Carbohydrate Research, 60 (1978) 353–366

KINETICS AND SUBSTRATE SPECIFICITY OF THE GLYCANASE ACTIVITY ASSOCIATED WITH PARTICLES OF *Klebsiella* BACTERIOPHAGE NO. 13*

HEINER NIEMANN†‡, HELMUT BEILHARZ, AND STEPHAN STIRM
Max Planck-Institut für Immunbiologie, Freiburg (German Federal Republic)
(Received December 16th, 1976; accepted for publication March 1st, 1977)

ABSTRACT

There is a glycanase activity associated with the particles of *Klebsiella* bacteriophage No. 13 which catalyzes hydrolysis of *O*-β-D-glucopyranosyl-(1 → 4)-β-D-mannopyranose linkages in *Klebsiella* serotype 13 capsular polysaccharide. The initial glycanase reaction velocity (at 37° and the optimum pH of 7–8) exhibits hyperbolical dependence on low concentrations of substrate, with K_m = 170 μM (in repeating units), and V_{max} = 140 nmol/min (glucose equivalents, for 10^{10} plaque-forming units). The reaction is inhibited by reaction products (type-13 penta- and deca-saccharides, one and two repeating-units), and also by the substrate at concentrations above ~1mM. Of seventy-four heterologous *Klebsiella*, and two other bacterial capsular heteropolysaccharides, as well as two substrate derivatives tested, only the *Klebsiella* serotype-2, -22, and -37 glycans were also depolymerized by the phage-13 enzyme. The reacting polysaccharides consist of repeating units, characteristically containing (*a*) chain 3*eq*,1*eq* → 4*eq*,1*eq* dihexopyranosyl sequences around the susceptible linkages, with equatorial hydroxyl and hydroxymethyl groups at C-2 and C′-5 next to them, and (*b*) branch carboxyl groups that may have to be located within a certain distance from these linkages.

INTRODUCTION

A wide variety of enzymic activities catalyzing different degradation reactions of host surface polysaccharides may be associated with bacterial virus particles. So far, esterases[1,2] (saponification of *O*-acetyl substituents), glycanases[3–8], and lyases[9,10] have been recognized; in the case of the eighty-one[11] *Klebsiella* capsular

*Bacteriophage-borne Enzymes in Carbohydrate Chemistry: Part II. For Part I, see Ref. 6.
†In partial fulfilment of the requirements for the degree of Dr.rer.nat. at Freiburg University. Some of the data were presented at the meeting of the Chemical Society Carbohydrate Group at Swansea, Great Britain, March 23–25, 1976.
‡Present address: Fred Hutchinson Cancer Research Center, Division of Biochemical Oncology, 1124 Columbia Avenue, Seattle, Washington 98104, U.S.A.

glycans and of glycanases alone, some sixty bacteriophages have now been isolated[12], carrying such activities with different substrate specificities.

Since pure bacteriophage particles are easily prepared in quantity[6,13], the "phage-borne" glycanases might prove useful to the carbohydrate chemist — if the principle of their substrate specificity were better understood. A comparison of different heteropolysaccharide structures, split or (although similar) not split by single phage glycanases should eventually lead to this understanding. We have therefore analyzed the substrate specificity of another glycanase (*cf.* Ref. 6) associated[14] with the particles of *Klebsiella* bacteriophage No. 13, *i.e.*, we have essentially identified the linkages split by this enzyme in four different *Klebsiella* capsular polysaccharides (serotypes K2[15], K13[14], K22[16], and K37[17,18]) and have inspected these glycan structures for homologies. We also report on the first[19] kinetic analysis of a polysaccharide depolymerization as catalyzed by complete bacteriophage particles.

Klebsiella bacteriophage No. 13 (originally[20] designated "KL 17") is a small virus[20] of Bradley's morphology group C[21]. It consists[22] of an isometric head (~50 nm in diameter) carrying a base plate (~32 nm in diameter, and 4 nm in thickness) with probably six attached spikes (roughly 6 × 10 nm). In morphology, phage 13 is thus very similar to *Klebsiella* phage 11[6,23,24], for which the host polysaccharide glycanase activity[6] was found to be associated with the viral spike organelles[23,24].

MATERIAL AND METHODS

Media and general techniques. — For the isolation of capsular polysaccharides, the bacteria were grown on $D_{1.5}$ agar[25], and phage 13 was propagated in CYG broth[14]; for all other purposes, Merck standard I medium was employed. The composition of the physiological, phosphate-buffered saline (PBS) has been given previously[26]. The general techniques for handling bacteria and bacteriophages were those of Kauffmann[27] and Adams[28].

Bacteriophage. — *Klebsiella* bacteriophage No. 13 (originally[20] designated "Kl 17", but subsequently[14] numbered in accordance with the K antigen of its new host) was kindly supplied by Dr. A. Przondo-Hessek, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland.

