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DEPOLYMERIZATION OF THE CAPSULAR POLYSACCHARIDE FROM *Klebsiella* K19 BY THE GLYCANASE ASSOCIATED WITH PARTICLES OF *Klebsiella* BACTERIOPHAGE ϕ19*†

MICHEL BEURRET‡ AND JEAN-PAUL JOSELEAU**

Centre de Recherches sur les Macromolécules Végétales, C.N.R.S., Université Grenoble I, B.P. 68, 38402 St. Martin d'Hères Cédex (France)

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ABSTRACT

The site of cleavage of the capsular polysaccharide from *Klebsiella* K19 by the endoglycanase associated with particles of *Klebsiella* bacteriophage ϕ19 was determined. The specific cleavage of the bond Rha*p*-(1→2)-Rha*p* provided a series of oligosaccharides having rhamnose at the reducing end. The enzyme is thus an α-rhamnosidase. Structural studies on the oligomers confirmed the sequence of the repeating unit of the polysaccharide from K19. The ^{1}H- and ^{13}C-n.m.r. spectra of the homologous series of oligosaccharides corresponding to one, two, three, and four repeat-units exhibit important differences that denote variation of conformation with chain length. The bacteriophage acted on modified forms of K19 polysaccharide to provide a series of linear oligomers, and emphasized the essential role of the negative charge on the uronic acid in the action of the glycanase.

INTRODUCTION

Selective depolymerization of extracellular, capsular polysaccharides from Gram-negative bacteria may be achieved by the use of bacteriophages. In the *Klebsiella*, several phages specific for a particular serotype have been isolated[1]. The exact mechanism of cleavage of the polysaccharide at a specific glycosidic bond is not clear. Two forms of the enzyme responsible for hydrolysis have been demonstrated[2], a phage-bond form and a soluble form. In earlier studies of the depolymerization of *Klebsiella* polysaccharides by bacteriophage-borne enzymes,

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‡Supported by a research grant from the Fonds National Suisse de la Recherche Scientifique. Present address: National Research Council of Canada, Division of Biological Sciences, Ottawa, Ontario, K1A OR6, Canada.

**To whom correspondence should be addressed.

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the "authenticity" of the homologous series of oligosaccharides obtained from polysaccharides containing acid-labile substituents has been emphasized.

In their comparative study of the action of the *Klebsiella* bacteriophage-associated depolymerases, Rieger-Hug and Stirm[1] reported that the phage ϕ19, specific of its host strain *Klebsiella* K19, cleaved the corresponding capsular polysaccharide, releasing oligosaccharides of average d.p. = 15, having a rhamnose group at the reducing end. They also suggested that ϕ19 could split the polysaccharide from strain K58. That the glycanase induced by a same phage may act on several different polysaccharides is not unusual, as demonstrated by the ability of *Klebsiella* bacteriophage ϕ13 to degrade the four different polysaccharides from Klebsiella K2, K13, K22 and K37, even though the four substrates do not have a true homology[3].

This paper reports on the mode of action of the glycanase associated with the phage ϕ19. Chemical studies and n.m.r. characterization of the oligosaccharides obtained from the K19 exopolysaccharide are described. Depolymerization by use of the bacteriophage is shown to be useful as a tool that simplifies the sequence determination of the repeating unit of this kind of bacterial polysaccharide.

RESULTS AND DISCUSSION

Phage ϕ19 is a phage of Bradley's type C[4], isolated by Rieger-Hug and Stirm[1] from sewage waters and purified by successive single plaque isolations. It was propagated on the host strain *Klebsiella* K19 in a nutrient-broth medium[5]. The optimal concentration of bacteria for which the maximal propagation of ϕ19 would be obtained was estimated from the growth curve of *Klebsiella* K19 (Fig. 1). At the onset of incubation, the curve shows a lag phase of about 45 min, followed by an exponential phase of ~1 h, ending with a stationary phase. At this point, the bacterial population was $\sim 10^9$ cells/mL. According to previous data, the optimal condition for infection of the bacteria by the phage was estimated to be at a concentration of 10^8 cells/mL (2.5×10^{10} cells in 250 mL), which corresponds to the beginning of the exponential-growth phase (Fig. 1). The initial quantity of virus particles of ϕ19 (3 particles per bacterial cell: 8×10^{10} plaque-forming units (p.f.u.) was sufficient to afford, in a single operation, a good, final bacteriophage concentration of 6×10^{12} p.f.u.

Assays of phage potency. — Phage ϕ19 was assayed by measuring the decrease of specific viscosity[6] of a solution of the K19 polysaccharide in 0.1M sodium chloride. Assays were conducted using two different titers of the bacteriophage (Fig. 2). At the higher concentration of 10^{13} p.f.u. for 1 g of polysaccharide (the recommended conditions for depolymerization), the decrease in viscosity was too rapid to permit good control of the oligosaccharides released. With the titer of 10^{12} p.f.u., the progressive loss of viscosity was slower and permitted ready recovery and separation of the oligosaccharides.

Preparation and separation of depolymerization products. — Depolymeriza-

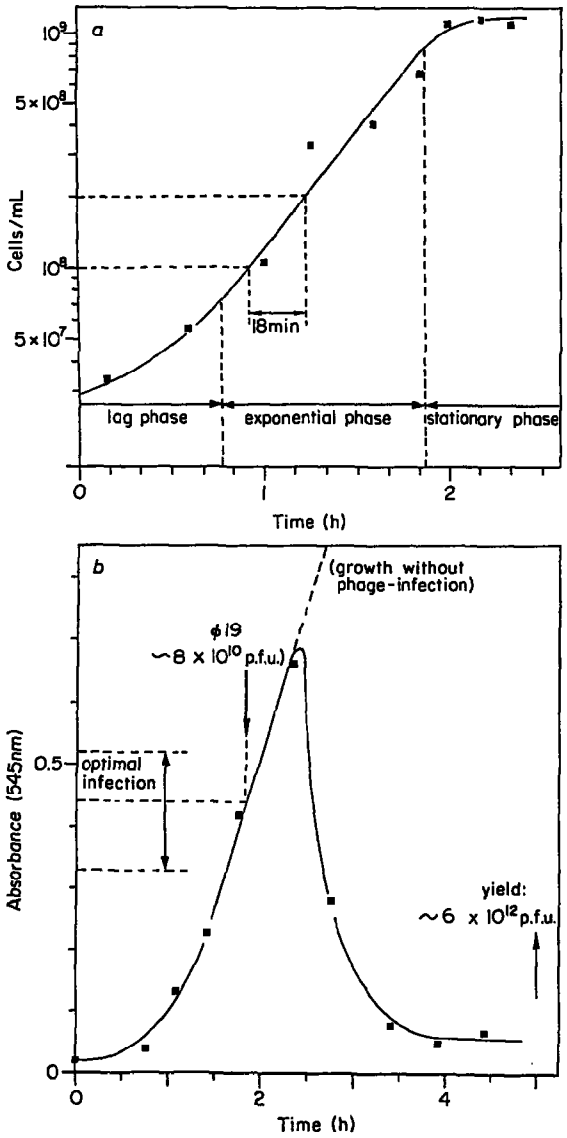


Fig. 1. Propagation of the bacteriophage $\phi 19$ on *Klebsiella* K19 cells. *a*, Growth curve of K19. During the exponential phase, the number of bacterial population was simultaneously monitored by measuring the absorbance at 545 nm. Broth volume: 100 mL. *b*, Phage-infection of K19. For optimal propagation, 3 viral particles (p.f.u.) per bacterial cell were added at the beginning of the exponential growth-phase. Broth volume: 250 mL.

tion of polysaccharide K19 by the bacteriophage $\phi 19$, and purification of the oligomeric products, were first performed according to Dutton *et al.*[7] (Fig. 3, scheme 1) in the presence of a phage suspension of high titer (10^{13} p.f.u. for 1 g of K19), without previous control of the specific viscosity. After 16 or 4 h of attack,

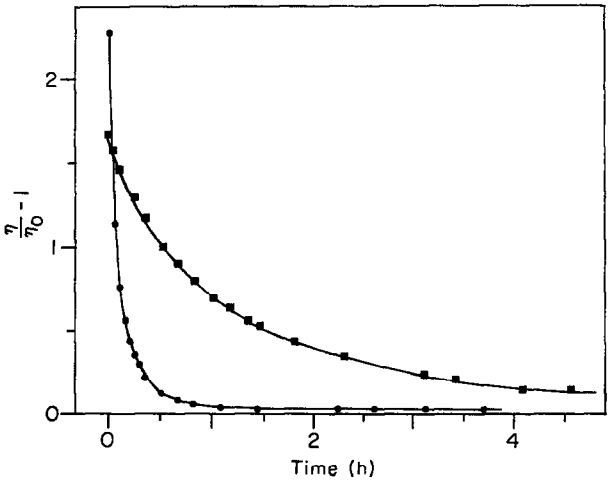


Fig. 2. Assays of phage potency as determined by measuring the decrease of specific viscosity of a solution of K19 polysaccharide (0.05 g at 2.5 g/L) in 0.1M NaCl. Two different concentrations of phage were assayed, 10^{13} p.f.u./g of K19 (—●—) and 10^{12} p.f.u./g of K19 (—■—).

the only oligomers observed after dialysis were **P1** (1 repeating unit) and **P2** (2 repeating units), although it could be shown that **P2** (and higher oligomers) did not dialyze completely out of the dialysis bag.

