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Klebsiella Serotype 25 Capsular Polysaccharide: Primary Structure and Depolymerization by a Bacteriophage-Borne Glycanase

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By partial acid hydrolysis, methylation and gas-liquid chromatography-mass spectrometry of the methylated monomers (as the alditol acetates), mass spectrometry of trimethylsilylated disaccharide alditols, as well as proton magnetic resonance, the primary structure of the *Klebsiella* serotype 25 capsular polysaccharide was elucidated. A glycanase activity, associated with the particles of newly isolated *Klebsiella* bacteriophage no. 25, was shown to catalyze the hydrolysis of the glycan.

So far, 81 K serotypes belonging to the genus *Klebsiella* have been recognized (31, 32). Roughly half of the different capsular polysaccharides carrying these serological determinants have been, or are being subjected to, structural analyses (see references 5, 7-9, 11-13, 41). All these glycans are rather similar—most of them contain either D-glucuronic acid (GlcUA) or, occasionally, D-galacturonic acid, as well as two to four of the following aldohexoses: D-glucose (Glc), D-galactose (Gal), D-mannose, L-fucose, and L-rhamnose (31). Therefore, this set of polysaccharides is well suited to a variety of comparative studies: for instance, by nuclear magnetic resonance (e.g., reference 1), on the substrate specificity of bacteriophage-borne glycanases (e.g., reference 42), on the chemical basis of the serological cross-reactions between *Klebsiella* K antigens (e.g., references 14, 15, 17) or between *Klebsiella* antigens and those of other bacterial genera (e.g., reference 18), or on polysaccharide conformation.

For these reasons, we have elucidated the primary structure of yet another *Klebsiella* capsular polysaccharide, the K serotype 25 glycan. In addition, we describe the depolymerization of this material by a glycanase activity associated with the particles of a newly isolated phage, *Klebsiella* bacteriophage no. 25.

MATERIALS AND METHODS

Media. Merck standard I broth was generally used for liquid and solid media, and an additional 0.3% (wt/vol) of Glc was added for the large-scale propagation of phage. For the production of capsular polysaccharide, *Klebsiella* 2002/49 was grown on $D_{1.5}$ agar (36).

Bacteria and bacteriophage. *Klebsiella* 2002/49 (O3:K25) (14, 33), the serological test strain for the *Klebsiella* K25 antigen (15, 24, 31), was used; it was kindly supplied by Ida Ørskov, World Health Organization International Escherichia Center, Statens Seruminstitut, Copenhagen.

Klebsiella bacteriophage no. 25 was isolated from Freiburg sewage. It forms plaques with acapsular halos (2, 40) on *Klebsiella* 2002/49.

***Klebsiella* serotype 25 capsular polysacharide—isolation, characterization, and partial debranching.** *Klebsiella* 2002/49 was grown on $D_{1.5}$ agar (overnight at 37°C, and then for 2 days at room temperature) and extracted with phenol-water (45). After sedimentation of the cell wall lipopolysaccharide, the acidic capsular polysaccharide was isolated from the aqueous phase by fractional cetyltrimethylammonium bromide precipitation (0.25 to 0.06 M NaCl) (22). After mild alkali treatment (4 h at 56°C in 0.25 N aqueous NaOH) (20, 22), the material was dialyzed against distilled water and lyophilized. Seven to eight milligrams of polysaccharide (sodium salt) was obtained from one 14-cm agar plate (250 mg of dry bacteria).

The analytical ultracentrifugation of the material was carried out in a Spinco model E instrument using an An-H-Ti rotor and Schlieren optics. For the measurement of the optical rotation (at 589 nm), a polarimeter (model 141, Perkin Elmer) was employed, and the equivalent weight of the glycan was determined by titration of an aqueous solution of the freeze-dried acidic form (obtained by passage over a Dowex 50/H^{+} column).

For partial debranching, a 1% (wt/vol) solution of K25 polysaccharide in 0.1 M aqueous trifluoroacetic acid (TFA) was heated at 100°C (compare reference 8). After 60 min, the residual polymer was precipitated with 5 volumes of ethanol, washed with ethanol, dissolved in distilled water, dialyzed against distilled water, and lyophilized. The yield was 65% (wt/wt).

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Klebsiella bacteriophage no. 25 – isolation, propagation, purification, and electron microscopy. Using *Klebsiella* 2002/49 as a prospective host, phage 25 was enriched from Freiburg sewage (39) and selected because of its plaque morphology, i.e., formation of acapsular haloes (2, 40). The virus was purified by 10 successive single-plaque isolations.

The propagation of phage 25 was carried out in an Eschweiler (Kiel, Germany) "Kleinfermenter Kiel": 10 liters of broth (with an additional 0.3% (wt/vol) of glucose added) was inoculated with *Klebsiella* 2002/49 and then incubated at 37°C with aeration (15 liters/min) and stirring (200 rpm). When the optical density at 660 nm had reached 1.3 in a 1-cm cell (about 8×10^8 colony-forming units per ml), 1 to 3 plaque-forming units (PFU) of phage 25 was added per bacterium. About 20 min later, lysis began. Incubation was continued for a total of 50 min after addition of virus, and the lysate was then clarified by centrifugation (10 min at $5,000 \times g$) after addition of 0.05% (wt/vol) sodium azide. The supernatant usually had a titer of 2×10 to -3×10^{10} PFU/ml.

The virus particles were purified from the lysates by consecutive precipitation with 10% (wt/vol) polyethylene glycol 6000 and isopycnic centrifugation (34, 42, 46). The phages banded around $\rho = 1.49$ g/ml; they were dialyzed against phosphate-buffered physiological saline, pH 7.3 to 7.4. Twenty-one percent of the PFU in the lysate were recovered in this manner. Upon addition of chloroform, the virus suspensions could be stored at 4°C for several months without appreciable loss of infectivity.