As previously detailed[6,14], phage 13 was propagated on *Klebsiella* 1470 (the serological test-strain for the K13 antigen) in CYG broth, concentrated by precipitation with polyethylene glycol 6000, and purified by isopycnic centrifugation.

Bacteria. — The serological test-strains[29] for the *Klebsiella* K1–K72, K74, K79, and K80 capsular antigens were used; the cultures were obtained from Dr. I. Ørskov, WHO International *Escherichia* Centre, Statens Seruminstitut, Copenhagen, Denmark.

Isolation and preparation of polysaccharides. — The *Klebsiella* capsular polysaccharides were isolated from the respective serological test-strains by the phenol–water–cetyltrimethylammonium bromide procedure[30], and mildly alkali-treated[14, 16,31].

Type-13 polysaccharide was carboxyl-reduced by the method of Taylor *et al.*[32]; the yield was >95%, with 86% reduction (as determined with carbazole–sulfuric acid[33]).

For partial debranching, the type-22 and 25 polysaccharides were heated at 100° in 0.1M aqueous trifluoroacetic acid, as detailed elsewhere[16,31].

The *Pneumococcal* type-III capsular polysaccharide was a gift from Dr. M. Heidelberger, New York University, School of Medicine, New York, U.S.A., and the hyaluronic acid was isolated from *Streptococcus pyogenes*, strain K56, as described previously[9].

Depolymerization of type-13 polysaccharide: conditions, kinetics, and inhibition.— The range of linear dependence of reducing power liberated on phage concentration, and on time, was tested as follows: at 37°, 10 μl of suspensions of pure phage-13 particles in 5mM sodium phosphate buffer of pH 7 [3.1×10^7 to 5×10^8, or 10^8 and 2.5×10^8 PFU (plaque-forming units); see Figs. 1a and 1b] were added to 100-μl portions of a 0.2% (w/v) aqueous solution of type-13 polysaccharide (lyophilized; 15% moisture), and 30 μl of 0.02M citric acid — 0.04M disodium phosphate buffer (pH 7.8). After 7 or 2–32 min (Figs. 1a and 1b), the glucose equivalents of reducing power were assayed by the method of Park and Johnson[34] (with a solution of ferric and monolauryl sulfate in 0.5%, v/v, sulfuric acid), employing a series of identical test mixtures with heat-denatured virus (15 min at 100°) as blanks.

For the evaluation of the pH stability of the viral enzyme (Fig. 1*c*), 10-μl samples with 5×10^9 PFU in 5mM phosphate buffer (pH 7) were adjusted to different pH values by the addition of 30 μl of 0.01M citric acid–0.02M disodium phosphate (pH 3.0 to 7.8), or 0.02M sodium hydrogen carbonate–carbonate (pH 7.5 to 10.7) buffers. After 30 min at 37°, the mixtures were cooled, and neutralized with 160 μl of 0.4M sodium phosphate buffer (pH 7). Portions (10 μl) were analyzed for reducing power after addition of 0.2 mg of substrate (lyophilized, 15% moisture; in 130 μl of water) and incubation at 37° for 7 min (blanks as above). The pH dependence of the enzymic reaction (Fig. 1*c*) was traced by adding 2.5×10^8 PFU (in 10 μl) to mixtures of 100-μl portions of an aqueous substrate solution (0.2%) and 30 μl of the citric acid–phosphate or carbonate buffers, incubating, and analyzing in the same manner.

For estimating the dependence of reaction velocity on substrate concentration, samples of type-13 polysaccharide (~0.09 to 6mM in repeating units; see Fig. 2) in 100 μl of water were mixed with 30 μl of 0.02M citric acid–0.04M disodium phosphate buffer (pH 7.8), and warmed to 37°. On addition of 10^8 PFU of pure virus (in 10 μl of 5mM phosphate buffer), and incubation at 37° for 7 min, the reducing power was determined (blanks as above).

For demonstrating product inhibition, 10-μl portions of phosphate buffer, containing 10^8 PFU of virus, were added to 30-μl samples of 5.6–237μM (see Fig. 2) solutions of type-13 phage degradation products P1 and P2 (see below) in 0.02M citric acid–0.04M phosphate buffer (pH 7.8). The mixtures, as well as identical series of blanks with heat-denatured phage, and controls containing no inhibitor, were incubated at 37° for 15 min. 100-μl portions of a 0.2% aqueous substrate solution

were then added, and the reducing power was determined after another 7 min at 37°.