However ϕ19 was very active, and nearly total depolymerization was achieved within 24 h. Analysis of the dialyzable material showed only the hexasaccharide repeating unit **P1**, to the exclusion of any higher oligomer, in an overall yield of 70% when the reaction was conducted on a preparative scale. Gel-permeation chromatography (g.p.c.) of the material retained in the dialysis bag showed, beside some residual polymer, a few oligosaccharides having d.p. higher than **P2** but no **P2**. The results are expressed in Table I.

Because the oligomers from the depolymerization of K19 are charged, Bio-Gel P2 could not be employed as for a neutral series for the separation of these oligomers of relatively low d.p. The partition coefficient K_d, which depends primarily on the hydrodynamic volume, is larger in the case of charged molecules, and Bio-Gel P6 was used for the separation[8]. Also, in order to suppress ionic repulsion between the solute charges and the residual charges of the polyacrylamide gel, which gives apparent low K_d values, the elution was performed with a solution of sodium nitrate[8] of low concentration (50mM).

The depolymerization of K19 and separation of the resulting acidic oligosaccharides was then performed according to Fig. 3, scheme 2, in which the dialysis step was omitted. The depolymerized material was filtered and then placed on top of a column of Bio-Gel P6 for separation. The purified oligomers were further desalted by g.p.c. on Bio-Gel P2 with water as eluent. In this assay, the phage concentration was lowered to 10^{12} p.f.u./g of K19 in order to give a longer depolymerization time, thus allowing a better appreciation of the mechanism of

TABLE I

PREPARATION AND SEPARATION BY G.P.C. OF THE DEPOLYMERIZATION PRODUCTS FROM K19 POLYSACCHARIDE

Oligomers[a]	K_d[b]	*Scheme 1*[c,d]			*Scheme 2*[c,e]
		Reaction time (h) 2	16	4	15
P1	0.47	100 (70)[f]	100	100	100 (31)
P2	0.25	—	7	15	74 (17)
P3	0.13	—	—	—	23 (3)
P4	0.08	—	—	—	11 (21)[g]
P5	0.04	—	—	—	11

[a]In percentage of the **P1** peak height. [b]Partition coefficient on Bio-Gel P6 with 10mM $NaNO_3$ as eluent. In subsequent experiments, g.p.c. separations were performed in 50mM $NaNO_3$ in order to suppress ionic repulsion between the solute and the gel[8]. [c]See Fig. 3. [d]Analytical: K19, 0.1 g at 1 g/L; ϕ19, 10^{13} p.f.u./g of K19; preparative: K19, 1 g at 2.2 g/L; ϕ19, 10^{13} p.f.u./g of K19. [e]Preparative: K19, 0.5 g at 5 g/L; ϕ19, 8.10^{11} p.f.u./g of K19. [f]Yield of purified and desalted oligomers, as percentage of the initial polymer. [g]Yield of **P≥4** on preparative scale as **P4** and **P5** were co-eluted on the preparative column.

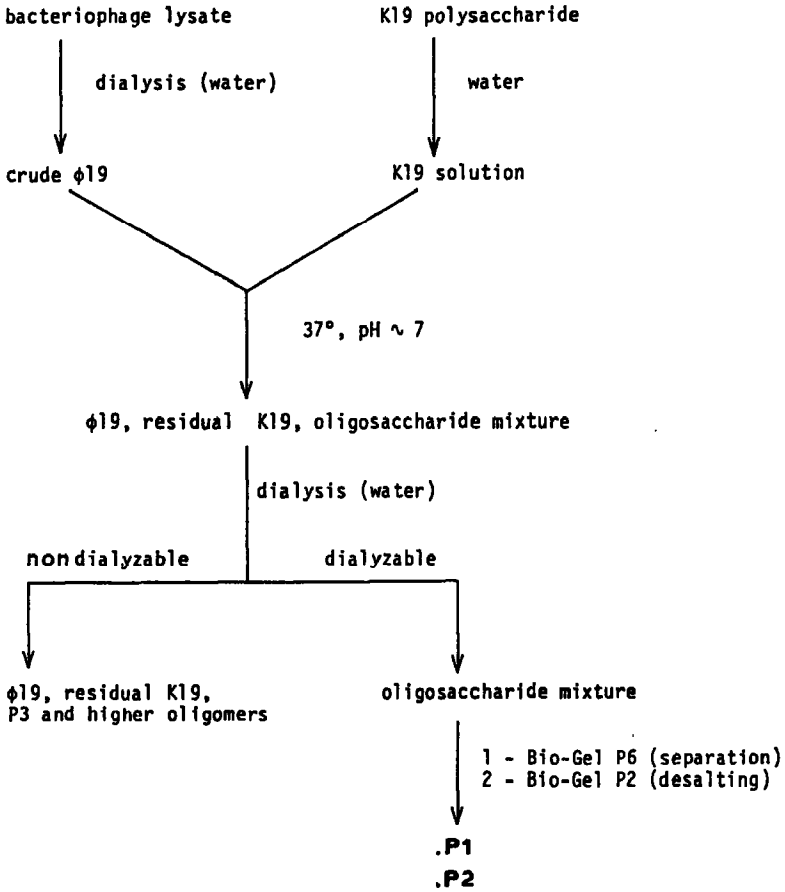
oligomer production by the bacteriophage. From the results in Table I it is clear that a more-controlled depolymerization was achieved, giving appreciable amounts of oligomers **P1** and **P2** and a small proportion of **P3**. The higher oligomers were not completely separated on the analytical column and were co-eluted on the preparative column. On the preparative scale, 0.5–1 g of K19 was submitted to the action of the bacteriophage and the separation was performed as before.

Determination of the size of the oligomers. — The plot of log K_d versus d.p. for a homologous series of oligomers gives a straight line. This was the case for the oligosaccharides released from K19 by the phage and chromatographed on Bio-Gel P6 (Fig. 4). In order to investigate oligomers higher then **P5**, the mixture was analyzed on Bio-Gel P10. Here the plot of log K_d *vs.* d.p. was linear up to **P3** and then a deviation was observed (Fig. 4). As the values of K_d observed with Bio-Gel P6 are less accurate than those measured on Bio-Gel P10 where the separation is in more-direct relation to the hydrodynamic volume, the deviation to the straight line could be related to change in conformation, starting from **P4**.

That **P1** corresponded to a hexasaccharide was well established by chemical analysis, n.m.r. spectroscopy, and also by the observation in f.a.b.–m.s. of unambigous quasimolecular ions (see later).