For electron microscopy (compare reference 39), a small volume of the virus suspension was dialyzed against 1% (wt/vol) aqueous ammonium acetate. Samples were placed on specimen grids coated with defatted carbon Holey films, negatively stained with uranyl acetate and formate, and observed with an electron microscope (Siemens Elmiskop IA).

Qualitative and quantitative monosaccharide analyses. For a qualitative identification of the monosaccharide constituents in oligo- and polysaccharides, the materials were hydrolyzed with 1 N H_2SO_4 at 100°C for 24 h, neutralized with $BaCO_3$, and subjected to descending paper chromatography (PC) with ethylacetate-pyridine-water (4:1:1, vol/vol/vol; solvent A) as in irrigant, as well as to paper electrophoresis (PE) (at 45 V/cm for 90 min) in pyridine-glacial acetic acid-water (10:4:86, vol/vol/vol; pH 5.3). Staining was carried out according to Trevelyan et al. (43).

The quantitative determinations of uronic acid were carried out directly in the polymers using the carbazole-sulfuric acid method (3) and GlcUA as a standard. The aldohexoses were determined in hydrolysates obtained by heating 0.2% (wt/vol) solutions of the materials in 0.5 N H_2SO_4 for 16 h (maximum of free Glc) or for 26 h (maximum of free Gal) at 100°C and subsequent neutralization with the same volume of 0.5 N NaOH. The two sugars were estimated enzymatically with fungal glucose oxidase (37) (EC 1.1.3.4; Boehringer no. 15755), or with galactose dehydrogenase from *Pseudomonas fluorescens* (44) (EC 1.1.1.48; Boehringer no. 15921), respectively, as well as by gas-liquid chromatography (GLC) of the alditol acetates (35). For this purpose, a Varian aerograph was used (model 15208, equipped with a flame ionization detector and a digital integrator, model 485), and glass columns (5 ft by 1/4 inch [152 by 0.64 cm]) filled with 3% (wt/wt) ECNSS-M on Gas-Chrom Q (100 to 120 mesh); nitrogen (28 ml/min) served as a carrier gas.

Methylation analyses. The methylations of intact, and of partially debranched, K25 polysaccharide (5- to 20-mg portions) were carried out by the procedure of Hakomori (16), as modified by Hellerqvist et al. (19). The methylated polymers were purified by passage over a column of Sephadex LH-20 (30 cm; by 1 cm^2) with chloroform (3 ml/h); the yield was about 75% (wt/wt).

For reduction-deuteration of the carboxyl ester groups in the methylated polymers, a solution of calcium borodeuteride (25) was first prepared by suspending 0.1 μmol each of $NaBD_4$ and $CaCl_2$ in 5 ml of dry tetrahydrofuran and stirring overnight at room temperature. Two milliliters of the supernatant was then mixed with 6 to 7 mg of methylated polymer. After stirring at room temperature overnight, 20 ml of water was added, and the mixtures were dialyzed against distilled water and lyophilized. The yield (wt/wt) was practically quantitative, and nearly 90% of the carboxyl ester groups were reduced in this manner (see Table 2).

All methylated products were hydrolyzed by sequential heating in 90% formic acid and in 0.25 N H_2SO_4 (19).

The methylated monomers were identified by PC of the free aldoses (38) and by combined GLC-mass spectrometry (GLC-MS) of the alditol acetates (4.27) – also with the help of suitable standards. For descending PC, butanone, saturated with 1% aqueous ammonia (solvent B), served as an irrigant; the chromatograms were sprayed with *p*-anisidine (1.5%)-trichloroacetic acid (5%). For GLC-MS, the mixtures of aldoses were reduced and acetylated, and first subjected to GLC on ECNSS-M as above; the retention times were recorded at a constant temperature of 155°C, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (2,3,4,6-Glc; $T = 1.00$ and to 2,3-Glc ($T = 5.39$) as internal standards. Instead of ECNSS-M, OV-225 (3%, wt/wt) on Chromosorb G-AW-DMCS (80 to 100 mesh) was exceptionally used for the separation of 2,3,6-Glc and 2,3,4-Glc (27) (see Table 2, footnote *g*). MS was carried out with a combined Finnigan GC (model 9500)/MS (model 3200E-003) instrument coupled to a model 600 Interactive Control and Graphic Output System. Again, ECNSS-M columns were employed (starting temperature, 155°C; temperature increment, 2°C/min), but with helium (25 ml/min) as a carrier gas. Electron impact ionization was used; the ionization potential was 72 eV, and the ionization current was 380 μA.

Isolation and analyses of the K25 aldobiouronic acid. Sixty milligram of K25 polysaccharide in 10 ml of 1 N H_2SO_4 was heated to 100°C for 150 min. Upon neutralization with barium carbonate and evaporation, the biouronic acid (mobility, relative to GlcUA, 0.73) was isolated by preparative PE (see above) and further purified and desalted by passage over a Sephadex G-10 column (95 cm by 2.3 cm^2; 7 ml/h) with a volatile buffer (pyridine-glacial acetic

acid-water, 10:4:1,000, vol/vol/vol; pH 4.5) and evaporation; 13 mg (22%, wt/wt) was obtained.

The digestion of the aldobiouronic acid with β-glucuronidase from *Helix pomatia* (EC 3.2.1.31; Boehringer no. 15472, free of α-glucuronidase activity) was carried out in a pH 4.5 acetate buffer (26): the products were tested by PC (solvent A) and by PE.

3-*O*-(β-D-glucopyranosyluronic acid)-D-galactose was obtained from *Escherichia coli* serotype 29 capsular polysaccharide (10, 30) as described by Choy et al. (10). It was separated from the K25 aldobiouronic acid by PC with ethyl acetate-glacial acetic acid-formic acid (98%)-water (18:3:1:4, vol/vol/vol/vol; solvent C).