Host range, and glycanase substrate specificity of phage 13. — The infective and enzymic host range of phage 13 was tested by placing 50-µl portions of a crude virus suspension (5×10^9 PFU) on freshly seeded lawns of the seventy-five *Klebsiella* strains listed above, and incubating at 37° overnight. In those cases where the bacterial lawn was affected, the experiment was repeated with a 1:10 dilution series of phage, in order to distinguish cross-infectivity (formation of single plaques) from a solely enzymic cross-reaction (no formation of single plaques). In the cases of cross-infectivity, the relative EOP (efficiency of plating)[28] was accurately titrated, and the simulation of the effect due to host lysogeny was excluded by plating a *Klebsiella* 1470 culture-filtrate.

For the qualitative evaluation of the substrate properties of different polysaccharides (see Table I), 2.5×10^8 PFU of pure phage-13 particles (in 10 µl of 5mM phosphate buffer), 30 µl of 0.02M citric acid—0.04M disodium phosphate buffer (pH 7.8), and 100-µl samples of 0.2% (w/v) aqueous solutions of the test glycans (lyophilized, 13–17% moisture) were mixed, and incubated at 37° for 15 min, and the reducing power determined (again using identical mixtures with heat-denatured virus for blanks).

For the quantitative characterization of substrate properties, the dependence of reaction velocity on polysaccharide concentration was recorded, as described above for the type-13 glycan.

Isolation and analysis of phage degradation products. — For exhaustive degradation, mixtures containing 2–3 mg of polysaccharide per ml of PBS, and 10^{10} PFU per mg of glycan were incubated at 37° for 48 h. As described previously[6,14], the oligosaccharides obtained were desalted by passage over Sephadex G10 with a volatile buffer and lyophilization, and separated by ion-exchange chromatography on DEAE-Sephadex A25.

High-voltage (45 V/cm) paper electrophoresis[35] of the degradation products was performed at pH 5.3; for further details, see Thurow *et al.*[6].

The procedure for determination of the size of the isolated oligosaccharides and for identification of their reducing-end sugars (labelling with $NaBH_4/NaBT_4$ and hydrolysis, followed by collection and radioactivity determination of the single monomers as the alditol acetates) has been described elsewhere[14].

For p.m.r. spectroscopy of the *Klebsiella* K2 repeating-unit tetrasaccharide, a 1.8% (w/v) solution in absolute deuterium oxide (Aldrich) was measured at 70° with a Bruker HFX-90 instrument (90 MHz) and a model 1074 Fabritek CAT.

RESULTS

Depolymerization of Klebsiella *serotype 13 capsular polysaccharide, as catalyzed by particles of bacteriophage No. 13.* — Incubation of whole virus particles of *Klebsiella* bacteriophage 13 (isolated and purified from the lysates by polyethylene glycol precipitation and isopycnic centrifugation[13,14]) with isolated host, *i.e.*, serotype-13

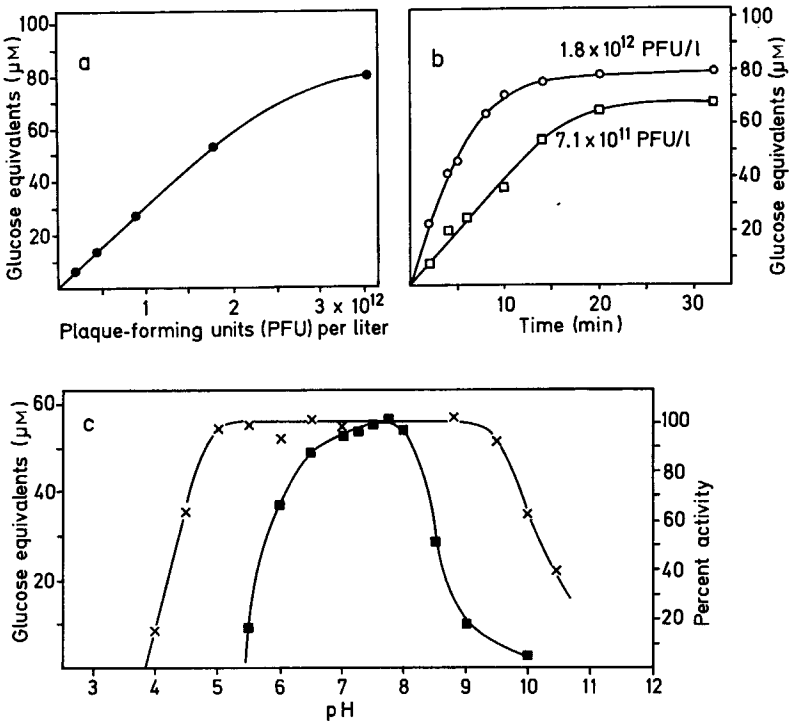

Fig. 1. Conditions of bacteriophage-catalyzed hydrolysis of *Klebsiella* serotype 13 capsular polysaccharide: (a) dependence of reducing power liberated on phage concentration: ~ 0.14% solutions of the glycan (1.36mM in repeating units) were incubated (7 min at 37° and pH 7.8) with increasing amounts of purified virus particles, and the reducing power (glucose equivalents) was determined; (b) dependence of reducing power liberated on time: polysaccharide solutions were incubated with 7.1×10^{11} (—□—), or with 1.8×10^{12} (—○—) PFU/l (plaque-forming units per liter); (c) pH stability and pH dependence of the viral glycanase activity: reducing power liberated by 1.8×10^{12} PFU/l, at pH 7 after pretreatment (30 min at 37°) of the viruses at various pH values (—x—), or running the reaction at different pH values (—■—). For further details, see Material and Methods.

