on the galacturonic acid, the methylated capsular polysaccharide was reduced with $LiAlH_4$, treated for a short period with formic acid at 100°, and then remethylated. G.l.c.–m.s. examination of the derived alditol acetates (Table I, column VIII) demonstrated that most of the pyruvate was removed. The appearance of the alditol acetates of 2,3,6- and 3,4,6-tri-*O*-methylgalactose shows that a portion of both the in-chain and branch residues linked to the galacturonic acid were cleaved. It is therefore not possible from this experiment to pinpoint the location of the side chain. However, the above data, coupled with the observation that the sum of the molar ratios of 2,3-di- and 2,3,4,6-tetra-*O*-methylmannose is approximately unity, indicate that the side chain cannot be composed of more than a single mannose residue. Fully methylated K43 polysaccharide was subjected to a β-elimination experiment[17] to give a nondialyzable product, analysis of which gave the results presented in Table I, column IX. From the amount of 2,3-di-*O*-methylmannose remaining it is clear that the reaction did not proceed to completion, but the decrease in this component, the others remaining constant, demonstrates that the side chain consists of only a single unit attached to O-4 of the uronic acid.

CONCLUSION

These experiments establish the structure of the *Klebsiella* K3 polysaccharide as shown in the Abstract, with the majority of the evidence being obtained from a study of the oligosaccharide **P1**. If the more traditional approach of concentrating on the examination of the polysaccharide had been adopted, it would still have been necessary to isolate some oligosaccharides in order to establish the sequence of the mannose and galactose residues. When a suitable bacteriophage is available the use of the viral endoglycanase provides an elegant and convenient alternative to chemical fragmentation.

EXPERIMENTAL

General methods. — Solutions were concentrated under diminished pressure at a bath temperature not exceeding 40°. Paper chromatography was performed on Whatman No. 1 paper, with the following solvent systems (v/v): (1) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water, (2) 8:2:1 ethyl acetate–pyridine–water, and (3) 2:1:1 1-butanol–acetic acid–water. After development, the spots were made visible with silver nitrate. Proton magnetic resonance spectra were recorded with a Bruker WH-400 spectrometer. Samples were exchanged (three or four times) by dissolving them in D_2O (99.7 atom %), then lyophilizing. Spectra were recorded for solutions in 99.7% D_2O containing acetone as an internal standard. The ^{13}C-n.m.r. spectra were recorded with the same spectrometer, and in the same manner.

Paper electrophoresis was performed in a Savant high voltage (5 kV) system (model LT-48A), with kerosene as the coolant. The buffer used was 5:2:743 (v/v) pyridine–acetic acid–water, pH 5.3. Strips (75 × 20 cm) of Whatman No. 1 paper were used for all runs, with the application of 15–25 mA for 1–1.5 h.

Optical rotations were measured at 23 ±2° on a Perkin–Elmer model 141 polarimeter, with a 1 cm cell. I.r. spectra were recorded with a Perkin–Elmer 457 spectrometer.

Analytical g.l.c. separations were performed using Hewlett–Packard models 5700 and 5890A and Packard–Becker model 417 instruments, all with flame-ionization detectors. Separations were carried out in stainless steel columns (1.8 or 2 m × 3 mm) packed with (A) 3% OV-225 or (B) 5% ECNSS-M, both on Gas-Chrom Q, or (C) 3% OV-17 or (D) 3% SP-2340, both on Chromosorb W, AW, DMCS. Column E was a fused silica capillary column, 30 m × 0.25 mm, J and W DB-225 (film thickness 0.25 μm).

Preparation and properties of Klebsiella *K3 polysaccharide.* — A culture of *Klebsiella* K3 was obtained from Dr. Ida Ørskov (Copenhagen). The polysaccharide, isolated as previously described[18], showed $[\alpha]_D$ +83° (*c* 1.0, water).

