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BACTERIOPHAGE-BORNE ENZYMES IN CARBOHYDRATE CHEMISTRY

PART I ON THE GLYCANASE ACTIVITY ASSOCIATED WITH PARTICLES OF *Klebsiella* BACTERIOPHAGE NO 11*

HORST THUROW†, HEINER NIEMANN, AND STEPHAN STIRM‡
Max Planck-Institut für Immunbiologie, Freiburg (Germany)
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ABSTRACT

The preparation and use of particles of *Klebsiella* bacteriophage No 11 are described A glycanase activity associated with the viruses catalyses the depolymerization of (alkali-treated) *Klebsiella* serotype 11 capsular polysaccharide, ultimately to a mixture of oligosaccharides consisting of one or two repeating units Mainly glucosidic bonds are hydrolysed The substrate specificity of the viral enzyme has been characterized by using derivatives of serotype-11 polysaccharide, as well as 81 heterologous, bacterial, capsular glycans It is concluded that the glycanase will (at least) also depolymerize all polysaccharides containing the unsubstituted chain-trisaccharide repeating-unit of its natural substrate

INTRODUCTION

As with many animal viruses, notably the *Myxoviruses*[1], enzymic activities catalysing a degradation of host cell-surface constituents may also be associated with bacteriophages Thus, most Vi phages[2–4], and certain *Escherichia coli* capsule bacteriophage particles[5], catalyse the hydrolysis of *O*- and *N*-acetyl groups substituting the host envelope-polysaccharides, and several other (also mainly *Enterobacteriaceae*) bacteriophages carry glycanase activities that depolymerize these acidic, capsular glycans[5–10], or, in some cases[11–14], the neutral glycans which are part of the cell-wall lipopolysaccharides of Gram-negative bacteria The murolytic enzymes, induced by many bacterial viruses[15], were originally thought to occur also as part of the virions[16,17] Later evidence[18], however, showed that, at least in the T-even group of *E coli* phages (but probably not in all cases[19,20]), the murolysins,

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†In partial fulfilment of the requirements for the degree of Dr rer nat at Freiburg University
‡To whom inquiries should be addressed

although produced under the control of the viral genomes, are not incorporated into the particles

Highly purified bacteriophage particles can be prepared in considerable amounts by using the simple techniques of precipitation with polyethylene glycol and isopycnic centrifugation[21] This, together with the facts that phages can be isolated for the majority of bacterial organisms[22] and that carbohydrates generally constitute a major part of bacterial surfaces, makes available a wide array of pure agents of potential value in carbohydrate chemistry

Klebsiella bacteriophage No 11 grows on *Klebsiella* 390 (03 K11), the serological test-strain for the *Klebsiella* type 11 capsular (K) antigen[23] The phage was isolated[24] from sewage, and selected because of its plaque morphology, which showed[6] that a host-capsule depolymerase activity is associated with it As reported elsewhere[6], the active center of this depolymerase is located in the virus spike-structures (organelles having a molecular weight of 155,000 $\pm$10,000, and consisting of one polypeptide chain of $\sim$62,500, and one of 94,000) and is part of the 62,500 sub-unit[25] We now describe the depolymerization of *Klebsiella* serotype 11 capsular polysaccharide[23], as catalysed by phage-11 particles, as well as some experiments on the substrate specificity of the viral enzyme

MATERIAL AND METHODS

Many of the materials and methods used have been described or cited in the preceding paper[23], including details of the phosphate-buffered, physiological saline (PBS) and most of the media, the isolation of *Enterobacteriaceae* capsular polysaccharides by the phenol–water–cetyltrimethylammonium bromide procedure, the analytical ultracentrifugation, and the methods employed for constituent and methylation analysis of oligosaccharides

P medium — Dialysable P-medium was prepared as follows[24]: solutions of 25 g of D-glucose (in 1000 ml of water), of 6 25 g of Casamino Acids (Difco B230), 0 4 g of L-tryptophan, 0 3 g of L-cysteine, 2 5 g of KH_2PO_4, 15 6 g of $Na_2HPO_4 \cdot 12H_2O$, 1 3 g of NH_4Cl, and 0 01 g of gelatin (in 1000 ml), of 5 0 g of $MgSO_4 \cdot 7H_2O$ and 0 01 g of $FeSO_4$ (in 100 ml), and, finally, of 0 5 g of $CaCl_2$ (in 10 ml) were sterilized separately and stored at 4° Before use, they were mixed in the volume proportions 20 80.1 0 1, and adjusted to pH 7 2

Bacterial strains — Besides the host strain, *Klebsiella* 390[23], the following capsular (or mucoid) strains were used for the substrate specificity experiments the other 71 serological *Klebsiella* K antigen test-strains as listed by Nimmich[26], the test strains for the *E coli* K antigens Nos 26, 28, 29, 30, 31, 34, 38, and 42[24], as well as *E coli* F9095/41M, which produces colanic acid[27] All strains were kindly supplied by Drs I and F Ørskov (WHO International *Escherichia* Center, Statens Seruminstitut, Copenhagen)