capsular polysaccharide (mildly alkali-treated), results in a depolymerization of the glycan, due to hydrolysis of β-D-glucosyl-(1→4)-β-D-mannose linkages[14] (*cf.* Table III).

As seen in Fig. 1*a* and 1*b*, the reaction exhibits a linear relationship between the degree of hydrolysis (*i.e.*, the development of reducing power) and the amount of virus (after 7 min at 37°) up to $\sim 1.8 \times 10^{12}$ PFU (plaque-forming units) per liter, or the time (with 7.1×10^{11}, or 1.8×10^{12} PFU/l) up to roughly 12 or 7 min. The phage-13 glycanase is stable (for 30 min at 37°) from about pH 5 to 9, and it exhibits an activity plateau between about pH 6.5 and 8 with a flat peak at pH 7.8 (Fig. 1*c*).

By determining the reducing power released after 7 min at 37° and at pH 7.8, the dependence of the initial reaction velocity on the concentration of type-13 polysaccharide was recorded. Fig. 2 shows the results, including a double-reciprocal (Lineweaver and Burk) plot of the data. At low concentrations of substrate, the

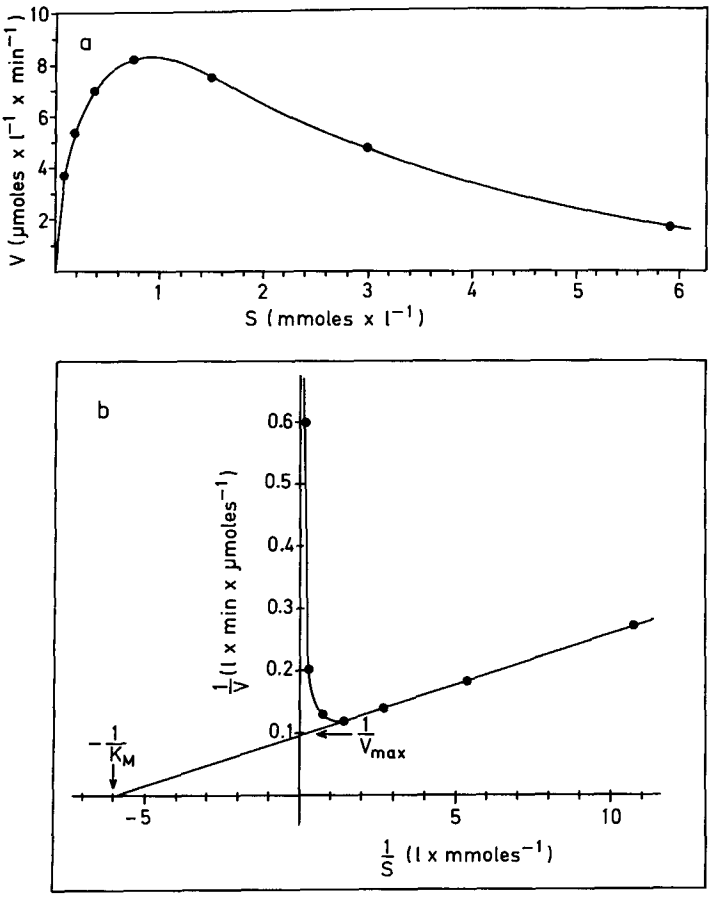

Fig. 2. Dependence of the initial velocity of phage 13-catalyzed hydrolysis on the substrate concentration: (a) μmoles of glucose equivalents of reducing power liberated per min by 7.1 × 10^{11} PFU/l (at 37° and at pH 7.8), as a function of the type-13 polysaccharide concentration (mmoles of repeating units per liter); (b) corresponding double-reciprocal plot. For details, see Material and Methods.

reaction follows simple, hyperbolical Michaelis–Menten kinetics, but is inhibited at higher concentrations of polysaccharide (above ~1mM, in repeating units).

In order to determine if the apparent substrate inhibition might be due merely to the increasing viscosity of the reaction mixtures, the reaction velocity at 1.56mM (see Fig. 2*a*) was also determined in the presence of 30% (v/v) of glycerol. Although this increased the specific viscosity, $(\eta - \eta_0)/\eta_0$, of the mixture by a factor of 2.85 (corresponding to a substrate concentration of ~2.9mM), the reaction velocity was decreased by 5% only (*cf.* ~34% between 1.56 and 2.9mM in Fig. 2*a*). In addition to the substrate, two oligosaccharide products, the type-13 repeating-unit pentasaccharide and the double-repeating-unit decasaccharide, also inhibited the viral enzyme; this is shown in Fig. 3.