For the MS analysis of the linkage position (23, 29), two aliquots of about 400 μg of aldobiouronic acid were reduced with $NaBH_4$, or with $NaBD_4$, in 0.01 N aqueous NaOH. After decationization with Dowex 50/H^+ and chasing of the boric acid by repeated evaporation with methanol, a solution of diazomethane in ether was added to both samples at 0°C until the yellow color remained. The mixtures were then stored at room temperature for 30 min and evaporated. The carboxyl acid methyl esters obtained were finally reduced with calcium borohydride in tetrahydrofuran (analogous to the description given above). After stirring at room temperature overnight, water was added to both samples. Cations and boric acid were again removed, and the final products were trimethylsilyated as detailed by Kärkkäinen (23). GC and combined GLC-MS were carried out as described above, but with columns of SE-30 (1%, wt/wt), on Chromosorb G-AW-DMCS (80 to 100 mesh) at 260°C.

Bacteriophage-catalyzed degradation of K25 polysaccharide. The time course of the depolymerization reaction was recorded as follows: 1.3×10^{12} PFU of purified phage 25 particles in 3 ml of phosphate-buffered saline was added to 200 mg of K25 polysaccharide in 50 ml. Three milliliters of the mixture was immediately transferred into an Ostwald viscosimeter, and the efflux time was recorded at intervals, while sequential samples were taken from the rest, and the reducing power was determined according to Imoto and Yagishita (21), with Glc as a standard. Both the viscosimeter and the rest of the mixture were kept at 37°C throughout.

For the isolation of phage degradation products, the incubation of 192 mg of polysaccharide with viruses was continued for 38 h. The mixture was then lyophilized, desalted in two portions by passage over the Sephadex G-10 column with the volatile buffer (data as above), lyophilized again, and adsorbed to a diethylaminoethyl-Sephadex A-25 column (31.5 cm by 1.75 cm^2), equilibrated with a 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.2. Elution (at 7 ml/h) was affected by addition of a linear 0 to 0.5 M NaCl gradient to the buffer. The effluent was analyzed with carbazole-sulfuric acid (3), and the peak fractions (see Fig. 3b) were pooled and desalted by passage over Sephadex G-10 in the volatile buffer (data as above) and lyophilization. Oligosaccharides P1, P2, and P3 were obtained in a yield of 64, 89, and 19 mg (in total, about 90%, wt/wt, from the polymer), respectively.

Analysis of the K25 repeating-unit tetrasaccharide. A solution of 19 μmol of $NaBH_4/NaB^3H_4$ (specific activity, 133 mCi/mmol) in 300 μl of 0.01 N NaOH (or in 300 μl of aqueous boric acid to give pH 8.0) was added to a mixture of 1.3 μmol of P1 and 2.5 μmol of xylose (internal standard) in 300 μl of water. After storage at room temperature for 6 h, excess $NaBH_4/NaB^3H_4$ was destroyed with acetic acid. Upon decationization and chasing of the boric acid, the residue was hydrolyzed, neutralized, and again brought to dryness. After reduction with $NaBH_4$ and acetylation, the mixture of partially labeled alditol acetates was subjected to GLC on ECNSS-M. Using the B channel of the Varian aerograph, and 1:10 splitting, the peracetyl-xylitol, -galactitol, and -glucitol were collected. The fractions were taken up on chloroform and counted with a Tri-Carb liquid scintillation spectrometer (Packard, model 2450) after addition of Scintigel.

Proton magnetic resonance (PMR). Solutions, 3.5 to 5% (wt/vol), of K25 repeating-unit tetrasaccharide P1 and of debranched K25 polysaccharide in absolute deuterium oxide were repeatedly evaporated and taken up in absolute deuterium oxide again (1). The spectra were run at 70°C, with use of a computerized Bruker HFX instrument set at 90 MHz and acetone or the sodium salt of 3-(trimethylsilyl)-propionic acid-d_4 as an internal standard.

RESULTS

***Klebsiella* serotype 25 capsular polysaccharide—isolation, characterization, and partial debranching.** As isolated from *Klebsiella* 2002/49 by the phenol-water-cetyltrimethylammonium bromide procedure (22, 45), followed by mild alkali treatment to break interchain linkages (see [20, 22, 41]), the *Klebsiella* K25 polysaccharide sedimented uniformly in the analytical ultracentrifuge ($s^{c}_{20} = 2.05 \times 10^{-13}$ s, in a 0.36% solution, wt/vol, in phosphate-buffered saline). Its quantitative sugar composition, optical rotation, and equivalent weight, as well as those of a partially debranched polymer derivative—obtained by mild hydrolysis with trifluoroacetic acid (TFA) (8)—are recorded in Table 1. It can be seen that K25 polysaccharide consists of Glc:Gal:GlcUA in a molar ratio approaching 2:1:1 and that the TFA-treated material has lost about 50% of one Glc equivalent.

***Klebsiella* bacteriophage no. 25—isolation, propagation, purification, and electron microscopy.** With *Klebsiella* 2002/49 as a host, phage 25 was isolated from sewage and selected because of its plaque morphology (acapsular halo), which showed (2, 40) that a host capsule depolymerase activity is associated with the virions. The bacteriophage was propagated in 10-liter batches and purified by polyethylene glycol precipitation and isopycnic centrifugation (46). Electron micrographs of the virus are shown in Fig. 1. It can be seen that phage 25 belongs to Bradley group B (6); it has an iso-

TABLE 1. *Composition and characteristics of Klebsiella serotype 25 capsular polysaccharide and a partially debranched derivative*

Determination	*Klebsiella* K25 polysaccharide	
	As isolated[a]	After mild TFA treatment (partial debranching)
Wt% (approximate molar ratio) of:		
Anhydro-D-Glc[b]	37.6 (2.02)	39.8 (1.51)
-Gal[b]	18.6 (1.00)	26.3 (1.00)
-GlcUA[c]	18.9 (0.94)	27.3 (0.96)
Water[d]	18.0	ND[e]
Sodium[f]	2.2	ND
Total	95.3	ND
Optical rotation $[\alpha]^{25}_{589}$	−40.7° (c = 0.3, water)	−22.4° (c = 0.6, water)
Equivalent weight	619[g]	ND

[a] Including treatment with mild alkali, i.e., possibly also saponification of *O*-acetyl substituents (compare [22, 41]), the original presence of which has not been determined.