Bacteriophage strain. — *Klebsiella* bacteriophage No 11[6], as propagated on *Klebsiella* 390 in P medium, was used exclusively

Isolation and homogeneity of phage particles — For all phage work, the standard procedures given by Adams[28] were used Stock suspensions of phage 11 in broth were obtained from $D_{1\ 5}$ agar plates by the confluent lysis method, and phage titrations were carried out by the agar overlayer technique

Purified phage particles were prepared as follows[6] 350- or 1000-ml wash-bottles having large, sintered-glass gas inlets, containing sterilized P-medium, were placed in a water bath at 37° and strongly aerated with a water pump, the inlets and outlets being stoppered with cotton wool. The medium was inoculated with a fresh colony of *Klebsiella* 390 and incubated under continued aeration until an extinction of 1 1 at 660 nm (1-cm cell) was reached The culture then contained $\sim 9 \times 10^7$ colony-forming organisms per ml, and was infected with ~ 3 PFU (plaque-forming units) of phage 11 per cell. After lysis, which occurred ~ 20 to 30 min later, the incubation was continued for another 30 to 60 min Foaming was prevented by reduction of the aeration and addition of a little silicon antifoam The lysates, which had a titer of 2×10^{10} to 4×10^{10} PFU/ml, were centrifuged at 5000 *g* (20 min), and 0 5M NaCl and 10% (w/v) of polyethylene glycol 6000 (Fluka, No 8/260)[21] were added to the supernatant After storage at 4° for 48 h, the phage particles were sedimented at 20,000 *g* (30 min) The pellets were taken up in as little PBS as possible (to a titer of 5×10^{11} to 10^{13} PFU/ml) Alternatively, the clarified lysates were concentrated to the same phage-titer by negative-pressure dialysis, and then centrifuged at 5000 *g* once more The concentrated virus-suspensions obtained by either method (1 vol) were placed on linear density gradients (2 vol) in Spinco swinging-bucket rotor tubes The gradients were obtained by dissolving 49 g of CsCl in 48 7 ml ($\rho = 1\ 63$ g/ml), and 9 8 g of CsCl in 47 2 ml ($\rho = 1\ 13$ g/ml) of a 0 1M Tris/HCl buffer (pH 7.5) containing 0 5% of NaCl and 0 1% of NH_4Cl, and mixing the two solutions with a linear-gradient mixing device After centrifugation for 2 h at 50,000 *g*, the opalescent phage-band at 1 50 g/ml (lowest band, this was clearly visible if $\sim 3 \times 10^{11}$ PFU or more had been applied to the gradient) was withdrawn with a syringe and dialysed against PBS In this manner, $\sim 10^{13}$ to $2\ 5 \times 10^{13}$ PFU of purified virus particles (50 to 60% of those in the crude lysate) could be obtained from 1 litre of culture, this was sufficient for the complete depolymerization of ~ 1 g of *Klebsiella* type 11 capsular polysaccharide If stored over chloroform at 4°, a phage suspension in PBS kept its enzymic activity for several months, although the plaque-forming activity decreased

The equipment and the techniques used for the electron optical visualization, as well as for the immunoelectrophoresis of phage-11 particles (against a rabbit serum obtained with crude, concentrated phage lysate) have been detailed previously[6 24]

Conditions of depolymerization — Under "standard conditions", a mixture of 2 mg of polysaccharide and $\sim 2 \times 10^{10}$ PFU (plaque-forming units) of purified phage particles per ml of PBS were incubated at 37° (up to 24 h for a maximum of depolymerization, see Results)

The loss of viscosity during the course of depolymerization was followed by running the reaction in an Ostwald viscosimeter at 37° and measuring the efflux time

at intervals, and the increase in reducing power by adding potassium ferricyanide reagent (0 5 g of $K_3[Fe(CN)_6]$ in 1 litre of 0 5M Na_2CO_3)[29] to consecutively withdrawn aliquots of the reaction mixture, and reading the extinction at 420 nm after 20 min at 100° (D-glucose serving as a standard). For the pH dependence of the depolymerization reaction, standard conditions were employed, but using 0 2M sodium acetate (for pH 3 6–5 5) and M/15 sodium phosphate (for pH 5 4–8 0) buffers as reaction media

The pH and temperature stability of the phage enzyme were tested by storing suspensions at different pH values or temperatures, and estimating the residual glycosidase activity by the methods described previously[6]

Purification of depolymerization products — Portions (150 mg) of *Klebsiella* type 11 oligosaccharides (obtained by phage degradation under standard conditions) in 1 ml of PBS were placed on a column (100 cm × 1 76 cm^2) of Sephadex G100 and eluted with a volatile water–pyridine–glacial acetic acid (1000 10 4) buffer (pH 4 5) at 4 ml/h, localizing the fractions containing carbohydrates with phenol–sulfuric acid[30] About 4% of the material (in terms of chromogen) appeared in the void volume, the rest, eluting between 135 and 165 ml, and showing partial separation of sub-fractions only, was combined and lyophilized Yield: above 90% (w/w of polysaccharide before phage action)