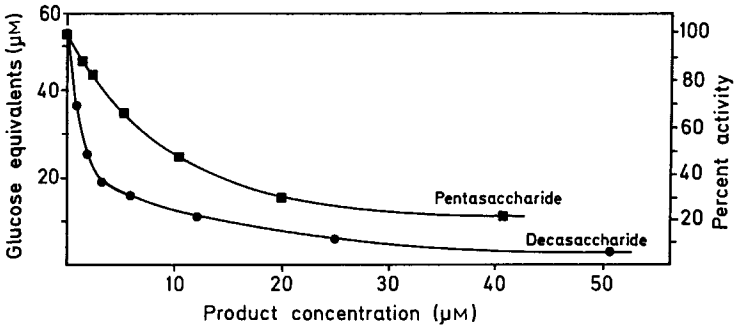

Fig. 3. Product inhibition of the phage 13-associated glycanase activity. An ~ 0.14% solution of the type-13 polysaccharide (1.36mM in repeating units) was incubated (7 min at 37° and pH 7.8) with 7.1 × 10^{11} PFU/l, after preincubation (15 min) of the viruses with increasing concentrations of the type-13 repeating-unit pentasaccharide P1 (—■—), or of the double-repeating-unit decasaccharide P2 (—●—); the reducing power liberated, and the inhibitor concentrations in the final mixtures are given. For further details, see Material and Methods.

TABLE I

SUBSTRATE SPECIFICITY OF THE *Klebsiella* BACTERIOPHAGE 13-ASSOCIATED GLYCANASE

Polysaccharide[a]	*Reducing-power liberated*[b] *(μM glucose equivalents)*	K_m *(μM)*[c]	V_{max} *(nmol/min)*[d]
Klebsiella capsular glycans			
type-13	76	170	140
type-2	67	25	50
type-22	2.3	500	25
type-37	5.2	170	15.5
type-13, carboxyl-reduced[e]	8.2		
type-22, partially debranched[f]	0		
type-25	0		
type-25, partially debranched[g]	0		
Pneumococcal capsular glycan			
type-III	0		
Streptococcal capsular glycan			
hyaluronic acid	0		

[a]For primary structures, see Tables III and IV. [b]Liberated within 15 min by 1.8 × 10^{12} plaque-forming units (PFU) per litre, from ~ 0.14% polysaccharide solution at pH 7.8 and 37° (*cf.* Fig. 1*b*). [c]At low concentrations (see Fig. 2*b*), and at pH 7.8 and 37°; glycan concentrations in μM repeating-units. [d]nmol glucose equivalents/min, liberated by 10^{10} PFU at pH 7.8 and 37°; values extrapolated as shown in Fig. 2*b*. [e]Reduced by the method of Taylor *et al.*[32] with 86% reduction of the glucuronic acid carboxyl-groups[33]. [f]Polymer material obtained[16] from type-22 glycan by partial acid hydrolysis, containing no (unsaturated) uronic acid and only 75% of the original glucose (*cf.* Table III). [g]Polymer material obtained[31] from type-25 glycan by partial acid hydrolysis, containing only ~ 75% of the original glucose (*cf.* Table IV).

Host range, and glycanase substrate specificity of phage 13. — High-titre suspensions of phage 13 were plated on seventy-four serological test-strains for *Klebsiella* capsular antigens other than K13 (*cf.* Ref. 6). In those cases where an effect of the viruses on the heterologous bacteria was observed, increasingly dilute suspensions of phage were additionally plated, to distinguish cross-infectivity plus enzymic cross-reaction (formation of single plaques with acapsular haloes[23,36] at lower concentrations) from a solely enzymic cross-reaction (capsule depolymerization at higher concentrations — no action, notably no plaque formation, at lower concentrations). Phage 13 did not affect any of the heterologous bacterial strains (relative efficiency of plating[28] $> 10^7$) with the exception of *Klebsiella* B5055 (*K2*), *Klebsiella* 1996/49 (*K22*), and *Klebsiella* 8238 (*K37*). Phage 13 depolymerizes all three capsules, but multiplies only on the K2 and K22 strains, with relative efficiencies of plating of 7 and 50, respectively (on *Klebsiella* 1470 versus these strains).