Velocity of the reaction. — A study of the velocity of the depolymerization was undertaken to determine the yield of each oligomers under well-defined conditions. As the viscosity of the polysaccharide solution decreased within a few hours, even with a phage concentration of low titer, the factor that seemed to be the most important in influencing the production of oligomers of a given size was

the actual concentration of the polysaccharide solution. The depolymerization was performed with two different concentrations of K19, 2.6mM (2.5 g/L) and 0.52mM (0.5 g/L) (calculated in repeating units: mol.wt. 960 for the sodium salt). In each instance, the release of the oligomers was monitored by g.p.c. measurements in the presence of an internal standard (Fig. 5). The depolymerization was followed for 20 days. The time of 19 h, which corresponded to complete loss of viscosity, also corresponded to the point of inflexion of the velocity of release of all oligomers from **P1** to **P5**. For the lower concentration of K19, the inflexion point was observed after 3.5–4 days of hydrolysis, but the curves are quite comparable for both concentrations of K19. It is noteworthy that the rates of appearance of **P1** and **P2** are linear at the beginning of the depolymerization, and then the oligomers higher than **P2** disappear and the concentration of **P2** decreases, whereas that of **P1** continues to increase. Aside from the quantity of **P1** produced from the residual polymer, the remainder of the **P1** thus arises from further splitting of **P2**. This shows that the glycanase from ϕ19 is able to hydrolyze **P2**, which is therefore the smallest oligomer accommodated by the enzyme.



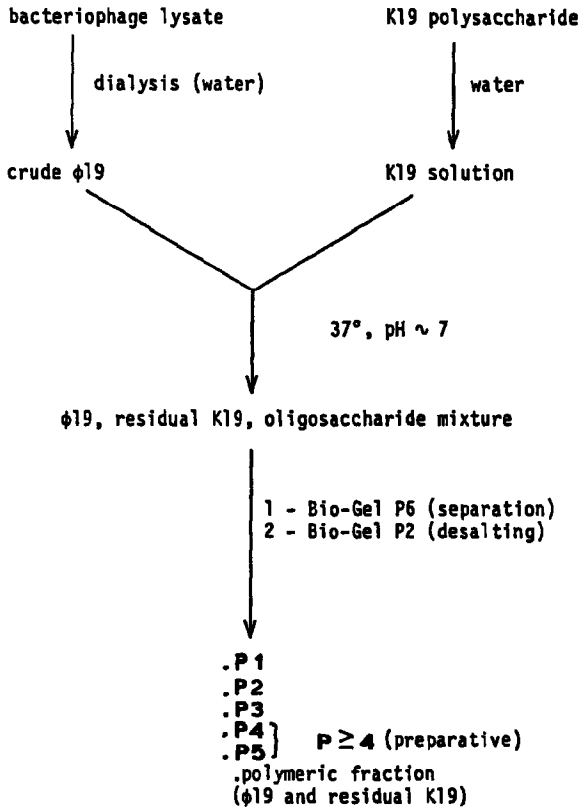


Fig. 3. Flow diagram for the preparation of oligosaccharides from K19 using the phage ϕ19. *Scheme 1*, according to Dutton *et al.*[7]; oligomers higher than **P2** did not dialyze out of the dialysis bag. *Scheme 2*, dialysis of the depolymerization products was omitted. On the preparative column, **P4** and higher members (**p≥4**) were not separated from the polymeric fraction. In all instances the saline eluent ($NaNO_3$) was removed by g.p.c. on Bio-Gel P2 with water as eluent.

Action of the bacteriophage ϕ19 on modified substrates. — Various studies have shown that the negative charge of the carboxyl group of uronic acid[2,9] residues or of pyruvate[1] substituents in the vicinity of the cleaved glycosidic bond has an important influence on the glycanase activity of phages. The carboxyl-reduced form of K19 (K19-CR)[10] was therefore submitted to ϕ19 under the same conditions as for the original polysaccharide. The solution of K19-CR underwent a drop of viscosity during the first 8 h of the attack; subsequently a very slow decrease of viscosity occurred during the following 8 days. As it is almost impossible to achieve total carboxyl-group reduction of the uronic acids in a polymer[11], the rapid decrease in viscosity observed may be ascribed to the action of the phage on surviving uronic acid residues in the polymer. Only a few cleavages would produce a drastic decrease in viscosity. It may be concluded that ϕ19 was not active on the carboxyl-reduced polymer, as no oligomer of low d.p. could be obtained.

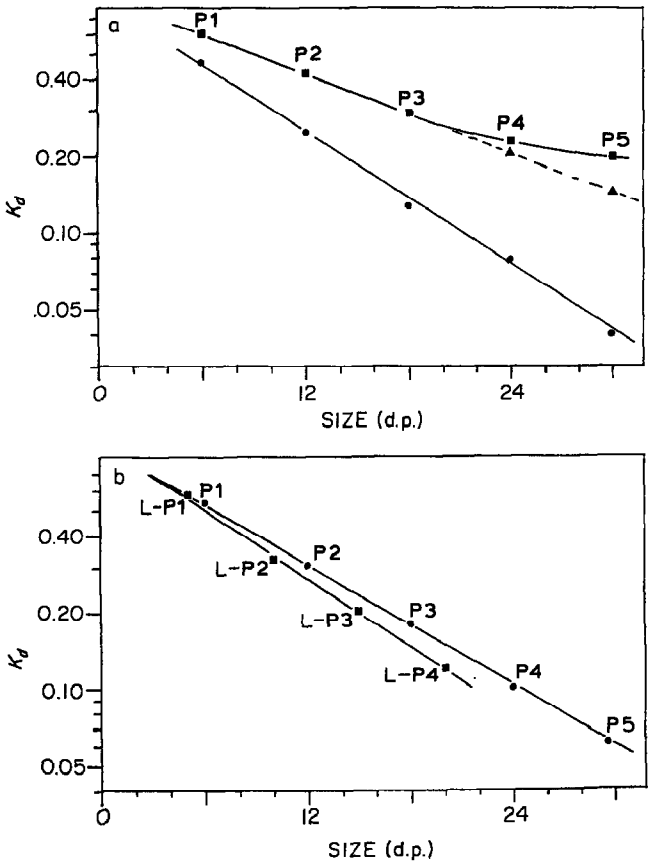


Fig. 4. Separation of the depolymerization products of K19 and K19-L by g.p.c. (analytical). *a*, Separation of the oligosaccharides from K19 on Bio-Gel P6 (—●—) and Bio-Gel P10 (—■—) with 10mM $NaNO_3$ as eluent; (—▲—) theoretical values. *b*, Separation of the oligosaccharides from K19 (—●—) and K19-L (—■—) on Bio-Gel P6 with 50mM $NaNO_3$ as eluent. K_d values are reported on a logarithmic scale.

Another modified substrate, the linear form of K19 (K19-L) obtained by controlled acid hydrolysis[10], was exposed to the action of ϕ19 in order to study the influence of the side chain upon the rate of hydrolysis. As in K19 the uronic acid residue is in the main chain, effective depolymerization of K19-L by ϕ19 could be expected. Indeed, g.p.c. analysis clearly showed that oligomers were released. Again this series of oligomers gave a linear plot of log K_d *vs.* d.p., with a slope greater than that of the original K19 series (Fig. 4). This difference may be ascribed to the difference between the hydrodynamic volume of a penta- and hexasaccharide bearing one negative charge. The velocity of hydrolysis (Fig. 5) indicated mainly linear production of all oligomers from L-**P1** to L-**P4**, with an inflexion of the curve after ~4–5 days of attack. The quantities of the higher oligomers L-**P3** and L-**P4**, then rapidly decreased, indicating that these oligomers