For further separation, 200-mg portions of the pre-purified oligosaccharides in 5 ml of a 0 05M Tris/HCl buffer (pH 7 2) were adsorbed on a column (60 cm × 1 76 cm^2) of DEAE-Sephadex A25 and eluted (18 ml/h) with a linear NaCl gradient (from 0 1 to 0 4M) in the same buffer The carbohydrates were localized as above, and the fractions belonging to single peaks (*cf* Fig 2) were combined, desalted by passage over a column (150 cm × 2 55 cm^2) of Sephadex G10 (elution at 12 ml/h with the volatile buffer mentioned above), and lyophilized Yield above 90% (in terms of phenol–sulfuric acid chromogen, as well as w/w of the pre-purified oligosaccharides) For the proportions of the single oligosaccharides, see Fig 2 and Results

Analyses of depolymerization products — Using the experimental details given previously[5], the reducing-end sugars in the oligosaccharides obtained by phage action were identified by labelling with $NaBH_4/NaBT_4$, hydrolysis, and high-voltage paper electrophoresis in a 0 2M arsenite buffer[31] of pH 9.6 Radioactive hexitols and gulonic acid were used as standards, and undepolymerized *Klebsiella* serotype 11 polysaccharide as a control The electropherogram strips were analysed with a Packard radiogram scanner For identification of the non-reducing-end sugar, oligosaccharide *P1* was oxidized with periodate, reduced with $NaBH_4$, and hydrolysed, and the hydrolysate was subjected to paper electrophoresis in pyridine–glacial acetic acid–water, using glucuronic and erythronic acids as standards For further experimental details, see preceding paper[23]

Oligosaccharides *P1* and *P2* were permethylated, and the methylated alditol acetates analysed by g l c –m s , as cited and detailed previously[23] The reduction

of methylated *P1* was carried out as described by Bjorndal and Lindberg[32], but using $LiBD_4$ instead of $LiAlH_4$ in tetrahydrofuran

The size of the phage degradation products was determined as follows At 4°, 50 µl of a 4% solution of $NaBH_4$ in 0 01M NaOH, and the same after decomposition of the borohydride with dilute acetic acid (control), were added to aliquots of an aqueous solution of oligosaccharide containing 0 4 µmole each After 18 h at 4°, the excess borohydride was decomposed also in the test sample, and the boric acid was removed from both by repeated evaporation with methanol After hydrolysis [0 5M H_2SO_4, 100°, 4 h, neutralization with dry Amberlite IR-410 (HCO_3^-) resin], both samples were analysed for D-glucose with D-glucose oxidase, and for D-glucitol with D-glucitol dehydrogenase from sheep liver (EC 1 1 1 14, Boehringer, No 15316), using the test conditions given by Bergmeyer *et al* [33]

Substrate specificity (and host range) of Klebsiella *phage 11* — Drops (50 µl) of a broth suspension of phage 11, containing 5×10^{10} PFU/ml, were placed on $D_{1\,5}$ agar plates freshly seeded with the strains listed above After 18 h at 37°, the plates were inspected for lytic actions As a control, one drop of the same suspension was incubated in the same manner on a lawn of *Klebsiella* 390 which had, however, already been grown for 18 h at 37° and then killed by heating to 60° for 90 min In the case of *E coli* B1161/42 (09 *K29*(A) H^-), the relative efficiency of plating on host versus test strain was accurately titrated[28]

Chemically modified type-11 polymers (for preparation and anlyses, see preceding paper[23]) as well as heterologous bacterial exo-polysaccharides (also isolated by the phenol–water–cetyltrimethylammonium bromide procedure[23]) were incubated with pure phage-11 particles for 24 h under "standard conditions", and the crude mixtures of products and viruses were then analysed for reducing power with the potassium ferricyanide reagent[29] (see above), using the unreacted polymers as controls The results were expressed in µg of reaction products equivalent to 1 µg of glucose in reducing power

RESULTS

Isolation and homogeneity of phage-11 particles — As isolated by precipitation with polyethylene glycol or negative-pressure dialysis, and isopycnic centrifugation, the particles of *Klebsiella* bacteriophage No 11 were homogeneous, as evidenced by immunoelectrophoresis against a rabbit serum obtained with crude, concentrated lysate, by electron microscopy, and by analytical ultracentrifugation

Conditions of virus-catalysed depolymerization of Klebsiella *serotype 11 capsular polysaccharide* — Originally, the depolymerizations of type-11 polysaccharide, as catalyzed by purified particles of phage 11, were carried out in "P buffer", *i e*, growth medium without glucose, amino acids, and gelatin. The addition of NH_4Cl, $MgSO_4$, $FeSO_4$, and $CaCl_2$, however, was then found to be unnecessary

As shown in Fig 1, the virus enzyme (which proved to be stable between pH 6 and 9, and up to at least 40°) showed a flat activity-optimum between about pH 5

and 7, and a temperature optimum around 33–37°, its action slowed down after 2–3 h at pH 7 and 37°, if 2×10^{10} plaque-forming units and 2 mg of polysaccharide per ml were incubated. All depolymerizations were then performed using these concentrations, 37°, and PBS of pH 7 as a reaction medium ("standard conditions")