A qualitative survey of the substrate specificity of the phage-13 glycanase was also obtained by incubating (15 min at 37° and pH 7.8; see Fig. 1*b*) solutions of different polysaccharides [the *Klebsiella* K13, K2, K22, and K37 glycans, as well as six others with similar primary structures (0.2%, w/v)] with 1.8×10^{12} PFU/l of pure virus particles, and determining the reducing power that developed. In those

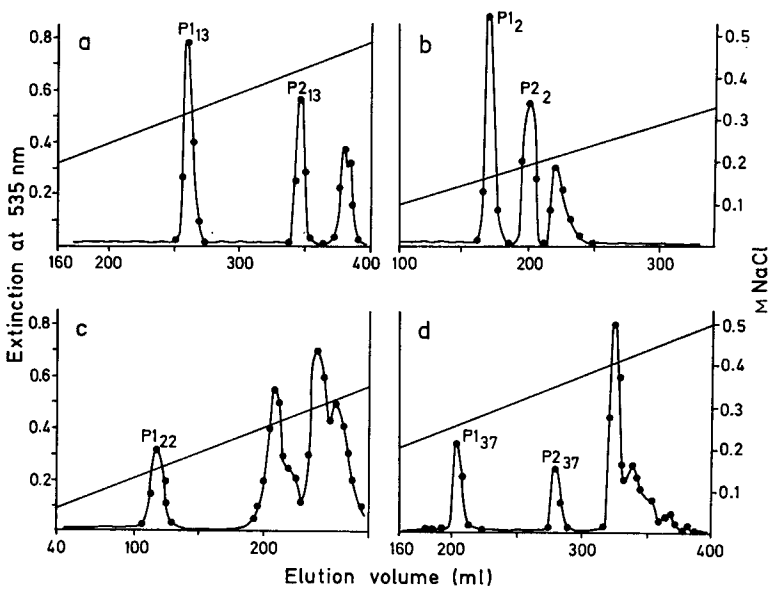

Fig. 4. Isolation of phage-13 glycanase depolymerization products. The oligosaccharides obtained by exhaustive virus-catalyzed hydrolysis of various *Klebsiella* capsular polysaccharides (2×10^{10}–3×10^{10} PFU/2–3 mg of mildly alkali-treated glycan/ml of PBS; 48 h at 37°), were desalted, absorbed onto a DEAE-Sephadex A25 column (31.5 cm × 1.75 cm²) from a 0.05M Tris/HCl buffer (pH 7.2), and eluted (7–9 ml/h) by addition of a linear NaCl gradient (——). The fractions were analyzed with carbazole-sulfuric acid[33] (extinction at 535 nm; —●—). (a) Depolymerization products obtained from serotype-13 polysaccharide (natural substrate, *cf.* Ref. 14); $P1_{13}$ and $P2_{13}$, one and two repeating-unit oligosaccharides. (b), (c), and (d), Products from type-2, type-22, and type-37 polysaccharides.

TABLE II

OLIGOSACCHARIDES OBTAINED FROM FOUR *Klebsiella* CAPSULAR POLYSACCHARIDES BY BACTERIOPHAGE 13-CATALYZED HYDROLYSIS

Designation of oligosaccharide[a] *(% yield)*[b]	*Mobility in paper electrophoresis*[c]	$[\alpha]^{22}_{589}$ *(c H_2O) (degrees)*	*Molar ratio of sugar constituents*[d] *(% radioact. incorp. from $NaBT_4$)*				*Size*[e]
			Glc	*Gal*	*Man*	*HexA*	
$P1_{13}$ (27.5)[f]	0.66	+ 108 (0.3)	2.11 (84.4)	1.0 (6.1)	0.73 (9.5)	0.86 (0)[g]	pentasacch.
$P2_{13}$ (20.5)[f]	0.71	+ 88 (0.1)	1.89 (88.1)	1.0 (2.5)	0.72 (9.4)	0.77	decasacch.
$P1_2$ (29)	0.41	+ 116 (0.5)	2.12 (99.3)	—	1.0 (0.7)	1.05	tetrasacch.
$P2_2$ (20)	0.46	+ 102 (0.3)					
$P1_{22}$ (12)	0.48	+ 60 (0.2)	1.92 (13.4)	1.0 (86.6)	—	0.92	tetrasacch.
$P1_{37}$ (16.5)	0.76	+ 58 (0.2)	1.75 (15.5)	1.0 (84.5)	—	0.55[h]	tetrasacch.
$P2_{37}$ (12)	0.85		1.81 (6.6)	1.0 (93.4)	—		octasacch.

[a]$P1_{13}$ = one repeating-unit oligosaccharide of the serotype-13 capsular glycan, etc.; *cf.* Fig. 4. [b]Percent weight of polysaccharide (lyophilized). [c]At pH 5.3, relative to GlcA. [d]Neutral sugars determined by g.l.c. of the alditol acetates; hexuronic acid with carbazole–sulfuric acid. [e]Calculated from the radioactivity incorporated from $NaBT_4$, relative to xylose as the internal standard. [f]Results[14] included for comparison. [g]No radioactive gulonic acid detected by paper electrophoresis. [h]Lactic acid ether derivative of GlcA[17].

cases, where an enzymic cross-reactivity was observed, K_m and V_{max} were additionally estimated (*cf.* Fig. 2). The results are summarized in Table I.

