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PREPARATION OF OLIGOSACCHARIDES BY THE ACTION OF BACTERIOPHAGE-BORNE ENZYMES ON *Klebsiella* CAPSULAR POLYSACCHARIDES

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ABSTRACT

Depolymerization of bacterial, capsular polysaccharides by viral enzymes provides a convenient method for preparing oligosaccharides that correspond to one or more repeating unit(s) of the polysaccharide. Previous methods used for purifying bacteriophage particles, and also the procedures employed in the isolation and purification of the oligomers generated by the bacteriophage action, have been so modified as to provide a more direct route to the degradation products. Improved techniques, both for the propagation of bacteriophage and for the isolation of the oligosaccharides formed, are reported. These simplified methods make possible the use of bacteriophages as convenient "reagents" for the preparation of oligosaccharides on a gram scale. The acid- and base-labile substituents present in certain of the polysaccharides examined were seemingly unaffected by the conditions used for depolymerization. The methods are illustrated by degradation of the capsular polysaccharides from *Klebsiella* serotypes K17, K36, K46, K60, K63, and K74.

INTRODUCTION

Klebsiella bacteriophages are associated with an enzymic activity capable of depolymerizing the host capsular-antigens into oligosaccharides that correspond to one or more repeating unit(s) of the polysaccharide. In earlier work of this nature, Sutherland[1] used several phage-induced enzymes to degrade the capsular polysaccharide from *Klebsiella* K54, and, more recently, Stirm and co-workers have isolated a large number of such viruses, each of which is specific for depolymerizing the capsular polysaccharide produced by the host strain, and, in some cases, other capsular glycans of related structure[2]. These viral enzymes have since been utilized in structural studies on certain *Klebsiella* polysaccharides, particularly those containing acid-labile constituents[3–6].

The oligosaccharides generated by bacteriophage action are suitable for a

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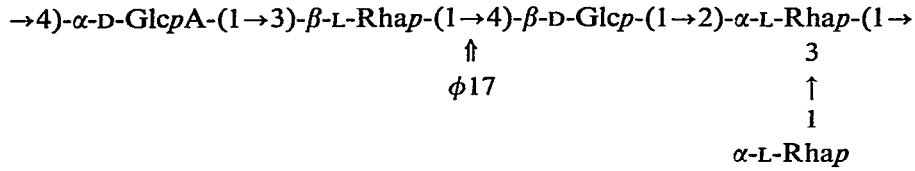
variety of comparative studies, not only in carbohydrate chemistry, but also in the fields of immunology and immunochemistry[7,8]. However, the standard techniques of virology used for obtaining highly purified virus particles with which to degrade the capsular antigens are not only time-consuming, but also result in considerable losses of bacteriophage. These procedures involve precipitation of the bacteriophage particles from the crude lysate with poly(ethylene glycol) 6000, and purification by isopycnic centrifugation using a linear density-gradient of cesium chloride. The recovery of purified virus particles is usually between 30 and 60% of those present in the crude lysate. Depolymerization is then conducted under "standard conditions" by incubating[3] a mixture of 2 mg of capsular polysaccharide and $\sim 2 \times 10^{10}$ plaque-forming units (PFU) of purified phage-particles in physiologically buffered saline (PBS) at 37°. The degradation products are now desalted, and separated from the bacteriophage and undegraded polysaccharide by successive passage[3] through columns of Sephadex G100, DEAE-Sephadex A25, and Sephadex G10.