Depolymerization products — By gel chromatography on Sephadex G100, the oligosaccharides produced by prolonged (*i e*, 10 or 24 h) phage action on native or mildly alkali-treated [removal of *O*-acetyl substituents and alteration of sedimenta-

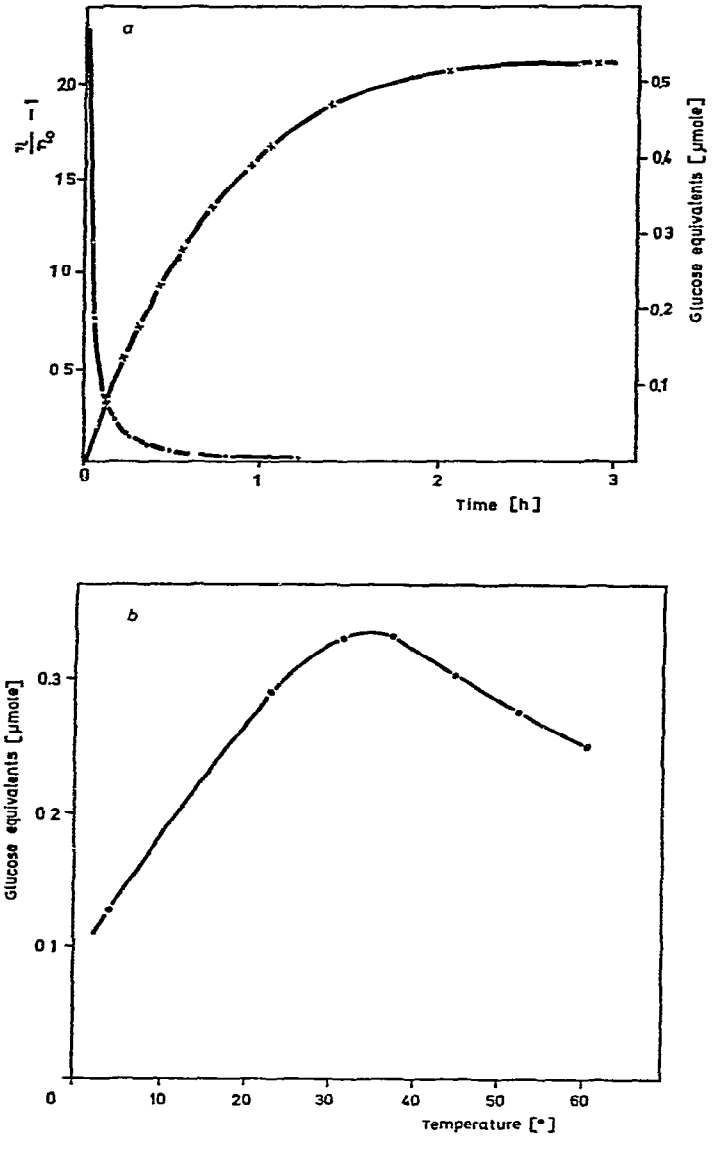

Fig 1

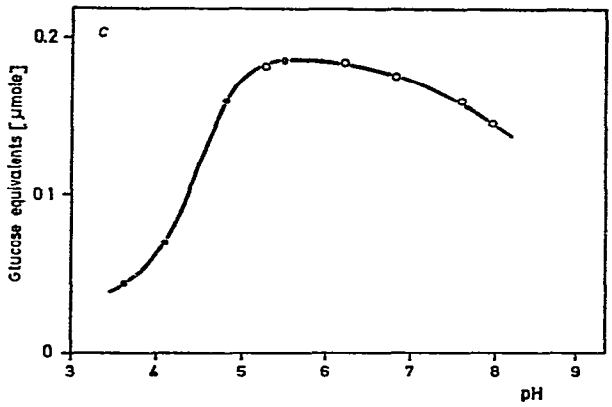

Fig 1 Conditions of depolymerization of *Klebsiella* serotype 11 capsular polysaccharide by catalysis of purified phage-11 particles *a*, Loss of viscosity (–●–●–●–) and increase in reducing power (–×–×–×–, glucose equivalents, µmole per ml) during incubation at 37° in PBS *b*, Temperature (in PBS), and *c*, pH (for buffers see Material and Methods) dependence of liberation of reducing power In all cases, ~2×10^{10} plaque-forming units, and 2 mg of polysaccharide per ml were incubated (1 h at 37° for *b* and *c*)

tion pattern[23] (see Discussion)] host capsular polysaccharide were separated from ~4% of undepolymerized material, as well as from the catalytic amounts of viruses The resulting mixtures of depolymerization products showed the same constituent composition as the polymers[23] (*e g*, D-glucose D-galactose D-glucuronic acid pyruvate *O*-acetyl = 1 00 1 77 1 07 0 91 0 41 found in the products from native glycan), except that no mannose was detected