Isolation and analysis of oligosaccharides obtained by phage degradation. — The four phage-13 glycanase substrates thus recognized, namely, the *Klebsiella* K13, K2, K22, and K37 polysaccharides, were exhaustively depolymerized (48 h at 37° with 10^{10} PFU/mg), and the products were isolated by ion-exchange chromatography. The d.p. was determined on the basis of radioactivity incorporated on treatment with $NaBT_4$ (relative to xylose as the internal standard). This also allowed the reducing end sugar (*i.e.*, the site of enzymic hydrolysis) to be identified. The results are summarized in Fig. 4 and Table II; for comparison, the data[14] for the type-13 oligosaccharides have been included.

As seen in Fig. 4 and Table II, phage-13 catalysis produces oligosaccharides with glucose as the reducing residue from *Klebsiella* serotype 2 capsular polysaccharide. Because the repeating unit of this glycan[15] contains two glucosyl residues (see Table III), this result does not unequivocally identify the site of enzymic cleavage. However, as these two glucosyl residues occur in different anomeric configurations, the point could be settled by p.m.r. spectroscopy of the type-2 repeating-unit tetrasaccharide.

The spectrum indicated ~2.5 equatorial [δ 5.33 ($J_{1,2}$ 4 Hz) and 5.25 (3.5 Hz)] and ~1.5 axial anomeric protons [δ 4.79 (<1 Hz) and 4.66 (8 Hz)] (*cf.* Ref. 15). These data clearly indicate cleavage of the β-glucosyl bond, as splitting of the α linkage would result in a tetrasaccharide having equatorial and axial anomeric protons in inverted ratio (~1.5:2.5).

DISCUSSION

Reaction kinetics. — At low concentrations of substrate, the initial velocity of hydrolysis of *Klebsiella* serotype 13 capsular polysaccharide, as catalyzed by the particles of bacteriophage No. 13, follows simple, hyperbolical Michaelis–Menten kinetics; at higher concentrations of substrate, the reaction is inhibited (Fig. 2). That this inhibition is not due merely to the increasing viscosity of the test mixtures follows from the findings that an increase in viscosity by addition of an inert agent has a negligible effect on the reaction, and that the depolymerizations of the *Klebsiella* type-2, type-22, and type-37 polysaccharides exhibit a rate decrease at about the same concentrations (0.7–1mM in repeating units; data not shown) — although the viscosities of the solutions vary considerably. Very much better inhibitors than the substrate are the products of phage 13-catalyzed hydrolysis. As seen in Fig. 3, the same (~38%) inhibition was effected by an additional 1500–1600 μmol of substrate (from 1.36 to 2.9mM in Fig. 2*a*) as by ~6 and ~1 μmol of the type-13 penta- and deca-saccharide, respectively. The latter product is a better inhibitor than the former, indicating the existence of an otimally inhibiting type-13 oligomer.

Substrate specificity. — Of seventy-five serological test-strains for different *Klebsiella* capsular antigens, three (K13, K2, and K22) support growth of phage 13, and their capsules, as well as that of serotype K37, are depolymerized by the phage enzyme. The kinetics of hydrolysis of the type-2, type-22, and type-37 polysaccharides are similar to those of the homologous type-13 substrate (data not shown, *cf.* Fig. 2), but with some variations of K_m, and with generally lower V_{max} values, notably for type-22 and type-37 (a fact which probably accounts for the larger average size of the oligosaccharides obtained from these two glycans) (Table I). In comparison with other bacteriophage-borne glycanases[4,5,6,36], the phage-13 enzyme is thus rather unspecific, and, because it allows the identification of a common region in a relatively large number of cross-reacting heteropolysaccharides, it was selected for the present study.

Isolation (Fig. 4), and labelling of the reducing-end sugar, of the various phage-13 degradation products (Table II), as well as p.m.r. spectroscopy of the type-13[14] and type-2 repeating-unit oligosaccharides led to the identification of the cleavage sites in the four heteropolymers (Table III) (the small amounts of other reducing sugars recorded in Table II are probably due to some β-elimination in the alkaline media of reduction and labelling, rather than to a lack of specificity, or a heterogeneity of the phage enzyme). The common chain-sequence in these four substrates is shown in Fig. 5. Several of the hydrolysis-resistant *Klebsiella* capsular