[b] Assigned to the D series by enzymatic determination.

[c] Assigned to the D series by enzymatic cleavage of the biouronic acid (see text).

[d] Loss of weight after 24 h at 50°C in vacuo over phosphorus pentoxide.

[e] ND, Not determined.

[f] Calculated from the GlcUA value.

[g] Note that 662 is calculated for a tetrasaccharide repeating unit containing three hexoses and one hexuronic acid.

metric head, 52 to 58 nm in diameter, and a noncontractile, cross-striated tail, most often 153 to 161 nm in length and about 10 nm in thickness. At the end of these tails, drop- or club-shaped appendages ("spikes") are occasionally visible.

Methylation analyses. *Klebsiella* K25 polysaccharide, as well as its partially debranched polymer derivative were permethylated (16, 19), and the carboxylmethyl ester groups were subsequently dideuterated-reduced with calcium borodeuteride (25). After hydrolysis, the methylated monomers were analyzed first as the free aldoses by PC (38) and then as the acetylated alditols by GLC-MS (4, 27). The results are summarized in Table 2. It can be seen that roughly equimolar amounts of 2,3,4,6-Glc (=2,3,4,6-tetra-*O*-methyl-D-glucose, etc.), 2,3,6-Glc, and 2,6-Gal were identified in the intact polymer before and, additionally, an equally equimolar amount of 3,4-Glc (dideuterated at C6) after carboxyl reduction-dideuteration. The partially debranched (TFA-treated) polymer still yielded equimolar amounts of 2,3,6-Glc and 2,6-Gal after methylation and carboxyl reduction-dideuteration. However, about half of the 2,3,4,6-Glc was lost and, of the 0.9 mol equivalent of GlcUA that was reduced and thus recognized, again, about half now appeared as 2,3,4-Glc instead of 3,4-Glc (both dideuterated at C6).

K25 aldobiouronic acid. More rigorous, but still partial, acid hydrolysis of K25 polysaccharide yielded an aldobiouronic acid. The material was isolated by preparative PE and found to consist of GlcUA and Gal; digestion with β-glucuronidase yielded the components, and the optimal rotation was $[\alpha]^{25}_{589}$ = +15° (c = 0.2, water).

The K25 aldobiouronic acid exhibited a mobility of 0.73 and an R_f of 0.47 relative to GlcUA in PE (at pH 5.3) and PC (solvent C), respectively—as compared with 0.66 (10, 30, 41) and 0.43 for 3-*O*-(β-D-glucopyranosyluronic acid)-Gal.

For MS analysis of the linkage position, two aliquots of the biouronic acid were reduced with sodium borohydride or with sodium borodeuteride, respectively. The pair of unlabeled, and of C′(1)-monodeuterated, biouronitols was esterified with diazomethane, carboxyl-reduced with calcium borohydride (25), trimethylsilylated (23), and subjected to GLC-MS (23, 29). The results are given in Fig. 2.

Bacteriophage-catalyzed degradation of K25 polysaccharide and analysis of the degradation products. A depolymerization of K25 polysaccharide occurred upon incubation of the material (at 37°C in PBS) with purified particles of phage 25 (Fig. 3a). The oligosaccharides obtained after exhaustive degradation were separated and isolated by ion exchange chromatography (Fig. 3b). Oligosaccharides P1, P2, and P3 were obtained with a yield of about 33, 46, and 10% (wt/wt polymer). An optical rotation of $[\alpha]^{25}_{589}$ = +1.9° (c = 1.8, water) was determined for P1.

The smallest oligosaccharide thus obtained, P1, was found to contain Glc, Gal, and GlcUA in a molar ratio approaching 2:1:1. For the identification of its reducing sugar and the determination of its size, P1 was reduced with $NaBH_4$/NaB_3H_4, hydrolyzed, reduced with $NaBH_4$, and acetylated, and the *O*-acetyl-hexitols were subjected to GLC. The ratio of unlabeled versus labeled hexitols (without gulonic acid) was found to approach 2:1, whereas the ratio of radioactive galactitol to radioactive glucitol was 4.7:1 or 13.0:1, depending on the pH (10 or 8) at which the reduction with $NaBH_4$/NaB^3H_4 was carried out.

PMR. Due to the viscosity even of dilute solutions, PMR spectra of the K25 polysaccharide were not obtained. Sufficiently concentrated solutions of the K25 repeating-unit tetrasaccharide (oligosaccharide P1), as well as of the partially debranched polymer, however,