The separation of the oligosaccharides obtained from mildly alkali-treated substrate was achieved by ion-exchange chromatography (Fig 2), or by paper electrophoresis in pyridine–glacial acetic acid–water (pH 5 3) (mobilities *P1*, 0 78, *P2*, 0 88, *P3*, 0 94, relative to glucuronic acid) *P1* was also checked for purity by p c with 1-butanol–glacial acetic acid–water ($R_{GAL} = 0\ 22$) Continued incubation of oligosaccharide *P3* with virus particles caused a very slow splitting in *P1* and *P2*, while isolated *P2* was not further affected Fig 2 also shows the ion-exchange chromatogram of the fragments produced from native type-11 polysaccharide Further incubation did not cause any change in the elution pattern The virus-catalysed degradation of native host-capsular glycan thus ultimately yielded some larger oligosaccharides not obtained from alkali-treated material The quantitative constituent analysis of *P1'* and *P2'* again showed the same composition (notably also the same *O*-acetyl content) as the native polymer

The reducing-end sugars produced by phage-11 action were identified[5] by labelling of the *crude* mixtures of depolymerization products with $NaBH_4/NaBT_4$, hydrolysis, and identification of the radioactive hexitols obtained by paper electrophoresis in an arsenite buffer[31] In this manner, glucose was found to be the dominant reducing-end sugar, but small amounts of radioactive galactitol and mannitol were

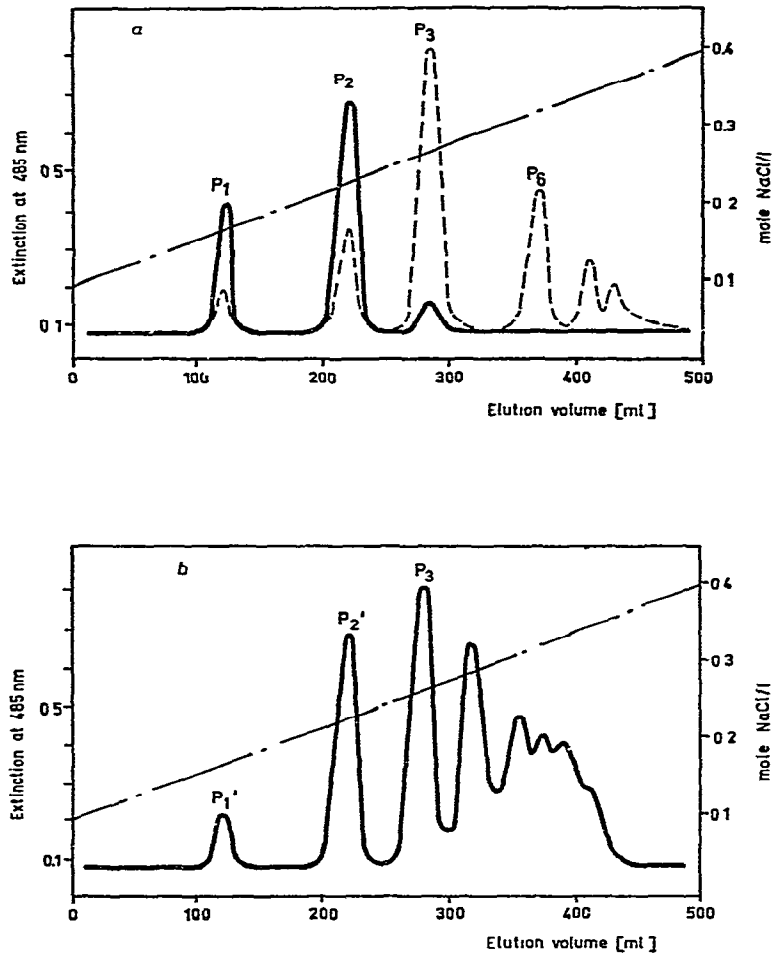

Fig 2 Ion-exchange chromatography of *Klebsiella* type 11 oligosaccharides obtained by phage depolymerization The material was adsorbed on a column of DEAE Sephadex A25 from a 0 05M Tris/HCl buffer (pH 7 2), and eluted with a linear 0 1 to 0 4M NaCl gradient in the same buffer The fractions were analysed with phenol–sulfuric acid[30], reading the extinction at 485 nm *a*, Alkali-treated type-11 polysaccharide after 10 (– – – –) and 24 (————) h of phage action (standard conditions) *b*, Native type-11 polysaccharide after 24 h of phage action In all cases, above 90% of the material applied (in terms of chromogen) was recovered

also detected By the same technique, fraction *P1*, as purified by ion-exchange chromatography (Fig 2), was found to consist of a mixture of oligosaccharides, ending, mainly, in reducing glucose, and, to a much smaller extent, in galactose No radioactive mannitol was obtained from *P1* Glucuronic acid (which is periodate-stable in the polymer[23]) could be identified as the major non-reducing-end sugar in fraction *P1* Thus, on oxidation of the material with periodate, followed by reduction with $NaBH_4$, hydrolysis, and paper electrophoresis in pyridine–glacial acetic acid–water,

considerable amounts of a compound having the same mobility as erythronic acid were detected, no glucuronic acid was detected