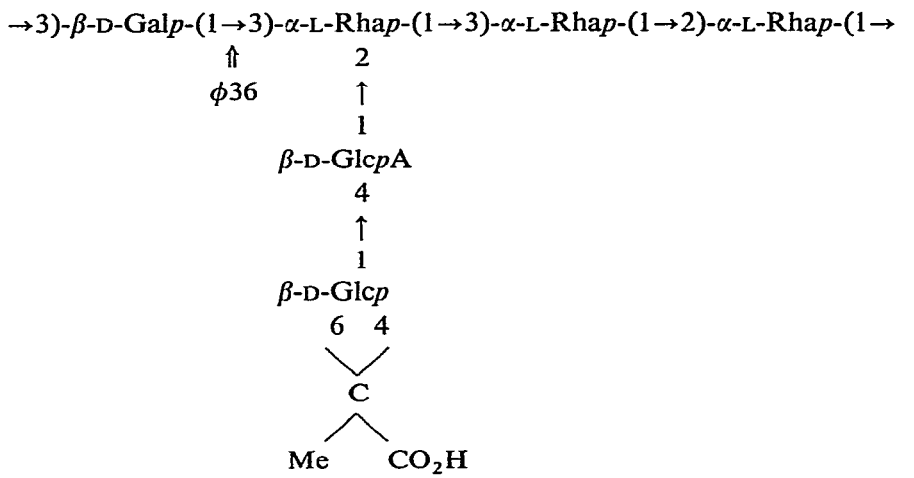
It should be noted that the procedure adopted by Sutherland[1], using partially purified viral-enzymes in a phosphate buffer and an aqueous solution of polysaccharide, with isolation of the products by dialysis and paper chromatography, provided a method simpler than those subsequently employed, but complete isolation and purification of all of the oligosaccharides could not be achieved.

For the characterization and study of a new bacteriophage, it is, of course, necessary to adopt a rigorous purification-procedure. When, however, it is desired to use the phage-borne enzyme as a reagent, certain simplifications are justified. Our interest in adapting bacteriophage degradations to the preparation of gram quantities of oligosaccharides has led to a series of experiments in which methods of propagation and purification of phage particles, as well as the degradation and isolation procedures[3], have been so modified as to provide a more direct route to the oligomeric products. The present studies were performed with six *Klebsiella* polysaccharides, comprising repeating units of different size and complexity (as shown), which were degraded with the respective, phage enzymes. These experiments could have been "standardized" by using the same polysaccharide and bacteriophage for each study. However, as our prime interest lies in utilizing the oligosaccharide products for further studies, it was more convenient to depolymerize several, distinct polysaccharides.

This paper is concerned with the different experimental conditions under which the depolymerization of capsular polysaccharides with phage-borne enzymes may be conducted. We initially tried to use crude bacteriophage and crude polysaccharide, but, because of a low yield of oligosaccharide and difficulties in purifying it, we then investigated other combinations of bacteriophage and polysaccharide of different degrees of purity. Method 4 reported here appears to give the best product for the least effort.



Klebsiella K17



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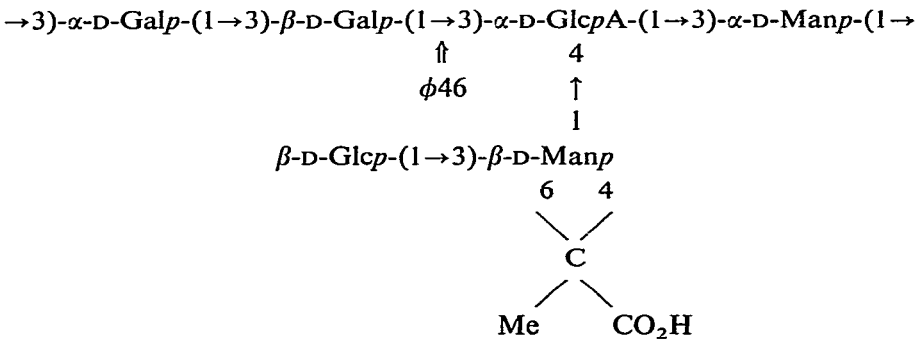
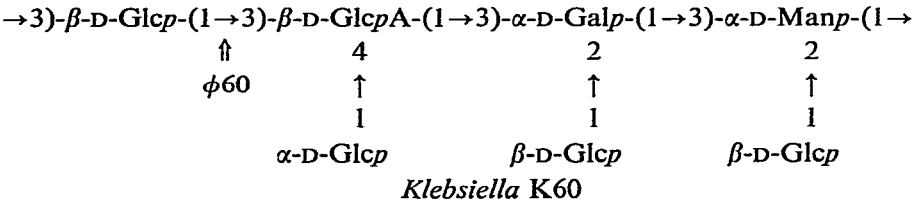