Bacteriophage depolymerization of polysaccharide K3. — Polysaccharide (1.0 g) was dissolved in 300 mL of partially purified lysate (method 4, ref. 7) con-

taining 1.38×10^{13} PFU, and the mixture was stirred at 28–30°. A small amount of chloroform was added to discourage bacterial growth. After 68 h the mixture was concentrated to 50 mL and dialyzed against distilled water (5 × 300 mL). The combined dialyzates were freeze-dried, the solid material obtained (2.1 g) was dissolved in water and passed through a column (2 × 25 cm) of Amberlite IR-120 (H^+) resin, and the effluent was freeze-dried. Three further treatments with resin were required to afford oligosaccharide (820 mg). Found: N, 0.63%. The product **P1** showed a single component on paper chromatography ($R_{cellobiose}$ 0.82, solvent 1) and on paper electrophoresis (M_{GalA} 0.83). Further purification of **P1** was effected by paper chromatography (solvent 1). The purified oligosaccharide (680 mg) had $[\alpha]_D$ +126.3° (*c* 1.7, water).

Analysis of the constituent sugars of native polysaccharide and **P1**. — Samples of K3 polysaccharide (20 mg) and **P1** (10 mg) in 3% methanolic hydrogen chloride were heated (oil bath) overnight under reflux. After neutralization (lead carbonate) and concentration, half of each sample was reduced (sodium borohydride in anhydrous methanol) and the product, after treatment with Amberlite IR-120 (H^+) resin and methanol to remove borate was further hydrolyzed with 2M trifluoroacetic acid (TFA). The other half of each sample was directly hydrolyzed with 2M TFA (16 h, 100°). Each of the four samples was freed of TFA, converted into a mixture of peracetylated aldononitriles[19], and examined by g.l.c. (column A at 200°).

P1 *alditol.* — **P1** (50 mg) in water (2 mL), reduced with sodium borohydride and subjected to the usual work up, afforded oligosaccharide alditol (40 mg). The oligosaccharide alditol was hydrolyzed with 2M TFA and the peracetylated aldononitriles were examined by g.l.c. (column A at 200°).

Partially hydrolyzed polysaccharide (**PH**). — Polysaccharide (140 mg) was hydrolyzed with 0.1M TFA (100°, 2 h) and then dialyzed. **PH**, recovered from the retentate by freeze-drying, had $[\alpha]_D$ +97° (*c* 2.0, water). It was hydrolyzed with 2M TFA, and the derived alditol acetates were examined by g.l.c. on column D.

Carboxyl-reduced K3 polysaccharide[16]. — Polysaccharide (200 mg) was dissolved in 25 mL of water, and the pH of the solution (2.50) was adjusted to 4.75 with 0.1M NaOH. Carbodiimide (*ca.* 1.40 g) was added and the solution was stirred, the pH being maintained at 4.75 with 0.1M HCl. After 90 min, $NaBH_4$ (2 g) was added and the pH of the solution being maintained at about 7 by the addition of 4M HCl. The mixture was dialyzed, the dialyzate was concentrated to about 25 mL, and a second reduction was carried out under the same conditions. The product, containing ~15% of residual galacturonic acid (carbazole), was analyzed by g.l.c. on column D.

Methylation analysis of **P1** *and its derivatives.* — (a) **P1** (20 mg) in anhydrous *N,N*-dimethylformamide (1 mL) was stirred for 2 h at <5° in the dark with silver oxide (200 mg) and methyl iodide (2 mL). Thereafter the mixture was stirred for 48 h at room temperature with a further addition of silver oxide and methyl iodide after 24 h. The fully methylated product was isolated in the conventional way and purified by passage through a column of Sephadex LH20. It was examined by m.s.,

and the derived partially methylated alditol acetates were examined by g.l.c. (columns B and E) and g.l.c.–m.s. (Table I, column I). (b) **P1** (30 mg) was methylated by the Hakomori[20] procedure and the product was reduced with $LiAlH_4$ in oxolane. Hydrolysis of the product with 2M TFA, reduction with sodium borohydride, and g.l.c.–m.s. analysis (columns B and E) of the acetylated, partially methylated alditols gave the results shown in Table I, column II. (c) **P1** alditol was methylated by the Hakomori method and the derived alditol acetates were examined by g.l.c.–m.s. (columns A, B and E). The data are recorded in Table I, column III.