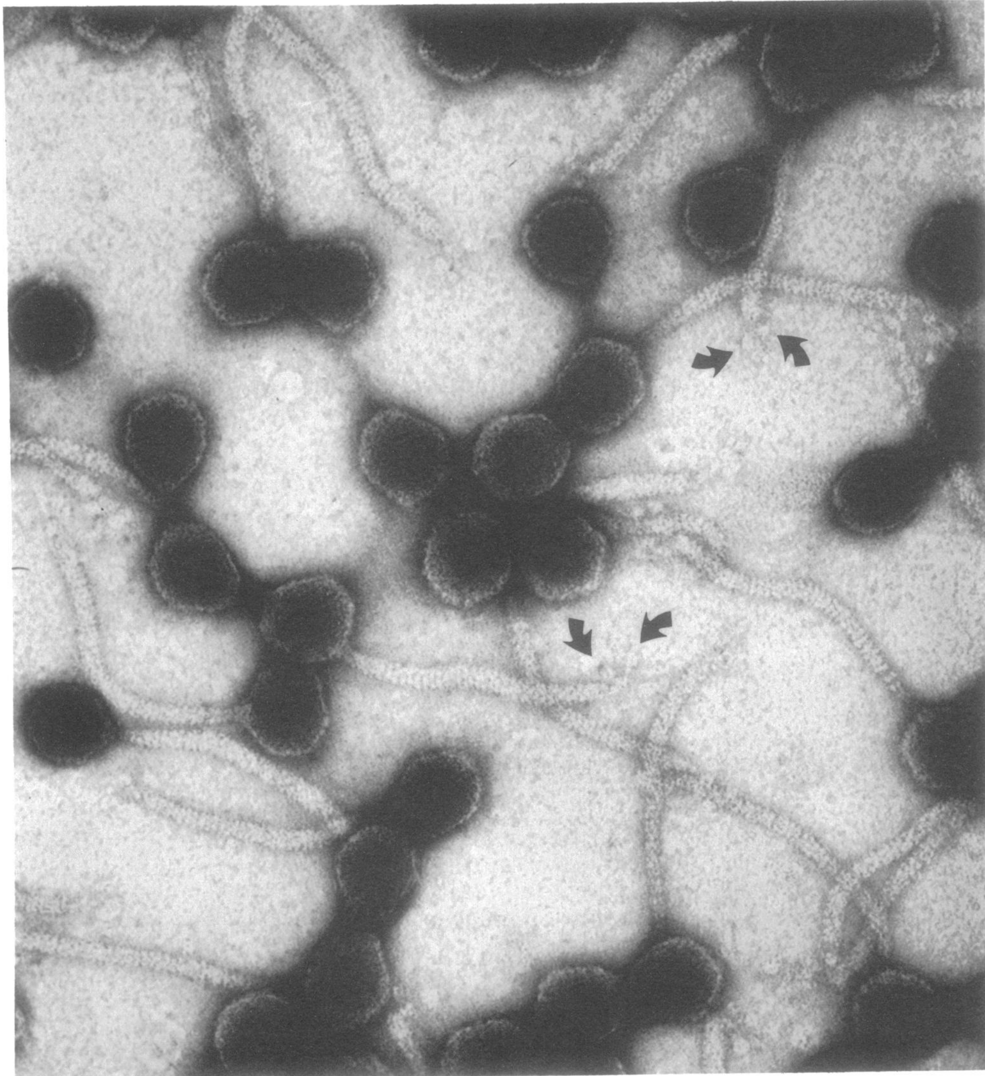

FIG. 1. *Electron micrograph of Klebsiella bacteriophage no. 25, negatively stained with uranyl acetate and formate (×256,000). Arrows point to tail tip appendages ("spikes").*

could be prepared, and the spectra were run at elevated temperature in absolute deuterium oxide (1). The spectrum of oligosaccharide P1 showed three signals of axial anomeric protons—β-linkages; Δ = 4.59, 4.75, and 5.01, respectively; $J_{1,2}$ = 7.0, 7.0, and 6.5 Hz, and one of an equatorial α-linkage; Δ = 5.25; $J_{1,2}$ = 3.2 Hz, with a ratio of peak integrals approaching 1.5:1:1:0.5. No signal was detected at α = 1.5 (pyruvate acetal protons; see [1]). The spectrum of the partially debranched polysaccharide, on the other hand, showed axial anomeric protons (β-linkages) only.

DISCUSSION

Klebsiella serotype 25 capsular polysaccharide, as isolated from *Klebsiella* 2002/49 (O3:K25), the serological test strain for the K25 antigen (14, 24, 31, 33), was found (Table 1) to contain Glc, Gal, and GlcUA, in agreement with the results of Nimmich (31). As evidenced by PMR (1), K25 glycan does not carry pyruvate acetal residues (also, W. Nimmich, personal communication).

K25 polysaccharide consists of tetrasaccharide repeating units. This follows from its equiv-

Table 2. *Identification and ratios of methylated aldoses obtained from Klebsiella serotype 25 capsular polysaccharide and its derivatives*

Methylated aldose[a]	R_f[b] of free aldose in PC		T[c] of alditol acetate in GLC		Primary fragments (m/e) found in alditol acetate						Ratio of alditol acetate peak integrals (presence of aldose spot with appropriate R_f)[d]		
	Literature value	Found	Literature value	Found	45	117	161	189	205	233	I	II	III
2.3.4.6-Glc	0.78	0.78	1.00	1.00[e]	+	+	+		+		0.9 (+)	0.8 (+)	0.4 (+)
2.3.6-Glc	0.56	0.57	2.50	2.35[f,g]	+	+				+	0.9 (+)	1.2 (+)	0.8[g] (+)
2.3.4-Glc	0.58	0.60	2.49	2.35[g]		+	+	(191)[h]		(235)[h]	− (−)	− (−)	0.4[g] (+)
2.6-Gal		0.24	3.65	3.68[i]	+	+		+			1.0[d](+)	1.0[d] (+)	1.0[d] (+)
3.4-Glc		0.32	5.27	5.29				(191)[j]			− (−)	0.9 (+)	0.5 (+)

[a] 2.3.4.6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc.

[b] R_f value of the free aldose relative to 2.3.4.6-Glc (R_f = 0.78) and 2.3-Glc (R_f = 0.28) in PC with the butanone-water azeotrope as an irrigant (solvent B) (38).

[c] Retention time of the alditol acetate relative to 2.3.4.6-Glc (T = 1.00) and 2.3-Glc (T = 5.39) in GLC on an ECNSS-M column (4, 27).

[d] I, Methylated K25 polysaccharide; the GlcUA derivative is not detected with the methods used. II, K25 polysaccharide, methylated, and then carboxyl reduced-dideuterated. III, Partially debranched K25 polysaccharide (see Table 1), methylated, and then carboxyl reduced-dideuterated. The ratio of the alditol acetate peak integrals in GLC is given (based on 2.6-Gal = 1.0); in addition, (+) and (−) designate the presence or absence of a spot with the appropriate R_f (and color) in PC of the aldoses.

[e] Inseparable from a standard, but separable from authentic 2.3.4.6-Gal.

[f] Separable from authentic 2.3.6-Gal.

[g] Separation of the two trimethyl alditol acetates was achieved by GLC on OV-225 (27).

[h] Dideuterated fragment found instead.

[i] Inseparable from authentic 2.6-Gal.

[j] Dideuterated fragment was also found.