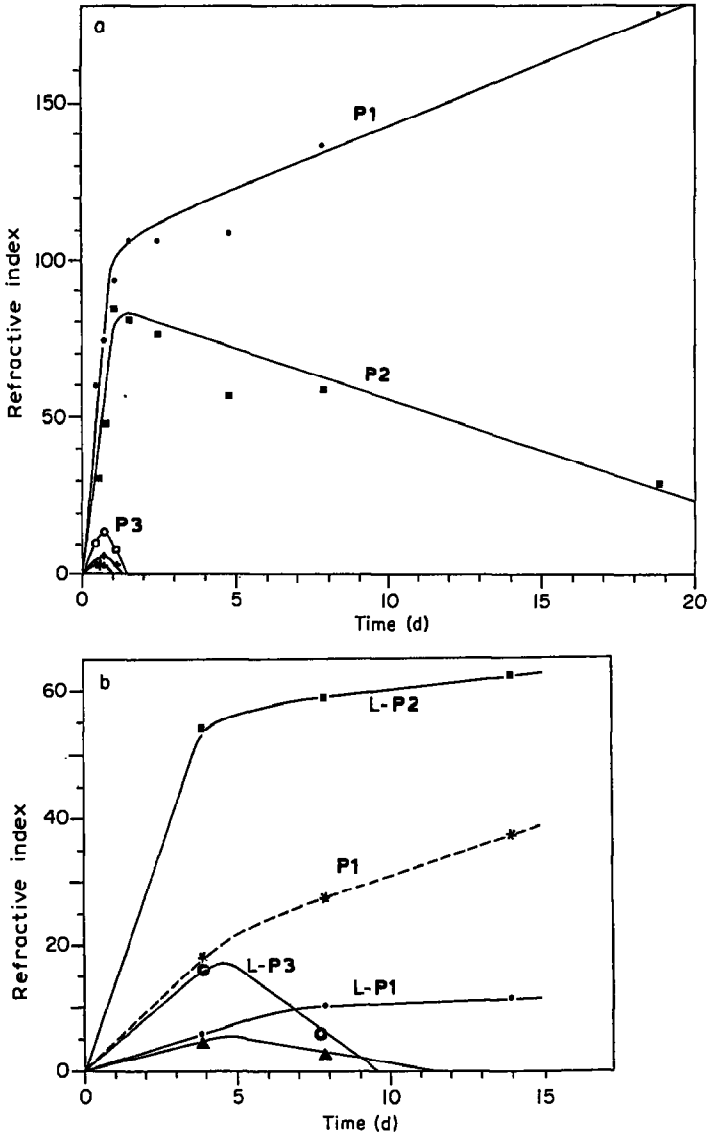


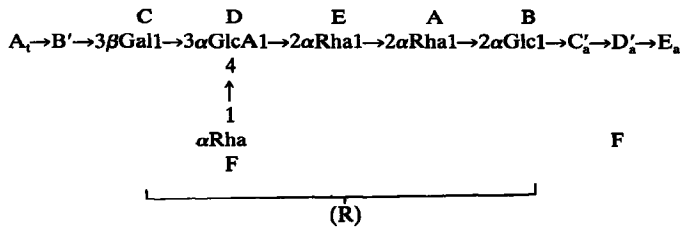
Fig. 5. Kinetics of release of the oligomers from K19 and K19-L during phage action. *a*, Oligosaccharides **P1** (—●—), **P2** (—■—), **P3** (—○—), **P4** (—+—), and **P5** (—*—) from the depolymerization of 2.6mM K19 (0.1 g at 2.5 g/L; ϕ19, 10^{12} p.f.u./g of K19). *b*, Oligosaccharides L-**P1** (—○—), L-**P2** (—■—), L-**P3** (—○—), and L-**P4** (—▲—) from the depolymerization of 7.37mM K19-L (0.09 g at 6 g/L; ϕ19, 2×10^{12} p.f.u./g of K19-L). The presence of residual amounts of **P1** (--*--) due to incomplete removal of the lateral nonreducing rhamnosyl groups[10] was also noted. In all instances the refractive index was checked in the presence of an internal standard.

TABLE II

^{1}H-N.M.R. DATA FOR THE OLIGOSACCHARIDES GENERATED BY PHAGE ACTION ON K19 POLYSACCHARIDE

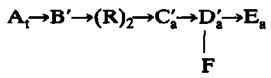
Compound	*δ[a] (p.p.m.)*	*^{3}J[b] (Hz)*	*Integral (proton)*	*Assignment[c]*	
P1	5.16	1.5	0.8	H-1 E_a	→2αRha-OH
A_t B′ C'_a D'_a E_a	5.06	4.0	1.1	H-1 B′	→2αGlc-
αRha1→2αGlc1→3βGal1→3αGlcA1→2Rha-OH	5.02	1.5	1.5	H-1 A_t	αRha-
4	5.01[d]	~3.5		H-1 D'_a	→3αGlcA-
↑	4.96[e]	3.5	0.7		4
1					↑
αRha	4.84	n.r.	0.5	H-1 E_a	→2βRha-OH
F	4.77	n.r.	1.3	H-1 F	αRha-
	4.77[d]	7.5	0.9	H-1 C'_a	→3βGal-
	4.74[e]	7.5			
	4.29	10.0	1.3	H-5 D'_a	→3αGlcA-
					4
					↑
	1.24	6.0	6.0	H-6 E_a, H-6 A_t	→2αRha-OH, αRha-
	1.21	6.0	3.0	H-6 F	
NaBH$_4$-reduced **P1** (**P1**-ol)	5.11	4.0	0.8	H-1 D'_{ol}	→3αGlcA-
					4
					↑
A_t B′ C D'_{ol} E_a	5.06	3.5	1.1	H-1 B′	→2αGlc-
αRha1→2αGlc1→3βGal1→3αGlcA1→2Rha-ol	5.02	1.5	0.9	H-1 A_t	αRha-
4	4.77	s	0.9	H-1 F	
↑	4.72	6.5	1.1	H-1 C	→3βGal-
1	1.24	6.0	3.0	H-6 A_t	αRha-
αRha	1.22	6.0	3.0	H-6 F	
F	1.20	6.0	3.0	H-6 E_{ol}	→2Rha-ol

P2



5.18	n.r.	1.1	H-1 E	→2αRha-
5.15	n.r.	0.6	H-1 E$_a$	→2αRha-OH
5.06	3.5	2.3	H-1 B, B′	→2αGlc-
5.02	n.r	3.5	H-1 A$_t$	αRha-
			H-1 A	→2αRha-
5.00	~4.0		H-1 D, D′$_a$	→3αGlcA-
				4
				↑
4.85	n.r.	0.4	H-1 E$_a$	→2βRha-OH
4.78[d]	~7.0	3.8	H-1 C′$_a$	→3βGal-
4.75	n.r.		H-1 F	αRha-
4.74[e]	~7.0		H-1 C, C′$_a$	→3βGal-
			H-6 E$_a$	→2Rha-OH
1.24	6.0	12.0	H-6 A, E	→2αRha-
			H-6 A$_t$	αRha-
1.22	6.0	6.0	H-6 F	

P3



5.17	1.5	2.4	H-1 E	→2αRha-
			H-1 E$_a$	→2αRha-OH
5.07	~3.5	3.0	H-1 B, B′	→2αGlc-
5.06	3.5			
5.03	n.r.	3.3	H-1 A$_t$	αRha-
			H-1 A	→2αRha-
5.01[d]	~4.0		H-1 D′$_a$	→3αGlcA-
4.96[e]	4.0	2.4	H-1 D, D′$_a$	4
				↑
4.84	n.r.	0.5	H-1 E$_a$	→2βRha-OH
4.78	n.r.	3.3	H-1 F	αRha-
4.77[d]	~7.0	2.7	H-1 C′$_a$	→3βGal-
4.74[e]	7.5		H-1 C, C′$_a$	
4.29	10.0	—	H-5 D, D′$_a$	→3αGlcA-
				4
				↑
			H-6 E$_a$	→2Rha-OH
1.25	6.0	18.0	H-6 A, E	→2αRha-
			H-6 A$_t$	αRha-
1.22	6.0	9.0	H-6 F	

TABLE II *(continued)*

Compound	*δ[a] (p.p.m.)*	*^3J[b] (Hz)*	*Integral (proton)*	*Assignment[c]*	
P≥4	5.16	n.r.	—	H-1 E	→2αRha
				H-1 E_a	→2αRha-OH
A_t→B′→(R($_{\geq 3}$→C'_a→D'_a→E_a	5.06	~3.5	—	H-1 B, B′	→2αGlc-
| F	5.05	3.5	—		
	5.02	n.r.	—	H-1 A_t	αRha-
				H-1 A	→2αRha-
	5.00[d]	~4.0	—	H-1 D'_a	→3αGlcA-
	4.95[e]	4.0	—	H-1 D, D'_a	4 ↑
	4.84	n.r.	—	H-1 E_a	→2βRha-OH
	4.77	n.r.	—	H-1 F	αRha-
	4.74	7.5	—	H-1 C, C'_a	→3βGal-
	4.26	10.0	—	H-5 D, D'_a	→3αGlcA- 4 ↑
				H-6 E_a	→2Rha-OH
	1.24	6.0	—	H-6 A, E	→2αRha-
				H-6 A_t	αRha-
	1.21	6.0	—	H-6 F	

[a]Chemical shift relative to internal acetone: 2.17 p.p.m. downfield from tetramethylsilane. [b]Coupling constant: $^3J_{1,2}$ for H-1; $^3J_{4,5}$ for H-5; $^3J_{5,6}$ for H-6; s = singlet; n.r., not resolved. [c]See text and ref. 10. [d]"β" twin signal due to the β anomer of the reducing rhamnose (E_a). [e]"α" signal due to the α anomer of the reducing rhamnose (E_a). In **P2** and higher oligomers, the signal of the internal residue (D, C) resonates at the same shift than the "α" signal (D'_a, C'_a). [f]The primed letters refer to signals that undrewent a shift by comparison with the original polymer.