Klebsiella K46



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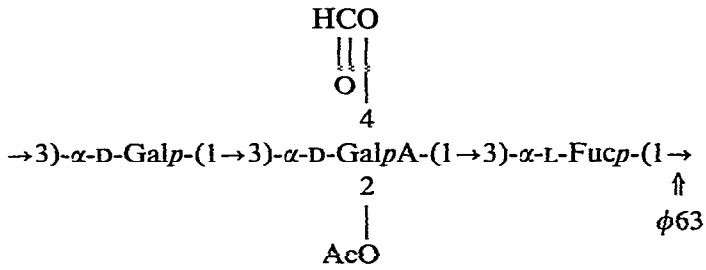
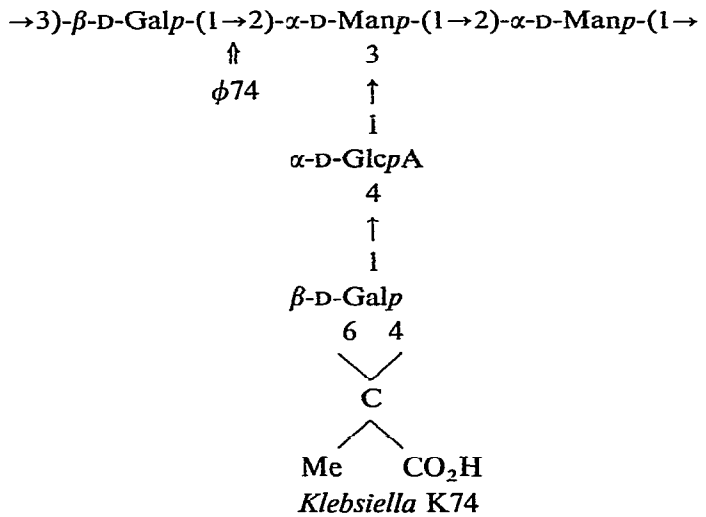


Klebsiella K63



Klebsiella K74

RESULTS

Klebsiella bacteriophages were isolated from sewage, purified by replating from single plaques, and propagated on their host strains by using nutrient broth as the medium[6]. Except in the case of bacteriophage ϕ63, propagation was continued on an increasing scale until the crude lysates contained a total of $\sim 10^{13}$ plaque-forming units, an amount sufficient to degrade one gram of polysaccharide. Bacteriophage ϕ63 was propagated on a large scale ($\sim$20 L) and then separated into two portions. The bulk of the crude lysate was partially purified by precipitation of the virus particles with poly(ethylene glycol) 6000, and the rest was stored in broth.

Capsular polysaccharides, grown and purified as previously described[9], were de-ionized before incubation with the corresponding bacteriophage according to the methods outlined next. For convenience, most reactions were allowed to continue for $\sim$3 days, although previous studies[3,7] had indicated that depolymerization is normally complete after $\sim$24 h. Even after 3 days, oligosaccharides smaller than one repeating unit could not be detected by paper chromatography. Because dilute solutions were used in these experiments, it was not practical to monitor the de-

TABLE I

EXPERIMENTAL CONDITIONS UTILIZED IN STUDIES ON BACTERIOPHAGE DEPOLYMERIZATION OF *Klebsiella* CAPSULAR POLYSACCHARIDES

Method	*K-Type*	*Poly-saccharide*	*Medium (mL)*	*Phage*	*Medium (mL)*[a]	*Reaction time (h)*	*Temperature (°C)*
1	63	agar	5000	broth	1000	72	37
2	63	PBS	350	PBS	25	67	37
3(a)	63	broth	175	broth	1200	67	37
3(b)	17	PBS	100	broth	660	67	37
3(c)	74	water	100	broth	990	67	37
4	46	water	150	water	300	24	37
4	60	water	110	water	300	24	37

[a]Volume containing $\sim 10^{13}$ PFU.

TABLE II

PROPORTIONS OF OLIGOSACCHARIDES ISOLATED FROM *Klebsiella* POLYSACCHARIDES FOLLOWING DEGRADATION WITH BACTERIOPHAGE

Method	*Poly-saccharide*	*References*	*Weight (g)*	*Phage (PFU)*	*Reaction volume (mL)*	*Oligosaccharides (mg)*			*Recovery (%)*
						P1	*P2*	*P3*	
1	K63	10, 11	n.d.[a]	1.0×10^{13}	1000	157	30	—	19
2	K63		1.0	1.0×10^{13}	375	188	131	9	33
3(a)	K63		1.0	1.0×10^{13}	1375	94	140	156	39
3(b)	K17	12	1.0	7.9×10^{12}	760	61	482	—	54
3(c)	K74	13	1.0	9.9×10^{12}	1100	180	160	172	51
4	K46	14	0.8	1.0×10^{13}	450	320	217	102	80
4	K60	15	1.0	1.0×10^{13}	410	441	226	93	76

[a]The yield of purified polysaccharide from 5 L of agar medium is usually 2–4 g. This may be somewhat higher in the case of K63, which is an exceptionally good producer of slime.

polymerization viscometrically, or by assay of the reducing power[3]. The conditions of hydrolysis used in each experiment are summarized in Table I, and the proportions of oligosaccharides thus generated are shown in Table II.