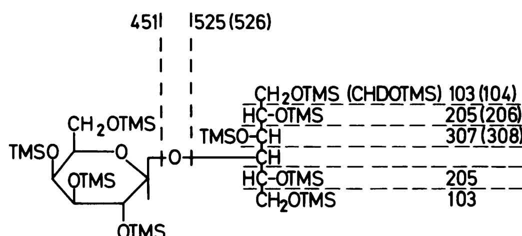

Fig. 2. *MS of the trimethylsilylated (TMS) aldobiitol obtained from K25 aldobiouronic acid by reduction of the aldehyde and carboxyl functions. Aliquots of K25 aldobiouronic acid were reduced with $NaBH_4$ or with $NaBD_4$, and the pair of H1-unlabeled and labeled biouronitols was esterified, carboxyl-reduced, trimethylsilylated, and subjected to GLC-MS (23). The materials that exhibited a retention time of 6.3 min (TMS lactitol, 5.6 min) on an SE-30 column at 260°C yielded the primary fragments indicated; the values in brackets show the alterations of the spectrum after monodeuteration at C1 of the galactose moiety.*

alent weight (Table 1), its quantitative sugar analysis (molar ratio Glc:Gal:GlcUA = 2:1:1 [Table 1]), from the results of methylation-carboxyl reduction-GLC-MS (equal amounts of four different methylated monomers [Table 2, column 2]), and from the direct isolation of the tetrasaccharide (P1) upon phage-catalyzed degradation of the polymer (Fig. 3).

The substitution pattern of the monomers in the K25 repeating unit can be concluded from the results of the methylation analyses summarized in Table 2 (columns 1 and 2). The unit consists of unsubstituted, i.e., branch terminal Glc, 4-substituted Glc, 2-substituted GlcUA, and 3,4-disubstituted Gal, which, therefore, must be the chain sugar that carries the branch.

The sequence of the monomers can be deduced as follows. The K25 aldobiouronic acid is 4-*O*-(β-D-glucopyranosyluronic acid)-Gal, as shown by the MS analysis of its reduced and trimethylsilylated derivatives (Fig. 2) and by its separation from the 3-substituted analogue in PE and PC, also by its sensitivity to β-glucuronidase and by its optical rotation. This leaves four possibilities: branches consisting of one Glc only, or of both Glc's, or of one or both Glc's and the GlcUA—the rest of the sugars constituting the chain in all cases. The first two, as well as the last, of these possibilities are ruled out by the results of partial debranching-methylation-carboxyl reduction: mild TFA hydrolysis yielded a residual polymer that had lost about 50% of one Glc equivalent (Table 1). Upon methylation-carboxyl reduction (Table 2, column 3), this material shows no loss of 3,4-substituted Gal (all branches are still present, either complete, or as stubs) and no apprecia-

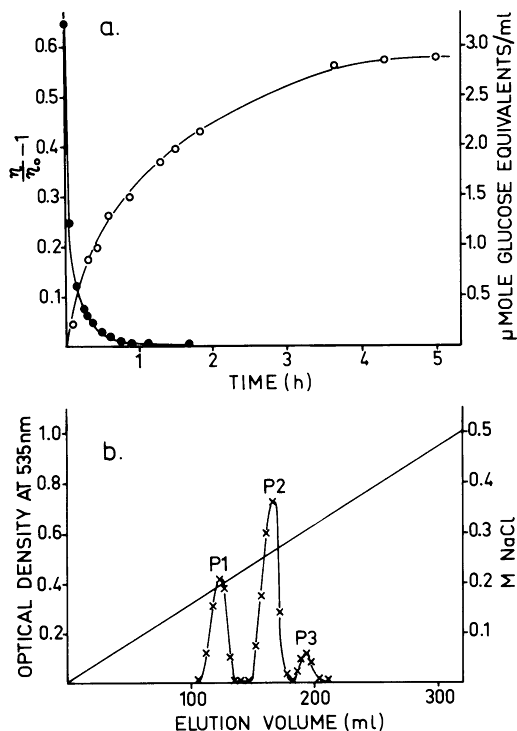

FIG. 3. *Degradation of Klebsiella K25 polysaccharide after incubation with particles of Klebsiella phage no. 25. (a) Time course of depolymerization. A solution containing 2.5 × 10^{10} PFU of purified phage particles and 4 mg of polysacchride per ml of phosphate-buffered saline was incubated at 37°C, and the loss of viscosity (η/η_0-1; ●), as well as the increase in reducing power (micromoles of glucose equivalents per milliliter; ○), was followed. (b) Separation of phage degradation products. The products obtained from 192 mg of polysaccharide after 38 h of incubation with virus were desalted and adsorbed to a diethylaminoethyl-Sephadex A-25 column from a 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.2. Elution was carried out by addition of a 0 to 0.5 M linear NaCl gradient (——) and analysis of the fractions with carbazole-sulfuric acid (3) (adsorption at 535 nm; ×).*

ble loss of 4-substituted Glc, but it does show loss of about half of the unsubstituted Glc, accompanied by a replacement of 2-substituted by unsubstituted GlcUA, demonstrating that the branches consist of 2-*O*-D-glucopyranosyl-D-glucopyranuronic acid.

The anomeric configurations of the sugars follow from the PMR data. The spectrum of the K25 tetrasaccharide gives evidence of roughly 0.5 mol equivalent of α-linkages only. Since no α-linkages were detected in the partially debranched polymer, it is clear that the small α contribution in P1 results from the cleavage of another β-linkage by the phage enzyme (and ensuing mutarotation), i.e., that all linkages in the K25 repeating unit are β.

As in similar cases (40, 42), K25 polysaccharide is depolymerized upon incubation with purified particles of *Klebsiella* bacteriophage no. 25 (Fig. 3), a virus that forms plaques with acapsular haloes, which was selected for this property (2, 40, 42). The smallest K25 oligosaccharide obtained by exhaustive depolymerization (P1) is a tetrasaccharide ending in reducing Gal. This follows from the results of labeling with $NaBH_4/NaB^3H_4$. The small amount of reducing Glc also detected by this approach is most likely a result of some β elimination during reduction with $NaBH_4/NaB^3H_4$ (β elimination is favored by the 3-substitution of the reducing Gal in P1), since its amount depends very much on the pH of the reduction medium.