Oligosaccharide fraction *P1* was also methylated, the methylated (and esterified) methyl glycosides obtained were reduced with $LiBD_4$, and this product was methylated again The methylated glycosides were hydrolysed, and the monomers analysed by g l c–m s as their alditol acetates The results are given in Table I It can be seen that methylated *P1* yielded approximately equal amounts of 2,4,6-tri-*O*-methylglucose (2,4,6-Glc), 2,4,6-tri-*O*-methylgalactose (2,4,6-Gal), and 2,3-di-*O*-methylgalactose (2,3-Gal), as well as a minute amount of 2,3,4,6-tetra-*O*-methylglucose (2,3,4,6-Glc), the peak ratio of which was ~0 05 (relative to 2,4,6-Glc = 1 00) Also about equimolar amounts of one additional compound (2,3-Glc or 2,3,6-Glc, respectively) were obtained from *P1* which had been reduced after methylation, or methylated once again after reduction Due to the deuterium label, both 2,3-Glc and 2,3,6-Glc could be identified as derivatives of the glucuronic acid residue by their mass spectra Methylation of *P2* yielded equal amounts of the same three *O*-acetyl-*O*-methylalditols as *P1*, and a trace amount of 2,3,4,6-Glc

TABLE I

IDENTIFICATION AND RATIOS OF *O*-ACETYL-*O*-METHYLALDITOLS OBTAINED FROM PERMETHYLATED OLIGOSACCHARIDE FRACTION *P1* AND ITS DERIVATIVES[a]

Alditol derivative[b]	T[c]		*Primary fragments found* (m/e)						*I*[d]	*II*	*III*
	Lit	*Found*	*45*	*117*	*161*	*205*	*233*	*261*	*Ratio of peak integrals*[f]		
2,3,4,6-Glc[e]	1 00	1 00[e]	+	+	+	+	•		~0 05	≲0 05	≲0 05
2,4,6-Glc	1 95	1 95	+	+	+		+		1 00[f]	1 00[f]	1 00[f]
2,4,6-Gal	2 28	2 27	+	+	+		+		0 86	0 99	0 97
2,3,6-Glc	2 50	2 53	+ (47)[g]	+	(+)		+ (235)[g]		—	—	1 04
2,3-Glc[e]	5 39	5 39[e]		+	+			+ (263)[g]	—	0 99	—
2,3-Gal	5 68	5 63		+	+			+	1 05	1 05	0 95

[a]Reducing *P1* (*cf* Fig 2*a*) was methylated directly [b]2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, etc [c]Retention time, relative to that of 2,3,4,6-Glc and 2,3-Glc on an ECNSS-M column at 170°[37] [d]I, Oligosaccharide fraction *P1*, methylated, II, oligosaccharide fraction *P1*, methylated, and then reduced with $LiBD_4$, III, oligosaccharide fraction *P1*, methylated, reduced, and remethylated [e]Standards [f]Peak ratio relative to 2,4,6-Glc [g]Doubly deuterated fragments found instead of the corresponding normal ones

TABLE II

SIZE OF OLIGOSACCHARIDES OBTAINED BY PHAGE ACTION

Oligosaccharide fraction[a]	*P1*	*P2*	*P3*	*P6*	*P1'*	*P2'*
D p[b]	1 04	2 09	3 17	5 76	1 05	1 91

[a]See Fig 2 [b]Degree of polymerization = (μmole of glucose/μmole of glucitol) + 1

The size of the isolated oligosaccharide fractions *Pn* (from mildly alkali-treated polysaccharide) and *Pn'* (from native polymer) was also estimated by reduction of each fragment with sodium borohydride, and enzymic determination of the resulting glucose–glucitol ratio The results are shown in Table II

Substrate specificity (and host range) of Klebsiella *phage 11* — The relative efficiency of plating[28] (on *Klebsiella* 390 versus test strain) of phage 11 on the 71 other encapsulated (or mucoid) *Klebsiella* and 9 *E coli* strains listed under Material and Methods was found to be above 10^9 in almost all cases A few plaques (relative efficiency of plating = 10^7–10^8) produced on *Klebsiella* K2, K7, and K59 were probably due to contamination, and were not further investigated Since, under the test conditions used, the amount of phages applied (2.5×10^9 PFU in 50 μl) was amply sufficient to cause a distinct decapsulation (largely increased transparency)[6] of a heat-killed lawn of *Klebsiella* 390, these results demonstrate a high degree of specificity *both* for the enzymic and the lytic activity of phage 11 One cross-reaction (relative efficiency of plating = 20–25), however, was found—with *E coli* B/161/42 (09 *K29*(A) H^-)

As listed in Table III, some derivatives of *Klebsiella* type 11 capsular polysaccharide (the preparation and analyses of which are reported in the preceding paper[23]) as well as several heterologous bacterial capsule or mucus polysaccharides were also tested directly for depolymerization by phage-11 particles (under "standard conditions", 24 h) It can be seen that of all the polymers thus investigated, only Smith-degraded type-11 glycan, and *E coli* serotype 29 capsular polysaccharide

TABLE III

ACTION OF PHAGE-11 PARTICLES ON CHEMICALLY MODIFIED *Klebsiella* SEROTYPE 11, AND ON HETEROLOGOUS BACTERIAL EXO-POLYSACCHARIDES