Method 1

Crude bacteriophage in broth was added directly to the slime polysaccharide grown on the surface of a nutrient, agar medium, and allowed to react for 24 h, following which, the liquid was decanted and incubated for a further 2 days at 37°. The depolymerization products were separated from the undegraded polysaccharide,

and also from the non-carbohydrate material, by the procedures outlined in the Experimental section.

Method 2

Partially purified bacteriophage was suspended in physiologically buffered saline (PBS), and the suspension was added to a solution of purified polysaccharide in the same medium. Depolymerization was allowed to proceed for ~3 days at 37°, following which, the solution was concentrated, the concentrate dialyzed, and the dialyzate freeze-dried. The oligosaccharides present in the dialyzate were extracted into pyridine, which was subsequently removed by distillation with water, and were isolated by preparative paper-chromatography.

Method 3

(*a*) Purified polysaccharide was dissolved in broth, and depolymerized with a crude solution of bacteriophage in broth for ~3 days at 37°, a small amount of chloroform having been added to prevent bacterial growth. The degradation products and low-molecular-weight constituents from the broth were separated from the bacteriophage and polymeric material by dialysis. The oligosaccharides in the dialyzate were isolated by preparative paper-chromatography, and further purified by ion-exchange chromatography.

(*b*) Purified polysaccharide was dissolved in PBS, and degraded with a crude solution of bacteriophage in broth for ~3 days at 37°, in the presence of chloroform.

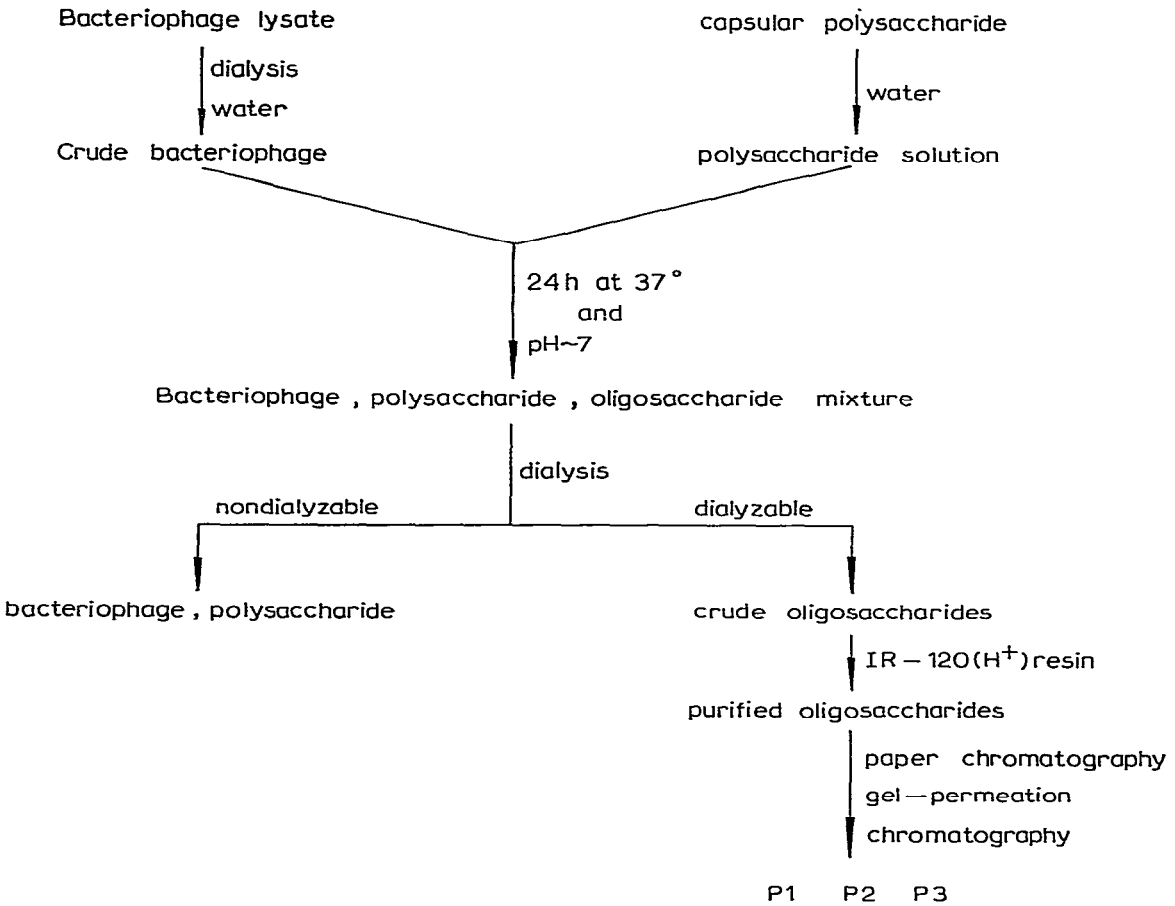
(*c*) Purified polysaccharide was dissolved in water, and degraded with a crude solution of bacteriophage in broth for ~3 days at 37°, in the presence of chloroform. The isolation and purification procedures used in (*b*) and (*c*) were as outlined in (*a*).