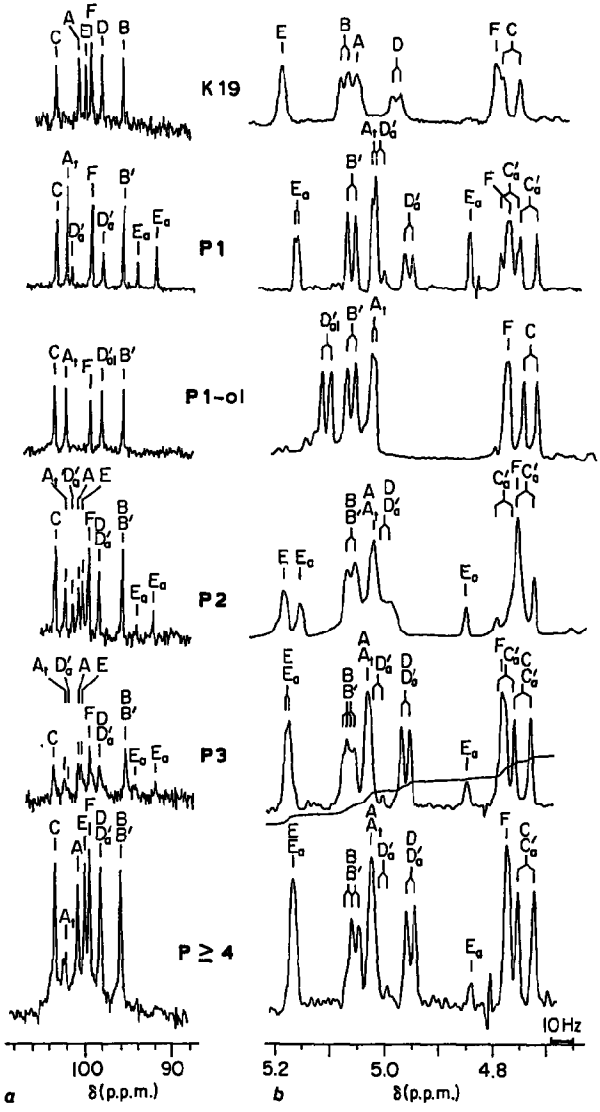


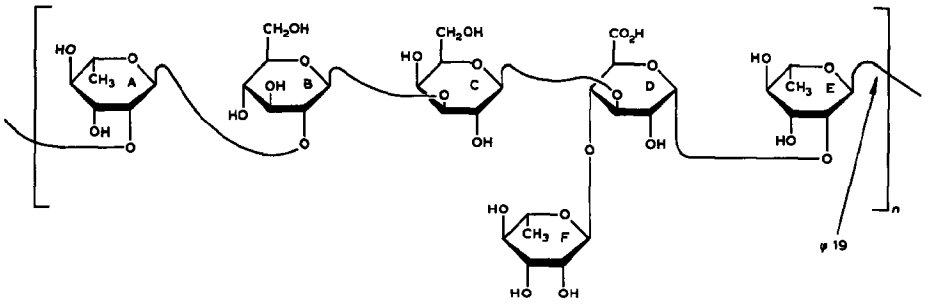
Fig. 6. N.m.r. spectra of the oligosaccharide generated by phage action on K19. *a*, ^{13}C-N.m.r.; chemical shifts (δ are relative to internal acetone, 31.07 p.p.m. downfield from tetramethylsilane. *b*, ^{1}H-N.m.r., chemical shifts (δ) are relative to internal acetone, 2.17 p.p.m. downfield from Me_4Si.

were further degraded to L-**P1** and L-**P2**. On the other hand, the rate of appearance of L-**P1** and L-**P2** suggests that the monomer and the dimer are released under a different pattern of hydrolysis than that of the original polysaccharide. The initial rate of production of the monomer unit L-**P1** was very much lower that of the dimer L-**P2**, but was also less than that of the trimer L-**P3**. In addition, after the inflexion at 4–5 days, the production of L-**P2** continues to increase at a rate

comparable to that of L-**P1**. This indicates that, contrary to the mode of cleavage of the original K19, the linear dimer L-**P2** is not further hydrolyzed to L-**P1** by the phage glycanase. Consequently, the linear oligomer of minimum structure accommodated by the enzyme is a trimer (L-**P3**). On the other hand, the absence of undegraded polymer after phage degradation shows that K19-L is as good a substrate for ϕ19 as the original K19.

^{1}H-N.m.r. spectra of the oligosaccharides. — Compared to that of K19 (ref. 10), the ^{1}H-n.m.r. spectrum of **P1** showed the six anomeric signals as in the spectrum of the polymer plus three extra signals arising from the reducing residue (Table II, Fig. 6). The better quality of the spectrum of the oligomer allowed measured on the polymer. The signal at 5.16 p.p.m., which integrated for 0.8 proton, corresponds to rhamnose (E_a) at the reducing end, H-1 of the β anomer proton, corresponds to rhamnose (E_a) at the reducing end, H-1 of the β anomer resonating at 4.84 p.p.m. These assignments were confirmed by examination of the spectrum of $NaBH_4$-reduced **P1**, (**P1**-ol), in which the signals at 5.16 and 4.84 p.p.m. (E_a) were absent. This result also demonstrated the hexasaccharide nature of **P1**. Two resonances are affected by the anomeric equilibration of the reducing residue; the H-1 signals of the β-galactose (C'_a) and that of the α-glucuronic acid (D'_a) residues appear as "twin" signals[12]. The two additional twin signals at 5.01 (D'_a) and 4.77 p.p.m. (C'_a), respectively, disappeared in the spectrum of **P1**-ol. This perturbation of two signals adjacent to the reducing end provides thus method for sequencing an oligosaccharide, by direct comparison of the spectra of its reduced ad non-reduced form. In **P1**-ol, the three signals of the CH_3 group of the two rhamnose (A_t,F) and the rhamnitol group (E_{ol}) are distinct, the signal of the rhamnitol being at 1.20 p.p.m.

In the spectrum of **P2** (Table II, Fig. 6), the reducing rhamnose residue (E_a) could identified from signals at 5.15 (α anomer) and 4.85 (β anomer) as in **P1**, whereas the signal at 5.18 p.p.m. (E) was that of the same rhamnosyl residue glycosidically linked at the junction between the two repeating units, as in the polymer. In the spectra of **P3** and in the those of the higher members (**P>4**), only the β anomer of the reducing rhamnose (E_a) was still detectable by a signal at 4.84 p.p.m. Another distinctive feature of the ^{1}H-n.m.r. spectrum of **P2** as compared to **P1** and the higher oligomers was the signal of glucuronic acid (D, D'_a), which appeared in **P2** at lower field (5.00 p.p.m.), partly overlapped by the signal of the second internal rhamnose residue (A), which is also present in this case as a terminal non-reducing end-group (A_t) (Fig. 6). Only a weak signal in **P3** at 5.00 p.p.m. (D'_a) showed the effect of the β anomer of the reducing rhamnose (E_a) on the contiguous neighboring sugars. The oligomer **P2** showed an additional difference from the other oligomers in the behavior of the signal of the terminal, nonreducing side-chain rhamnosyl group (F), which shows a slight displacement (to 4.75 p.p.m.) as compared with **P1** and **P3**. Similarly, the displacement of the shift of the signal of the α-glucose residue (B, B') observed in **P3** and **P>4** but not in **P1**, is another manifestation of the nonequivalence of the differently positioned repeating-units in these oligomers.