Capsular (or mucus) polysaccharide	*Reducing power[a] after incubation[b] with phage-11 particles*
Klebsiella serotype 11	
native	4 μg
alkali-treated	4
—, oxidized with periodate, reduced with $NaBH_4$, and subjected to "Smith hydrolysis"[c]	4
—, dimethyl ester[c]	>400
—, —, reduced with $NaBH_4$[c]	>400
Klebsiella serotype 7	>400
E coli serotype 26, 28, 30, 31, 34, 38, 42, or M (colanic acid)	>400
E coli serotype 29	6
Pneumococcus serotype III[d]	>400

[a]Expressed in μg of depolymerization products equivalent to 1 μg of glucose in reducing power Before phage action, all polymers showed values above 400 μg [b]All polysaccharides were incubated for 24 h with phage-11 particles under "standard conditions" [c]For preparation, analyses, and structures, see preceding paper[23] and Table IV [d]Gift from Dr M Heidelberger, New York University, College of Medicine

were also degraded by the viral enzyme—to reducing fragments of roughly the same size as those obtained from native and alkali-treated host material

DISCUSSION

The results presented in Fig 2*a*, together with the finding that fraction *P3* can, albeit very slowly, be split further to *P1* and *P2*, show that catalysis by pure, whole virions of *Klebsiella* bacteriophage No 11 causes a quantitative depolymerization of mildly alkali-treated *Klebsiella* serotype 11 capsular polysaccharide, ultimately to a mixture of fragments *P1* and *P2*

Inspection of the structure of type-11 polysaccharide[23] (see Table IV, the alkali-treated material, in contrast to the native product as isolated from the bacteria, does not carry one *O*-acetyl residue on every second repeating unit, and it sediments uniformly in the analytical ultracentrifuge[23]), together with the results of reducing- and non-reducing-end sugar identification in its split products, of methylation analysis of *P1* (Table I) and *P2*, and, finally, of quantitative comparison of reducible and non-reducible glucose in *P1* and *P2* (Table II), leads to the following further conclusions (*1*) *P1* and *P2* mainly consist of a tetrasaccharide (one repeating-unit), and an octasaccharide, respectively, produced by cleavage of chain β-D-glucosyl-(1→3)-β-D-glucuronic acid linkages (*2*) The viral enzyme also catalyses the splitting of a few chain α-D-galactosyl-(1→3)-β-D-glucose bonds Thus, *P1* and *P2* each consists of mixtures of oligosaccharides—ending in reducing glucose or galactose, respectively—, the molar ratio of which is estimated to be ~20 1 from the results of methylation of *P1* (peak ratio of 2,3,4,6-Glc to dideuterated 2,3-Glc, or 2,3,6-Glc, respectively), and from the ratio of radioactive glucitol and galactitol obtained after reduction with $NaBH_4/NaBT_4$ Since a separation and identification of the minor products was not carried out, the results do not allow an estimate of the distribution of tri-, tetra-, and penta-, and of hepta-, octa-, and nona-saccharides, respectively, amongst them (block versus random cleavage of galactosyl relative to glucosyl bonds) That the multiplicity of products results from a lack of specificity of the viral enzyme, rather than from a micro-heterogeneity of the substrate (*e g*, occasional replacement of a glucose by another chain galactose residue), follows from the detection not only of reducing galactose, but also of non-reducing-end glucose in the minor products (*3*) The phage enzyme catalyses a hydrolysis and not a β-elimination as do several lyases active on other acidic polysaccharides, *e g*, on hyaluronic acid[34], pectic acid[35], and Vi polysaccharide[36] This follows conclusively from the detection of the (dideuterated) fragments having *m/e* 263 and 235 in the mass spectra of 2,3-Glc and 2,3,6-Glc, respectively, from methylated–reduced, and methylated–reduced–remethylated *P1* (Table I) (*4*) The results presented in Fig 2*a* and Table II show that fragments of four and five repeating-units do not occur during phage degradation of alkali-treated type-11 polysaccharide, and that *P1* and *P2* are formed in about equimolar amounts Together with the rapid loss of viscosity, and comparatively slow development of reducing power seen in Fig 1*a*, this indicates that the virus

TABLE IV

STRUCTURAL COMPARISON OF POLYSACCHARIDES WHICH ARE DEPOLYMERIZED OR NOT DEPOLYMERIZED BY THE *Klebsiella* BACTERIOPHAGE 11 GLYCANASE