The methods utilized thus far all involve the separation of oligosaccharides from other materials of low molecular weight (mainly salts and amino acids*) present in the reaction medium, a step that may be eliminated by using the procedure described next.

Method 4

A crude solution of bacteriophage in broth was concentrated to a small volume, dialyzed overnight, and then added to an aqueous solution of purified polysaccharide. Depolymerization was allowed to proceed for ~24 h at 37°, chloroform being added to prevent bacterial growth; the mixture was then concentrated, and the concentrate dialyzed against distilled water. The process of concentration and dialysis was repeated twice more, the dialyzates were combined and concentrated, and the concentrate was treated with a cation-exchange resin, and freeze-dried. The mixture of oligosaccharides obtained was then separated into pure components by using the methods of paper, and gel-permeation, chromatography. The procedure is outlined in Scheme 1.

*From denaturation of peptides present in the nutrient broth.



Scheme 1. Flow diagram for the preparation of oligosaccharides using bacteriophage-borne enzymes.

Recovery of phage

In one set of experiments, a batch of *Klebsiella* K36 polysaccharide[16] was degraded according to method 2, and, when hydrolysis was judged to be complete, the solution was centrifuged at 100,000*g*. The recovered pellet of phage was resuspended in PBS, and used to depolymerize a second batch of polysaccharide. It was found possible to repeat this sequence twice more with only a small loss of activity.

DISCUSSION

From the results shown in Table II, it is evident that high yields of oligosaccharides may be obtained by using a solution of crude bacteriophage particles to degrade the corresponding, purified polysaccharide. Degradation may be achieved with broth, buffered saline, or water as the reaction medium, but the last is preferable, as it decreases the degree of contamination with salts and other low-molecular-weight

substances, the removal of which is often difficult. It should be noted that all three media have a pH that is close to neutral; previous studies by Stirm *et al.*[3] showed that viral enzymes exhibit an activity optimum in the pH range of 5–7.

The recovery of oligosaccharides using Method 1 was extremely poor, probably due to the facts that: (*a*) the amount of bacteriophage added was sufficient to depolymerize only a small proportion of the polysaccharide generated by the bacteria; (*b*) the oligomers generated were highly contaminated with salts and other low-molecular-weight materials; and (*c*) oligosaccharides could have been absorbed into the agar medium. During this study it became apparent that reasonable amounts of oligomeric material released from the K63 polysaccharide could be desalted by conversion into its trimethylsilyl derivative and extraction of the ether into hexane. However, as this method of purification depends mainly on the solubility of the oligosaccharides in pyridine, it is limited to oligomers soluble in this solvent.