^{13}*C-N.m.r. spectra of the oligomers (Table III, Fig. 6).* — Compared to the spectrum of the original K19 polymer[10], the spectrum of **P1** showed two main differences, with the disappearance of the two signals at 101.2 (A) and 100.5 p.p.m. (E). The former was shifted to 102.5 p.p.m. (A_t) because of cleavage of the glycosidic bond at C-2 of this rhamnosyl residue (see later). The latter appeared as a set of two signals at 94.5 and 92.4 p.p.m. (E_a), corresponding to the anomeric equilibrium of the reducing rhamnose residue. Here again, the glucuronic acid residue (D'_a) is affected by the anomeric equilibrium of its neighboring reducing residue: a "twin" signal corresponding to the β anomer of the reducing rhamnose (E_a) appeared at 101.9 p.p.m. (D'_a). The signals of those two residues (E_a, D'_a) were assigned according to the relative intensities of the peaks, by reference to the ^{1}H-n.m.r. spectrum of **P1**. The large difference observed in the chemical shifts of the α and β anomers of rhamnose is certainly due to the oligomer substituent at C-2 of this residue (E_a). As in the proton spectrum of the reduced form, the CH_3 signals of the rhamnose residues and of the rhamnitol in **P1**-ol are distinct.

In contrast to observations in the ^{1}H-n.m.r. spectra of **P2** and the higher oligosaccharides, their ^{13}C-n.m.r. spectra were very similar to that of the original polymer[10]. In each case, the spectra incorporated the signals of **P1** plus those of the polymer K19, the first becoming more and more discrete as the d.p. increased.

Structural analysis of oligomer **P1** *by chemical methods and mass spectroscopy.* — Total acid hydrolysis of **P1** gave a sugar composition completely identical to that of the polymer[10] (L-rhamnose:D-galactose:D-glucose = 2.8:1.0:1.2), thus confirming that ϕ19 does not cleave K19 into oligomers smaller than the repeating unit. This result also provides evidence that the capsular polysaccharide from *Klebsiella* K19 is effectively made up of the repeating sequence of a hexasaccharide.

Methylation of **P1** and modified **P1** (defined later) not only confirmed the sequence of the repeating unit of K19 (ref. 10), but also established the position of the site of cleavage by the phage as being at the Rha*p*1→2Rha*p* linkage (Table IV). The modified forms of **P1** used for this study were the $NaBD_4$-reduced **P1** (**P1**-ol) and the carboxyl-reduced **P1** (**P1**-CR) and **P1**-ol-CR, where the carboxyl group had been reduced by $LiAlH_4$ after permethylation. It may be noted that a small amount of 2,3,4,6-tetra-*O*-methylglucose appeared alongside the expected derivatives, together with 3,4-di-*O*-methylrhamnose (in only residual amount in

TABLE III

^{13}C-N.M.R. DATA FOR THE OLIGOSACCHARIDES GENERATED BY PHAGE ACTION ON K19 POLYSACCHARIDE

Compound	*δ[a] (p.p.m.)*	*Assignment[b]*	
P1	103.6	C-1 C	
A_t B′ C D'_a E_a	102.5[c]	C-1 D'_a	→3αGlcA-
αRha1→2αGlc1→3βGal1→3αGlcA1→2Rha-OH	101.9[d]		4
4			↑
↑	99.8	C-1 F	αRha-
1	98.4	C-1 A_t	
αRha	96.1	C-1 B′	→2αGlc-
F	94.5	C-1 E_a[e]	→2βRha-OH
	92.4		→2αRha-OH
	62.0	C-6 B′	→2αGlc
	61.6	C-6 C	→3βGal-
	17.5	C-6 A_t, F	αRha-
		C-6 E_a	→2Rha-OH
$NaBH_4$-reduced **P1** (**P1**-ol)	103.8	C-1 C	→3βGal-
A_t B′ C D'_{ol} E_a	102.6	C-1 D'_{ol}	→3αGlcA-
αRha1→2αGlc1→3βGal1→3αGlcA1→2Rha-ol			4
4			↑
↑	99.8	C-1 F	αRha-
1	98.5	C-1 A_t	
αRha	96.2	C-1 B′	→2αGlc-
F	62.0	C-6 B′	
	61.6	C-6 C	→3βGal-
	61.0	C-1 E_{ol}	→2Rha-ol
	19.4	C-6 E_{ol}	
	17.6	C-6 A_t, F	αRha-

P2

$A_t \rightarrow B' \rightarrow 3\beta Gal1 \rightarrow 3\alpha GlcA1 \rightarrow 2\alpha Rha1 \rightarrow 2\alpha Rha1 \rightarrow 2\alpha Glc1 \rightarrow C \rightarrow D'_a \rightarrow E_a$

4
↑
1
αRha
F

F

(R)

103.7	C-1 C	→3βGal-
102.6[c]	C-1 D'_a	→3αGlcA-
101.8[d]		4
101.1	C-1 D	↑
100.6	C-1 E	→2αRha-
100.0	C-1 F	αRha-
98.9	C-1 A_t	
96.2	C-1 A	→2αRha-
	C-1 B, B′	
94.6	C-1 E_a[e]	→2βRha-OH
92.7		→2αRha-OH
62.1	C-6 B, B′	→2αGlc-
61.7	C-6 C	→3βGal-
	C-6 E_a	→2Rha-OH
17.6	C-6 A, E	→2αRha-
	C-6 A_t, F	αRha-

P3

$A_t \rightarrow B' \rightarrow (R)_2 \rightarrow C \rightarrow D'_a \rightarrow E_a$

F

103.7	C-1 C	→3βGal-
102.6[c]	C-1 D'_a	→3αGlcA-
102.1[d,f]		4
100.9	C-1 D	↑
100.6	C-1 E	→2αRha-
99.7	C-1 F	αRha-
98.7	C-1 A_t	
95.6	C-1 A	→2αRha-
	C-1 B, B′	→2αGlc-
94.6[f]	C-1 E_a[e]	→2βRha-OH
92.3		→2αRha-OH
62.0	C-6 B, B′	→2αGlc
61.3	C-6 C	→3βGal-
17.5	C-6 A, A_t, E, E_a, F	

P≥4

$A_t \rightarrow B' \rightarrow (R)_{\geq 3} \rightarrow C \rightarrow D'_a \rightarrow E_a$

F

103.8	C-1 C	→3βGal-
102.5[c]	C-1 D'_a	→3αGlcA-
101.2	C-1 D	4
		↑

TABLE III *(continued)*

Compound	*δ*[a] *(p.p.m.)*	*Assignment*[b]	
	100.3	C-1 E	→2αRha-
	99.7	C-1 F	αRha-
	98.4	C-1 A_t	
	96.0	C-1 A	→2αRha-
		C-1 B, B′	→2αGlc-
	61.8	C-6 B, B′	
	61.4	C-6 C	→3βGal-
	17.5	C-6 A, A_t, E, E_a, F	

[a]Chemical shift relative to internal acetone: 31.07 p.p.m. downfield from Me_4Si. [b]See text, ref. 10, and footnote *f*, Table II. [c]"α" signal due to the α anomer of the reducing rhamnose group (E_a). Assignment: according to the relative intensity of the peak, by reference to the ^{1}H-n.m.r. spectrum. [d]"β" twin signal due to the β anomer of the reducing rhamnose group (E_a). Assignment: see footnote *c*. [e]The signals of the α and β anomers were assigned according to the relative intensities of the peaks, by reference to the ^{1}H-n.m.r. spectrum. [f]Residual signal.