Polysaccharide	*Structure*
	Hydrolysed by phage action
Klebsiella, serotype 11[23] (native or alkali-treated)	Pyruvate(acetal) 4/\6 α-D-Gal*p* 1↓4 3)-β D Glc*p*-(1→3)-β D-GlcUA*p*-(1→3)-α-D-Gal*p*-(1→
(after "Smith degradation")[a]	3)-β D Glc*p*-(1→3)-β D-GlcUA*p*-(1→3)-α-D-Gal*p*-(1→
E coli, serotype 29[38,39]	Pyruvate(acetal) 4/\6 β D Glc*p* 1↓2 α D-Man*p* 1↓4 2)-α-D-Man*p* (1→3)-β D-Glc*p*-(1→3)-β D-GlcUA*p*-(1→3)-α-D-Gal*p*-(1→
	Not hydrolysed by phage action
Klebsiella, serotype 11[23] (after esterification and reduction with $NaBH_4$)[b]	Pyruvate(acetal) 4/\6 α D-Gal*p* 1↓4 3)-β-D Glc*p*-(1→3)-β D-Glc*p*-(1→3)-α-D-Gal*p*-(1→
Pneumococcus, serotype III[40]	4)-β-D Glc*p*-(1→3)-β D-GlcUA*p*-(1→
Klebsiella, serotype 24[41]	β D-Man*p* 1↓4 2)-α-D-Man*p* (1→3)-β-D Glc*p*-(1→2)-α D-GlcUA*p*-(1→3)-α-D-Man*p* (1→
Klebsiella, serotype 54[42,43]	β-D Glc*p* 1↓4 6)-β D Glc*p* (1→4)-α-D GlcUA*p* (1→3)-?-L-Fuc*p*-(1→
Klebsiella, serotype 56[44]	α-L-Rha*p* Pyruvate(acetal) 1↓2 4/\6 3)-α-D-Gal*p*-(1→3)-β-D-Gal*p* (1→3)-β D-Glc*p*-(1→3)-β-D-Gal*p* (1→
E coli, serotype 30[45]	3)-?-D-Man*p*-(1→2)-β-D-GlcUA*p*-(1→3)-?-D-Gal*p*-(1→

[a] 85 to 90% of the branch pyruvate-galactose residues removed [b] 92% of the glucuronic, but only 23% of the pyruvic acid carboxyl groups were reduced upon esterification with diazomethane, and reduction with $NaBH_4$

enzyme acts by an endo-mechanism, rapidly cleaving the polymer into fragments of $3n$ repeating units, and that *P3* thus finally formed, is then much more slowly split into *P1* and *P2* Without knowledge about the conformation of the substrate also, an explanation for these observations cannot be given However, model building of type-11 polysaccharide did not exclude a helical conformation with a dodecasaccharide repeating-unit

As seen in Fig 2*b*, phage degradation of native type-11 glycan yields considerable amounts of fragments larger than *P1* and *P2*, and these fragments are not degraded further by prolonged enzyme action This phenomenon cannot be due to a protection against enzymic hydrolysis by *O*-acetyl groups, since the same amount of acetyl as in the polymer was also found in *P1'* and *P2'*. A possible explanation is offered by the hypothesis (see Ref 23) that, in native *Enterobacteriaceae* capsular polysaccharides, the single glycan strands are cross-linked by alkali-labile (possibly uronic acid ester) bonds If this were true, the larger fragments obtained from native type-11 polysaccharide might comprise these bonds

For an evaluation of the substrate-specificity experiments (see Table III and Results), the structures of those polymers that were depolymerized by phage-11 action, as well as some of those which were not, are compiled in Table IV The following conclusions can be drawn (*1*) The phage enzyme acts on larger oligo- and polysaccharides only (*P3* is split further, *P2* not) (*2*) The viral glycanase generally is highly specific (of 81 heterologous bacterial exo-polysaccharides, only one—*E coli* type 29—was found to cross-react) It is even more specific than the population of K11 antibodies produced by rabbits upon injection[23] of *Klebsiella* 390 (*3*) Phage-11 depolymerase does not hydrolyse all polysaccharides containing the β-D-Glc*p*-(1→3)-β-D-GlcUA*p*-disaccharide (no depolymerization of *Pneumococcus* type III polymer), but it can be expected to act upon all glycans containing the unsubstituted *Klebsiella* type 11 chain trisaccharide-repeating-unit (as in Smith-degraded material) In this specificity for the chain rather than the branch constituents, the enzyme differs strikingly from K11 antibodies[23] (*4*) The phage enzyme tolerates some substitution on C-4 of the glucuronic acids (as in *Klebsiella* type 11, and *E coli* type 29 capsular polysaccharides), but it does not tolerate reduction of the carboxyl groups of these residues (*5*) The glycanase will not depolymerize polysaccharides containing chain trisaccharides which differ from those in Smith-degraded type-11 polymer by (several) minor alterations only—such as single changes of anomeric configuration, substitution of one hexose for another, or alteration of one substitutional position (no action on *Klebsiella* type 24, 54, and 56, nor on *E coli* type 30, or *Pneumococcus* type III capsular polysaccharide) A further specification of this statement obviously must await the structural analyses of additional capsular polysaccharides

Finally, the results presented confirm some of the conclusions reached in the preceding paper[23] on the structure of *Klebsiella* type 11 polysaccharide (*1*) The capsular glycan does not contain mannose (after phage degradation, this sugar is separated off with the small amount of undepolymerized material) (*2*) The chain glucose is linked to position 3 (and thus the branch galactose to position 4) of the

glucuronic acid, and the pyruvate exclusively to positions 4 and 6 of the branch galactose [see methylation analysis of *P1* (Table I) and *P2*] (*3*) Free oligosaccharides can be directly methylated upon (short) treatment with the Hakomori reagent (peak ratio of 2,4,6-Glc to the other alditols in Table I)

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