Methods 2 and 3 necessitate separation of the oligosaccharide products from salts and other dialyzable material, a process that may be eliminated altogether by dialysis of the crude, bacteriophage lysates before addition to an aqueous solution of the polysaccharide (Method 4). Oligosaccharides may then be separated from the bacteriophage and undegraded polysaccharide by dialysis, and readily purified by passage through a cation-exchange resin. This method has since been employed in studies on the polysaccharide[17] from *Klebsiella* K70, again resulting in a high yield of degradation products (>70%). The quantities of oligosaccharides produced in this way are comparable to those obtained by Stirm and co-workers on using a dialyzable P-medium with which to propagate the bacteriophage.

Procedures that are used to separate mixtures of oligosaccharides into their pure components depend primarily on the size and nature of the oligomers themselves. In the case of *Klebsiella* K63, for example, the products were readily separated by preparative paper-chromatography, whereas the larger oligomers released from the K60 polysaccharide were better separated by using a column of Bio-Gel P-4. The separation of higher oligomers from their low-molecular-weight counterparts may, in some instances, be achieved by use of dialysis tubing having a low cut-off point (~2000).

The relative proportions of oligosaccharides generated by the enzyme action are related to the initial concentration of the polysaccharide, as is evident from the results obtained for the K63 polysaccharide [see Table II, Methods 2 and 3(a)], where a higher concentration of polysaccharide yielded mainly the monomer of the repeating unit (P1), but, at lower concentration, the oligosaccharides corresponding to two (P2) and three (P3) such units preponderated. Behavior of this type was also noted in the other depolymerization experiments, and may be of value in future studies, as it has recently been shown that a minimum of two repeating units is needed in order to obtain a substantial representation of the serological specificity[7] of a bacterial polysaccharide. Furthermore, the higher oligomers may be subjected to a number of nondegradative studies, and ultimately be depolymerized to give the monomeric product.

The procedures used in this study, particularly those outlined in Method 4, therefore provide a relatively simple, and more-convenient, procedure for the preparation of large quantities of oligosaccharides generated by the action of viral enzymes on capsular polysaccharides. Neither the acetal substituents present in the polysaccharides from K46 and K74, nor the acetate and formate groups in the K63 polysaccharide, were found to be removed to any great extent under these conditions.

The ability to recover the phage enzyme by centrifugation suggests that it may be possible to improve on the currently accepted requirement of 10^{13} PFU per gram of polysaccharide to be degraded, but no attempt has been made here to determine the practical maximum.

EXPERIMENTAL

General methods. — Bacteriophages were isolated from sewage, and purified by successive replating of single plaques on their respective host-strains. Phages were propagated in broth [peptone (5 g), beef extract (3 g), and sodium chloride (2 g) per liter of water] until $\sim 10^{13}$ PFU had been obtained (see Table I). Crude lysates were stored over chloroform at 4° until used in the depolymerization experiments. In all reactions involving the use of broth, chloroform was added to the mixture to prevent bacterial growth.

In the case of phage ϕ63, propagation was continued until ~20 L of crude lysate had been obtained. The bulk of this solution (12 L), containing $\sim 4 \times 10^{13}$ PFU, was treated with poly(ethylene glycol) 6000, and the precipitated material was isolated by centrifugation. The partially purified ϕ63 ($\sim 2 \times 10^{13}$ PFU) was resuspended in PBS (50 mL), and the suspension used to degrade the K63 polysaccharide according to Method 2. The rest of the crude lysate was stored at 4° until utilized as described in Method 3(a).

Preparative paper-chromatography was performed with Whatman 3MM paper, and freshly prepared 2:1:1 1-butanol–acetic acid–water as the solvent. Oligosaccharides were made visible by development of the chromatograms with *p*-anisidine spray[18] or alkaline silver nitrate reagent[19]. Gel-permeation chromatography was performed in columns (85 cm × 2.5 cm) of Bio-Gel P-2 or Bio-Gel P-4, with 500:5:2 water–pyridine–acetic acid as the eluant.

Depolymerization reaction-mixtures were incubated in a thermostatically controlled water-bath at 37°. Solutions were evaporated under diminished pressure in a rotary evaporator at a bath temperature not exceeding 40°.