TABLE IV

METHYLATION ANALYSIS AND β-ELIMINATION OF THE REPEATING UNIT **P1** FROM K19 POLYSACCHARIDE

Methylated sugars[a]	T[b] *program A*[c]	T *program B*	*Original* (**P1**)	*Carboxyl-reduced*[c] (**P1**-*CR*)	*Alditol*[d] (**P1**-*ol*)	*Carboxyl-reduced alditol*[c] (**P1**-*ol-CR*)	*α-Elimination of permethylated* **P1**-*ol*[f]
1,3,4,5-Rha-*1d*[g]	0.24	0.25	—	—	0.5	0.8	1.2
2,3,4-Rha	0.50	0.54	1.9	1.6	1.8	1.8	1.4[h]
3,4-Rha	0.79	0.83	0.4	0.8	0.1[i]	0.1[i]	0.1[i]
2,3,4,6-Glc	1.00	1.00	0.2[i]	0.2[i]	0.3[i]	0.3[i]	0.5[i]
3,4,6-Glc	1.46	1.37	0.9	1.1	0.8	1.0	0.9
2,4,6-Gal	1.66	1.52	1.0	1.0	1.0	1.0	1.0[j]
2,4,6-Gal-*1d*			—	—	—	—	
2,4-Glc	2.31	1.95	—	0.2[k]	—	0.4[k]	—
2-Glc	2.84	2.27	—	0.6[l]	—	0.4[l]	—

[a]In molar proportions of the alditol acetate derivatives. Values are corrected by use of the effective, carbon-response factors given by Sweet *et al.*[16]. [b]Retention times relative to 2,3,4,6-Glc. [c]Reduction by $LiAlH_4$ after permethylation. [d]$NaBD_4$-reduced **P1**. [e]See Experimental section. [f]Reduction by $NaBD_4$ after mild acid hydrolysis of β-eliminated permethylated **P1**-ol. [g]1,3,4,5-Rha-*1d* = 2-*O*-acetyl-1,3,4,5-tetra-*O*-methyl-1-deuterio-rhamnitol, etc. [h]High value due to incomplete β-elimination of the lateral, non-reducing rhamnose. [i]See text. [j]Mixture of deuterated and non-deuterated derivatives due to incomplete cleavage of the galactosyl bond during mild-acid hydrolysis of β-eliminated permethylated **P1**-ol. [k]Residual value arising through slight hydrolysis of the lateral, non-reducing rhamnose during treatment by Amberlite IR-120 (H^+) resin before methylation. [l]Low value because of incomplete reduction of carboxyl groups. See also footnote [k].

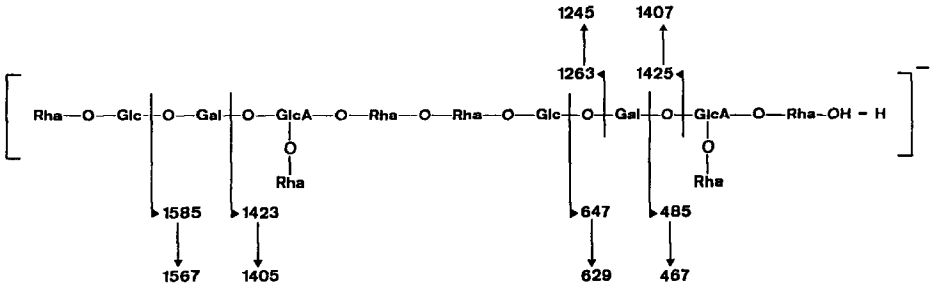


Fig. 7. Fragmentation pattern of **P2** by f.a.b.–m.s. (negative mode). Quasimolecular ion: $[M - H]^-$ at *m/z* 1893. Primary fragments shown *inter alia*[14] were further dehydrated to give ions of *m/z* 1407, 1245, 1567, 1405, 629, and 467.

the carboxyl-reduced and β-eliminated samples). This result provides evidence for a second site of cleavage by φ19, namely the Rha*p*1→2Glc*p* bond. This cleavage gave rise to an isomeric series of oligomers that could not be separated in the g.p.c. analyses, and these were not further investigated. The branched structure and the length of the side chain was again confirmed by base-caalyzed β-elimination performed on permethylated **P1**-ol. After mild acid hydrolysis, the residual methylated oligomer was reduced with sodium borodeuteride, and then analyzed conventionally (Table IV). The presence of 2,4,6-tri-*O*-methylgalactose-*1-d* confirmed the direct attachment of galactose to O-3 of the glucuronic acid residue. The foregoing methylation analysis results thus confirmed the structure of **P1** and also brought definitive evidence for the sequence of the polymer K19 (ref. 10).

The recent development of the f.a.b. technique in mass spectroscopy is well adapted for the study of complex oligosaccharides[13]. Both the positive and negative f.a.b.–m.s. spectra of **P1** provided unambiguous values for the corresponding quasimolecular ions, $[M + Na]^+$ of *m/z* 1001 and $[M + H]^+$ of *m/z* 979 (with **P1** as the sodium salt, mol.wt. 978), and $[M - H]^-$ of *m/z* 955. Similarly, the $[M - H]^-$ of *m/z* 1893 was in agreement with the structure of **P2**. In addition most of the fragments detected in the negative f.a.b.–m.s. spectra of **P2** suggested sequential degradation of the oligosaccharide, progressing from both ends of the molecule[14] (Fig. 7). This is exemplified by the fragments at *m/z* 1425 and 1263, which can only be explained by a cleavage from the reducing end, and by the series of ions at *m/z* 1585, 1423, 647, and 485, which are due to the loss of fragments that include the sugar at the non-reducing end.

CONCLUSION

According to the kinetics of release of the oligomers from K19 by φ19, it seems that, after a random attack, the oligosaccharides of higher d.p. are further rehydrolyzed to the monomeric repeating-unit **P1**. The glycanase is therefore able to cleave **P2**, which constitutes the smallest oligomer recognized by the enzyme.

This is a characteristic feature of the phage ϕ19 as compared to the specific phages of *Aerobacter aerogenes* DD 45 (ref. 2), and *Klebsiella* K11 (ref. 6), which were shown to be unable to cleave the dimers **P2**, the trimers **P3** being the minimum structures that could be accommodated by the enzymes. The efficient depolymerization of the linear polysaccharide K19-L seems to be a generalization of the ability of the phage glycanases to retain full activity on linear substrates[6], provided that the main chain retains the charged residue. This last point is well demonstrated by the failure of the phage to depolymerize the carboxyl-reduced substrate K19-CR. The necessity of the negative charge has been reported repeatedly for other phage systems[2,3,6,9], and the 0–10% activity observed was undoubtedly due to residual, unreduced carboxyl groups.

The site of cleavage by the glycanase of ϕ19 demonstrates that the phage-induced enzyme is an α-rhamnosidase. The fact that it was a rhamnosidase had already been suggested by Rieger-Hug and Stirm[1]. They also suggested that the capsular polysaccharide from *Klebsiella* serotype K58 should be cleaved by the same bacteriophage ϕ19, as these authors observed lysis plaques created by ϕ19 on a culture of K58 cells. Our attempts to depolymerize K58 with ϕ19 gave no cleavage of the polysaccharide, and yet the suspension of ϕ19 was able to form lysis plaques with *Klebsiella* K58, the relative efficiency of plating (E.O.P.) being ~50 times less than that observed in *Klebsiella* K19. Other unusual attributes have already been reported[3] with bacteriophages of *Klebsiella*. The phage ϕ13 was able to depolymerize K13, K2, K22, and K37 polysaccharides, but did not form lysis plaques on K37. The resistance of polysaccharide K58 to splitting by phage ϕ19-associated α-rhamnosidase is not surprising, as there is no rhamnose in K58 polysaccharide[15]. Even the second discrete glycanase activity detected through the hydrolysis products of K19 could not be responsible for a possible depolymerization of K58, as it was also characterized as an α-rhamnosidase. Another enzyme, bound to the phage particles, must therefore cause the infection of K58 cells.

EXPERIMENTAL

General methods. — G.l.c. analyses of permethylated **P1** and derivatives were performed with a Hewlett–Packard 5710 A instrument fitted with dual flame-ionization detectors. The spectrometer was coupled to a Hewlett–Packard 3390 A integrator. A glass column (3 mm × 2 m) packed with 3% OV17 on Chromosorb W-AW DMCS (100–120 mesh) was used, with a carrier-gas (nitrogen) flow rate of 60 mL/min. The programs used were: (A) from 170° for 4 min, and then at 1°/min to 220°; and (B) from 150° for 4 min, and then at 1°/min to 220. G.l.c.–m.s. was performed as previously described[10] for routine analyses. A special program was necessary to detect the tetra-*O*-methylated rhamnitol derivative from **P1**-ol: from 140° for 4 min, and then at 1°/min to 220°. ^{13}C-N.m.r. spectra of the oligosaccharides were recorded at 303K. See also preceding paper[10] for other general methods, total acid hydrolysis, and n.m.r. spectroscopy.

Propagation of the bacteriophage ϕ19. — For all phage work, the standard procedures given by Adams[17] were used. Phage titrations were carried out by the agar overlayer technique.