Methylation analysis of the polysaccharide and its derivatives. — (a) Polysaccharide K3 (100 mg) in the acid form was methylated under Hakomori conditions and then by treatment with Purdie reagents in the presence of *N,N*-dimethylformamide. Successive hydrolysis of the methylated product with 2M TFA, reduction with sodium borohydride, and acetylation with pyridine and acetic anhydride afforded a mixture of partially methylated alditol acetates. Examination of the alditol acetates by g.l.c. on column A gave the results shown in Table I, column IV. (b) Carboxyl-reduced polysaccharide and partially hydrolyzed polysaccharide were similarly methylated. G.l.c.–m.s. analysis of the derived alditol acetates (column C) gave the results shown in columns VI and VII, respectively. (e) Methylated polysaccharide (50 mg) was reduced with $LiAlH_4$ in anhydrous oxolane. A portion of the product was converted into the alditol acetates and examined by g.l.c. (column A) and g.l.c.–m.s. The results are shown in column V. (d) Methylated, $LiAlH_4$-reduced polysaccharide (20 mg) in formic acid (2 mL) was heated for 15 min on a boiling water bath. The reaction mixture was concentrated to dryness, dissolved in *N,N*-dimethylformamide (1 mL), and methylated with methyl iodide and silver oxide. The isolated product was hydrolyzed (2M TFA), and the derived alditol acetates were examined by g.l.c.–m.s. (columns B and E). The results are shown in Table I, column VIII.

Base-catalyzed degradation of K3 polysaccharide[7]. — Methylated K3 polysaccharide (120 mg) was stirred overnight with 2M potassium methylsulfinylmethylide. The reaction was stopped by the addition of ice and water, the solution was neutralized with 50% acetic acid and dialyzed against running water, and the product was isolated by lyophilization. The β-eliminated polysaccharide was hydrolyzed with 50% acetic acid (5 mL, 1 h, 100°) and dialyzed. The non-dialyzable, polymeric product was hydrolyzed, first with 90% formic acid and then with 2M TFA. The results of the g.l.c. examination (column D) of the derived alditol acetates are shown in Table I, column IX.

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REFERENCES

1 I. ØRSKOV, *Acta Pathol. Microbiol. Scand.*, 38 (1956) 375–384.
2 W. NIMMICH, *Acta Biol. Med. Ger.*, 26 (1971) 397–403.
3 W. NIMMICH, *Z. Med. Mikrobiol. Immunol.*, 154 (1968) 117–131.
4 J.-P. JOSELEAU, F. MICHON, AND M. VIGNON, *Carbohydr. Res.*, 101 (1982) 175–185.
5 J. P. KAMERLING, B. LINDBERG, J. LÖNNGREN, AND W. NIMMICH, *Acta Chem. Scand., Ser. B*, 29 (1975) 593–598.
6 H. PAROLIS, L. A. S. PAROLIS, AND G. G. S. DUTTON, *Carbohydr. Res.*, 152 (1986) 249–259.
7 G. G. S. DUTTON, J. L. DI FABIO, D. M. LEEK, E. H. MERRIFIELD, J. R. NUNN, AND A. M. STEPHEN, *Carbohydr. Res.*, 97 (1981) 127–138.
8 H. THUROW, H. NIEMANN, AND S. STIRM, *Carbohydr. Res.*, 41 (1975) 257–271.
9 J. L. DI FABIO, G. G. S. DUTTON, AND H. PAROLIS, *Carbohydr. Res.*, 133 (1984) 125–133.
10 J. L. DI FABIO, D. N. KARUNARATNE, AND G. G. S. DUTTON, *Carbohydr. Res.*, 144 (1985) 251–262.
11 G. G. S. DUTTON AND M. PAULIN, *Carbohydr. Res.*, 87 (1980) 119–127.
12 Y. M. CHOY, G. G. S. DUTTON, AND A. M. ZANLUNGO, *Can. J. Chem.*, 51 (1973) 1819–1825.
13 U. ELASSER-BEILE, J. FRIEBOLIN, AND S. STIRM, *Carbohydr. Res.*, 65 (1978) 245–249.
14 P. A. J. GORIN, *Adv. Carbohydr. Chem. Biochem.*, 38 (1981) 13–104.
15 P. J. GAREGG, B. LINDBERG, AND I. KVARNSTROM, *Carbohydr. Res.*, 77 (1979) 71–78.
16 R. L. TAYLOR AND H. E. CONRAD, *Biochemistry*, 11 (1972) 1383–1388.
17 B. LINDBERG, J. LÖNNGREN, AND J. L. THOMPSON, *Carbohydr. Res.*, 28 (1973) 351–357.
18 Y. M. CHOY AND G. G. S. DUTTON, *Can. J. Chem.*, 51 (1973) 198–207.
19 G. D. MCGINNIS, *Carbohydr. Res.*, 108 (1982) 284–292.
20 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.