Method 1

Each of two stainless-steel trays (60 × 40 cm) was charged with a sucrose-rich medium[14] (2.5 L), and then inoculated with K63 bacterial culture (50 mL), pre-incubated overnight at 37°. The trays were then covered, and bacterial growth was allowed to proceed for 3 days, following which, 500 mL of crude ϕ63 ($\sim 5 \times 10^{12}$ PFU) was added to each tray and spread evenly over the surface. After 24 h, the

liquid was decanted, and the surface of the agar was rinsed with small amounts of water, in order to remove any residual polysaccharide. The solution was then incubated for 2 days at 37°, in order to allow complete depolymerization. At this stage, the sucrose absorbed from the polysaccharide medium was decomposed by the addition of baker's yeast (1 g), and 24 h later, the solution was centrifuged. The clear, supernatant liquid was freeze-dried, and the residue was successively extracted with cold and hot methanol. Evaporation of the combined, hot extracts yielded 1.9 g of crude material, which was shown by paper chromatography to contain two components. Gel-permeation chromatography of a sample (0.25 g) on Bio-Gel P-2 yielded 25 mg of the trisaccharide (P1), together with a trace of the hexasaccharide (P2). Separation of a further sample (0.48 g) by preparative paper-chromatography gave both oligosaccharides P1 (64 mg) and P2 (20 mg).

The bulk of the material (1.2 g) was treated with the (trimethylsilyl)ating agents [pyridine (35 mL), hexamethyldisilazane (10 mL), and chlorotrimethylsilane (6 mL)], and then water was added and the *O*-trimethylsilyl derivatives were extracted into hexane. Evaporation of the extract, and subsequent removal of the Me_3Si groups with methanol, yielded a carbohydrate-rich fraction which was separated into pure components P1 (68 mg) and P2 (10 mg) by preparative paper-chromatography.

Method 2

Purified K63 polysaccharide (1 g) was dissolved in PBS (350 mL), and depolymerized with partially purified ϕ63 in PBS (25 mL) for 67 h. The resulting mixture was concentrated, the concentrate dialyzed against distilled water (3 × 500 mL), and the combined dialyzates were freeze-dried. The residue was extracted with pyridine (3 × 100 mL), the extracts were combined, and the pyridine was evaporated in a rotary evaporator by co-distillation with water, to yield a carbohydrate-rich fraction that was separated by preparative paper-chromatography into the proportions of oligosaccharides shown in Table II.

Methods 3(a)–3(c)

Solutions of the purified polysaccharides from K-types 63, 17, and 74 were each depolymerized by using a crude solution of the corresponding phage in broth. The media and volumes used in each experiment are shown in Table I; the oligomers generated in these reactions were separated from high-molecular-weight material by dialysis, and separated by preparative paper-chromatography. De-ionization of the individual components with Amberlite IR-120 (H^+) resin yielded the purified oligosaccharides (see Table II).

Method 4

Crude lysates (1 L) of bacteriophages ϕ46 and ϕ60 (containing $\sim 10^{13}$ PFU) were concentrated to a small volume, and the concentrates dialyzed against tap water for ~18 h before being added to an aqueous solution of the capsular polysaccharide. Depolymerization was conducted for 24 h, the mixture was evaporated to dryness,

the residue was dissolved in the minimum volume of water, and the solution dialyzed against distilled water (3 × 800 mL). The dialyzates were combined, and concentrated, and the concentrates were treated with Amberlite IR-120 (H^+) resin, and freeze-dried. The oligosaccharides generated in each reaction were separated by gel-permeation chromatography on Bio-Gel P-4, and, in some cases, further purified by preparative paper-chromatography. Traces of amino acids still present after these procedures were eliminated by a second treatment with the resin.

Recovery of phage. By following Method 2, K36 polysaccharide (1 g in 200 mL of PBS) was incubated at 37° with 30 mL of a solution of ϕ36 containing 2×10^{12} PFU.mL^{-1}. The viscosity fell to that of the saline solution within 4 h, and the reaction was allowed to proceed for 5 days. The average $\overline{d.p.}$, as determined by colorimetric measurement of the reducing power[3] (galactose standard-curve), was 8. The phage was recovered as a pellet by centrifugation at 100,000*g*, resuspended in PBS with gentle stirring, and two-thirds of the suspension was added to K36 polysaccharide (400 mg) in PBS (100 mL). The viscosity dropped within 3 h to that of the saline solution and the $\overline{d.p.}$ was 8. Similar results were obtained by recovery, and reincubation with two further batches of polysaccharide.

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