The results are summarized in Fig. 4.

It is also worth noting that the unaltered amount of Glc in oligosaccharide P1 shows that the phage enzyme acts by hydrolysis and not by elimination and that—analogous to similar cases (2, 40)—the spike-shaped organelles occasionally visible at the tail tips of phage 25 particles (Fig. 1) may constitute the depolymerase.

Further, the results presented in Fig. 4 lead to an understanding of the structural homologies responsible for the serological cross-reactions between *Klebsiella* K25 and *Klebsiella* K8 (14, 15), as well as *Pneumococcus* type II and type V (18) capsular antigens: both *Klebsiella* K8 and *Pneumococcus* type II polysaccharides contain branch GlcUA residues—terminally linked α-(1 → 4) to Gal in the former case and α-(1 → 6) to Glc in the latter case—whereas (1 → 2)-GlcUA-(1 $\xrightarrow{\beta}$ 3) → has been reported to be a structural element of the *Pneumococcus* type V glycan (18).

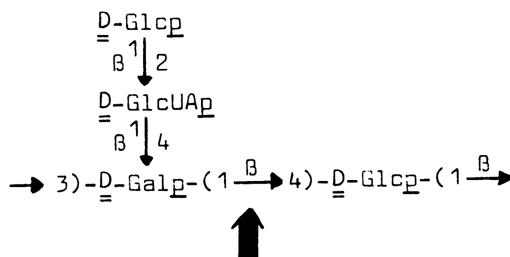

FIG. 4. *Primary structure of the repeating unit of Klebsiella serotype 25 capsular polysaccharide and site of hydrolysis by the glycanase associated with particles of Klebsiella bacteriophage no. 25. Abbreviations: Glcp, Glucopyranose; GlcUAp, glucuronic acid (pyranoside); Galp, galactopyranose.*