Suspensions of phage ϕ19 of high titer were prepared from samples of 2×10^{10} p.f.u./mL. Propagations were conducted in 250 mL of nutrient-broth medium (Standard I Nutrient Broth, Merck) under standard procedures[5–7]. The bacterial culture was infected with 3 viral particles per cell (8×10^{10} p.f.u.), at the beginning of the exponential growth phase when an absorbance of 0.44 was reached (that is 2.5×10^{10} cells at 10^8 cells/mL). Lysis occurred after 35 min and was complete 90 min later (Fig. 1). Incubation was pursued for ~1 h, and then the phage lysate was concentrated, centrifuged (2000*g* for 20 min) to remove the cell debris, and then dialyzed against distilled water. The average yield was 6×10^{12} p.f.u. in each experiment. The crude bacteriophage suspensions were stored at 4° in the presence of chloroform or sodium azide to prevent bacterial contamination. They were used directly for the depolymerization experiments without further purification[7].

Assays of phage potency. — Viscosity experiments were conducted at 37° in a thermostat-heated Ubbelhode cell with an automatic MS-Fica viscometer. The decrease of specific viscosity of a solution of K19 polysaccharide (0.05 g at 2.5 g/L in 0.1M NaCl) was monitored in the presence of either 10^{13} or 10^{12} p.f.u./g of K19 (Fig. 2). To prevent bacterial growth, NaN_3 was added to a final concentration of 0.02%. In each case the initial value was obtained by measuring the specific viscosity of blank solutions containing heat-denaturated viral particles (15 min at 100°). The same experimental conditions were applied to the carboxyl-reduced form of K19 (K19-CR)[10] and to K58 polysaccharide. No significant loss of specific viscosity was observed in either case. Only a slight drop of viscosity, attributable to incomplete reduction of the carboxyl groups, was noted during the first hours of the attack of K19-CR.

Gel-permeation chromatography. — Analytical g.p.c. was performed on a column (2.5 cm × 1 m) packed with Bio-Gel P6 (200–400 mesh), and eluted[8] with 50mM $NaNO_3$ (unless otherwise stated)) at a rate of 30 mL/h. In one case, Bio-Gel P10 (200–400 mesh) was used instead of Bio-Gel P6. Samples corresponding to 10 mg of the original polysaccharide were injected. Preparative g.p.c. was conducted on a column (4.5 cm × 1 m) packed with Bio-Gel P6 (200–400 mesh), and eluted with 50mM $NaNO_3$ at a rate of 70 mL/h. Samples corresponded to 0.25 g of the original polysaccharide. In all instances, g.p.c. was performed at 25° and NaN_3 was added to the eluent to a find concentration of 0.02%.

Partition coefficients (K_d) were determined through the relation $K_d = (V_e - V_0)/V_T - V_0)$, where V_e is the elution volume of the solute, V_0 the dead volume corresponding to the elution volume of the polymeric fraction excluded by the gel, and V_T the total volume corresponding to the elution of water in the saline eluent (or to the elution of an excess of $NaNO_3$).

The oligosaccharides were desalted on a column (4.5 cm × 1.5 m) packed

with Bio-Gel P2 (200–400 mesh) that was eluted at 65° with degassed water at a rate of 140 mL/h.

In all experiments a Milton-Roy mini-pump was used that allowed constant rates of elution. A Waters R 401 differential refractometer was used for detection.

Depolymerization of polysaccharide K19. — All experiments were conducted at 37° with crude bacteriophage lysates and solutions of K19 polysaccharide in water (Fig. 3). A final concentration of 0.02% NaN_3 was necessary to prevent bacterial growth. The reactions were stopped by heat-denaturation of the viral particles (15 min at 100°) before separation of the depolymerization products. In analytical g.p.c., the amounts of the different oligosaccharides were estimated through their refractive index by reference to the **P1** peak height.

The first experiments were performed according to Dutton *et al.*[7] (Fig. 3, scheme 1) on 0.1 g of K19 (at 1 g/L) with 10^{13} p.f.u./g of K19, and stopped at various times (4, 16, or 24 h). G.p.c. analyses showed no oligomers higher than **P2** because these were retained in the dialysis bag. A preparative experiment conducted during 24 h (K19, 1 g at 2.2 g/L; ϕ19, 10^{13} p.f.u./g of K19) gave only **P1** (yield: 70% of the original material). Results are shown in Table I.

In order to recover the higher oligomers, the dialysis step was then omitted (Fig. 3, scheme 2). A preparative experiment conducted during 15 h (K19, 0.5 g at 5 g/L; ϕ19, 8×10^{11} p.f.u./g of K19) afforded the purified and desalted oligosaccharides **P1**, $[\alpha]_D^{20}$ +71.2° (*c* 2.85, water), **P2**, $[\alpha]_D^{20}$ +62.4° (*c* 1.56 water), **P3**, $[\alpha]_D^{20}$ +38.4° (*c* 1.32, water), and a polymeric fraction **P≥4**, in the yields expressed in Table I.

Kinetics of depolymerization of K19 and K19-L. — Experiments were conducted at 37° for two different concentrations of K19, namely 2.6 or 0.52mM (0.1 g at 2.5 or 0.5 g/L), in the presence of 10^{12} p.f.u./g of K19. During the reaction, the changes of the concentrations of the different oligomers were estimated through their refractive index, NaN_3 acting as an internal standard, K_d 0.88 in 50mM NaO_3. Results of the first experiment are given in Fig. 5. For the lower concentration, the rate of appearance of each oligomer was lower but the curves were very much the same.

In the same way, the linear form of K19 (K19-L)[10] was submitted to the action of ϕ19 (K19-L, 0.09 g at 6 g/L; ϕ19, 2×10^{12} p.f.u./g of K19-L). The results are shown in Fig. 5.

Methylation analysis. — The original **P1** (30 mg) and its $NaBD_4$-reduced form **P1**-ol (30 mg) were dissolved in dimethyl sulfoxide, and methylated in the presence of methylsulfinyl sodium by a modified Hakomori[18] method according to Sandford and Conrad[19]. The permethylated oligosaccharides were recovered by partition between chloroform and water. In each case, one half of the material was carboxyl-reduced overnight with lithium aluminium hydride ($LiAlH_4$) in boiling tetrahydrofuran. A portion of each peralkylated oligosaccharide was then subjected to total acid hydrolysis with formic acid (90%, 1 h at 100°), and then with trifluoroacetic acid (2M, 3 h at 100°). The resulting partially methylated sugars were

analyzed as their alditol acetate derivatives by g.l.c.–m.s. (Table IV). The tetra-*O*-methylated rhamnitol derivative (1,3,4,5-Rha-*1d*), corresponding to the reducing end of **P1**-ol, was characterized by its fragmentation pattern in g.l.c.–m.s. analyses. Significant peaks appeared at *m/z* 46 (29%), 59 (100), 103 (33), 147 (3), 162 (14), and 206 (5) for the primary fragments, and at *m/z* 71 (36), 72 (37), 87 (32), 88 (17), 101 (16), 102 (18), 129 (36), 130 (32), and 146 (29). Part of the permethylated **P1**-ol (10 mg) was carefully dried, and then dissolved in a mixture (3 mL) of Me_2SO and 2,2-dimethoxypropane (19:1 v/v), and *p*-toluenesulfonic acid (0.5 mg) was added. Uronic acid degradation (β-elimination) was performed by adding methylsulfinyl sodium (3 mL). The mixture was kept under nitrogen for 16 h at 20°, and then made neutral with 50% acetic acid, and diluted with water. The degraded product was extracted by partition between chloroform and water. Mild acid hydrolysis was then conducted in the presence of 25% acetic acid for 1 h at 100°. After reduction with $NaBD_4$, the mixture was totally hydrolyzed and derivatized as usual. The results are expressed in Table IV. The deuterated 2,4,6-tri-*O*-methylgalactose-*1-d* (m.s. fragments at *m/z* 45, 118, 161, 234, and 277 appeared together with the non-deuterated derivative (m.s. fragments at *m/z* 45, 117, 161, 233, and 277) because of incomplete cleavage of the galactosyl bond during mild acid hydrolysis.

F.a.b.–mass spectrometry. — Samples of **P1** and **P2** were dissolved in a 1-thioglycerol matrix. No addition of sodium salts was needed because traces of $NaNO_3$ were still present in the purified oligosaccharides. Spectra (positive and negative mode) were performed with a VG Analytical ZAB-HF mass spectrometer fitted with a Ion-Tech FAB 11 NF atom-gun. The atom-gun was operated at 8 kV with argon as the bombarding gas.

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