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LITERATURE CITED

1. **Bebault, G. M., Y. M. Choy, G. G. S. Dutton, N. Funnel, A. M. Stephen, and M. T. Yang.** 1973. Proton magnetic resonance spectroscopy of *Klebsiella* capsular polysaccharides. J. Bacteriol. 113:1345–1347.
2. **Bessler, W., E. Freund-Mölbert, H. Knüfermann, C. Rudolph, H. Thurow, and S. Stirm.** 1973. A bacteriophage-induced depolymerase active on *Klebsiella* K11 capsular polysaccharide. Virology 56:134–151.
3. **Bitter, T., and H. M. Muir.** 1962. A modified uronic acid-carbazole reaction. Anal. Biochem. 4:330–334.
4. **Björdal, H., C. G. Hellerqvist, B. Lindberg, and S. Svensson.** 1970. Gas-Flüssigkeits-Chromatographie und Massenspektrometrie bei der Methylierungsanalyse von Polysacchariden. Angew. Chem. 82:643–674.
5. **Björndal, H., B. Lindberg, J. Lönngren, M. Mészáros, J. L. Thompson, and W. Nimmich.** 1973. Structural studies of the capsular polysaccharide of *Klebsiella* type 52. Carbohydr. Res. 31:93–100.
6. **Bradley, D. E.** 1967. Ultrastructure of bacteriophages and bacteriocins. Bacteriol. Rev. 31:230–314.
7. **Choy, Y. M., and G. G. S. Dutton.** 1973. Structure of the capsular polysaccharide of *Klebsiella* K-type 56. Can. J. Chem. 51:3021–3026.
8. **Choy, Y. M., G. G. S. Dutton, and A. M. Zanlungo.** 1973. The structure of the capsular polysaccharide of *Klebsiella* K-type 24. Can. J. Chem. 51:1819–1825.
9. **Choy, Y. M., and G. G. S. Dutton.** 1974. The structure of the capsular polysaccharide of *Klebsiella* K-type 72; occurrence of 3,4,-*O*-(1-carboxyethylidene)-L-rhamnose. Can. J. Chem. 52:684–687.
10. **Choy, Y. M., F. Fehmel, N. Frank, and S. Stirm.** 1975. *Escherichia coli* capsule bacteriophages. VI. Primary structure of the bacteriophage 29 receptor, the *E. coli* serotype 29 capsular polysaccharide. J. Virol. 16:581–590.
11. **Curvall, M., B. Lindberg, J. Lönngren, and W. Nimmich.** 1975. Structural studies of the capsular polysaccharide of *Klebsiella* Type 81. Carbohydr. Res. 42:73–82.
12. **Curvall, M., B. Lindberg, J. Lönngren, and W. Nimmich.** 1975. Structural studies of the capsular polysaccharide of *Klebsiella* type 28. Carbohydr. Res. 42:95–105.
13. **Dutton, G. G. S., A. M. Stephen, and S. C. Churms.** 1974. Structural investigation of *Klebsiella* serotype K7 polysaccharide. Carbohydr. Res. 38:225–237.
14. **Edwards, P. R., and M. A. Fife.** 1952. Capsule types of *Klebsiella*. J. Infect. Dis. 91:92–104.
15. **Edwards, P. R., and W. H. Ewing.** 1968. Identification of *Enterobacteriaceae*. Burgess Publishing Co., Minneapolis.
16. **Hakomori, S.** 1964. A rapid permethylation of glycolipid and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. J. Biochem. (Tokyo) 55:205–207.
17. **Heidelberger, M., W. Nimmich, J. Eriksen, G. G. S. Dutton, S. Stirm, and C. T. Fang.** 1975. Cross-reactions of *Klebsiella*. Acta Pathol. Microbiol. Scand. Sect. C 83:397–405.
18. **Heidelberger, M., and W. Nimmich.** 1976. Immunochemical relationships between bacteria belonging to two separate families: *Pneumococci* and *Klebsiella*. Immunochemistry 13:67–80.
19. **Hellerqvist, C. G., B. Linberg, S. Svensson, T. Holme, and A. A. Lindberg.** 1968. Structural studies on the O-specific side-chains of the cell wall lipopolysaccharide from *Salmonella typhimurium* 395 MS. Carbohydr. Res. 8:43–55.
20. **Hungerer, D., K. Jann, B. Jann, F. Ørskov, and I. Ørskov.** 1967. Immunochemistry of K antigens of *Escherichia coli*. 4. The K antigen of *E. coli* O9:K30:H12. Eur. J. Biochem. 2:115–126.
21. **Imoto, T., and K. Yagishita.** 1971. A simple activity measurement of lysozyme. Agric. Biol. Chem. (Tokyo) 35:1154–1156.
22. **Jann, K., B. Jann, F. Ørskov, I. Ørskov, and O. Westphal.** 1965. Immunchemische Untersuchungen an K-Antigenen von *Escherichia coli*. II. Das K-Antigen von *E. coli* O8:K42(A):H$^-$. Biochem. Z. 342:1–22.
23. **Kärkkäinen, J.** 1969. Determination of the structure of disaccharides as *O*-trimethylsilyl derivatives of disaccharide alditols by gas-liquid chromatography-mass spectrometry. Carbohydr. Res. 11:247–256.
24. **Kauffmann, F.** 1966. The bacteriology of *Enterobacteriaceae*. Munksgaard, Copenhagen.
25. **Kollonitsch, J., O. Fuchs, and V. Gábor.** 1955. Alkaline-earth borohydrides and their applications in organic syntheses. Nature (London) 175:346.
26. **Levvy, G. A., and C. A. Marsh.** 1960. β-Glucuronidase, p. 397–407. *In* P. D. Boyer, H. Lardy, and K. Myrbäck (ed.), The enzymes, vol. 4. Academic Press Inc., New York.
27. **Lindberg, B.** 1972. Methylation analysis of polysaccharides. Methods Enzymol. 28B:178–195.
28. **Lindberg, B., J. Lönngren, U. Rudén, and W. Nimmich.** 1975. Structural studies of the capsular polysaccharide of *Klebsiella* type 59. Carbohydr. Res. 42:83–93.
29. **Lönngren, J., and S. Svensson.** 1974. Mass spectrometry in structural analysis of natural carbohydrates. Adv. Carbohydr. Chem. Biochem. 29:41–106.
30. **Nhan, L.-B., B. Jann, and K. Jann.** 1971. Immunochemistry of K antigens of *Escherichia coli*. The K29 antigen of *E. coli* O9:K29(A):H$^-$. Eur. J. Biochem. 21:226–234.
31. **Nimmich, W.** 1968. Zur Isolierung und qualitativen Bausteinanalyse der K-Antigene von *Klebsiellen*. Z. Med. Mikrobiol. Immunol. 154:117–131.
32. **Nimmich, W., and W. Münter.** 1975. Ein neuer *Klebsiella* Serotyp K81. Z. Allg. Mikrobiol. 15:127–129.
33. **Ørskov, I.** 1954. O antigens in the *Klebsiella* group. Acta Pathol. Microbiol. Scand. 34:145–156.
34. **Rieger, D., E. Freund-Mölbert, and S. Stirm.** 1975. *Escherichia coli* capsule bacteriophages. III. Fragments of bacteriophage 29. J. Virol. 15:964–975.
35. **Sawardeker, J. S., J. H. Sloneker, and A. Jeanes.** 1965. Quantitative determination of monosaccharides as their alditol acetates by gas-liquid chromatography. Anal. Chem. 12:1602–1604.
36. **Schlecht, S., and O. Westphal.** 1966. Wachstum und Lipopolysaccharide (O-Antigen-)-Gehalt von *Salmonellen* bei Züchtung auf Agarnährböden. Zentralbl. Bakteriol. Parasitenkd. Infektionkr. Hyg. Orig. 200:241–259.
37. **Schlubach, H. H., and K. Repenning.** 1959. Glucosewert-Bestimmung mit Glucoseoxidase. Angew. Chem. 71:193.
38. **Smith, F., and R. Mongtomery.** 1959. The chemistry of plant gums and mucilages. Reinhold Publishing

Corp., New York.

39. **Stirm, S., and E. Freund-Mölbert.** 1971. *Escherichia coli* capsule bacteriophages. II. Morphology. J. Virol. 8:330-342.
40. **Thurow, H., H. Niemann, C. Rudolph, and S. Stirm.** 1974. Host capsule depolymerase activity of bacteriophage particles active on *Klebsiella* K20 and K24 strains. Virology 58:306-309.
41. **Thurow, H., Y. M. Choy, N. Frank, H. Niemann, and S. Stirm.** 1975. The structure of *Klebsiella* serotype 11 capsular polysaccharide. Carbohydr. Res. 41:241-255.
42. **Thurow, H., H. Niemann, and S. Stirm.** 1975. Bacteriophage-borne enzymes in carbohydrate chemistry. Part I. On the glycanase activity associated with particles of *Klebsiella* bacteriophage no. 11. Carbohydr. Res. 41:257-271.
43. **Trevelyan, W. E., D. P. Procter, and J. S. Harrison.** 1950. Detection of sugars on paper chromatograms. Nature (London) 166:444-445.
44. **Wallenfels, K., and G. Kurz.** 1962. Über die Spezifität der Galaktosedehydrogenase aus *Pseudomonas saccharophila* und deren Anwendung als analytisches Hilfsmittel. Biochem. Z. 335:559-572.
45. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure, p. 83-91. *In* R. L. Whistler and M. L. Wolfrom (ed.), Methods in carbohydrate chemistry, vol. 5. Academic Press Inc., New York.
46. **Yamamoto, K. R., B. M. Alberts, R. Benzinger, L. Lawhorne, and G. Treiber.** 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large scale virus purification. Virology 40:734-744.