TABLE III

PRIMARY STRUCTURES OF *Klebsiella* CAPSULAR POLYSACCHARIDES HYDROLYZED BY BACTERIOPHAGE-13 ACTION, AND SITES OF CLEAVAGE

Serotype	*Structure*
K13[14, a]	pyruvate (acetal) / \ 3 4 D-Gal*p* $\beta^1\downarrow4$ D-GlcA*p* β $\alpha^1\downarrow3$ β α →3)-D-Glc*p*-(1→4)-D-Man*p*-(1→4)-D-Glc*p*-(1→ ↑
K2[15]	D-GlcA*p* β $\alpha^1\downarrow3$ β α →3)-D-Glc*p*-(1→4)-D-Man*p*-(1→4)-D-Glc*p*-(1→ ↑
K22[16, b]	XUA*p* $\beta^1\downarrow6$ D-Glc*p* $\alpha^1\downarrow4$ β β →3)-D-Gal*p*-(1→4)-D-Glc*p*-(1→ ↑
K37[17, 18, c]	YUA*p* $\beta^1\downarrow6$ D-Glc*p* $\alpha^1\downarrow4$ β β →3)-D-Gal*p*-(1→4)-D-Glc*p*-(1→ ↑

[a]For identification of cleavage site, see also Niemann *et al.*[14]. [b]XUA = 4-deoxy-*threo*-hex-4-enuronic acid[16]. [c]YUA = 4-*O*-[(S)-1-carboxyethyl]-D-glucuronic acid[17].

polysaccharides (*e.g.*, those of serotypes K11[26], K20[38], K38[39], K47[40], and K54[41]) contain chain sequences that differ in one or two details only, for example, single alterations of anomeric configuration, or of position of substitution. However, since they all exhibit differences in side-chain structure, these data do not prove that the chain requirements noted in Fig. 5 are also necessary.

In addition to the chain homology, the four substrates are characterized by branches (on positions 4 or 3′ of the sugars on either side of the susceptible linkage) containing hexuronic acids (Table III). The carboxyl groups of these residues are also necessary for the glycanase-13 substrate property. This follows from the relative resistance to hydrolysis of the type-13 glycan after carboxyl-reduction (the velocity of hydrolysis is proportional to the hexuronic acid content), and from the enzyme

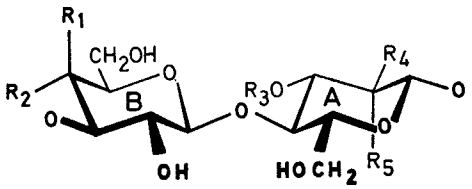

Fig. 5. Chain homology in substrates of *Klebsiella* phage-13 glycanase. The same torsional angles[43] as those accepted for hyaluronic acid, chondroitin, etc.[43–45] ($\varphi_{BA} = \psi_{BA} = 160°$, $\varphi_{AB} = \psi_{AB} = 220°$) were assumed. The central glycosidic linkage of the 3*eq*,1*eq*→4*eq*,1*eq* dihexopyranoside sequence shown is hydrolyzed by the viral enzyme (*cf.* Table III).

Serotype of substrate[a]	R_1	R_2	R_3	R_4	R_5
K13[14]	H	OH	D-Gal*p*-(1→4)-D-GlcA*p*-(1→ (β, α; 3 4; pyruvate (acetal))	H	OH
K2[15]	H	OH	D-GlcA*p*-(1→ (α)	H	OH
K22[16, b]	XUA*p*-(1→6)-D-Glc*p*-(1→ (β, α)	H	H	OH	H
K37[17, 18, c]	YUA*p*-(1→6)-D-Glc*p*-(1→ (β, α)	H	H	OH	H

[a] *Klebsiella* capsular polysaccharides of different K serotypes, *cf.* Table III. [b] XUA = 4-deoxy-*threo*-hex-4-enuronic acid[16]. [c] YUA = 4-*O*-[(S)-1-carboxyethyl]-D-glucuronic acid[17].

susceptibility of the type-2 polysaccharide which differs from type-13 only by the absence of the pyruvyl-galactose branch termini (*i.e.*, also by their carboxyl groups) (Tables I and III). The above conclusion is further corroborated by the resistance to hydrolysis of partially debranched type-22 glycan (Tables I and III), as well as by analogous findings in other phage-glycanase systems[5,6].

TABLE IV

PRIMARY STRUCTURES OF SOME SUBSTRATE ANALOGUES NOT HYDROLYZED BY *Klebsiella* BACTERIOPHAGE-13 ACTION

Bacterial capsular polysaccharide	*Structure*
Klebsiella, type-25[31]	D-Glc*p* (β1↓2) D-GlcA*p* (β1↓4) →3)-D-Gal*p*-(1→4)-D-Glc*p*-(1→ (β, β)
Pneumococcus, type-III[37]	→3)-D-GlcA*p*-(1→4)-D-Glc*p*-(1→ (β, β)
Streptococcal hyaluronic acid[9]	→3)-D-GlcNAc*p*-(1→4)-D-GlcA*p*-(1→ (β, β)

However, the presence both of the chain homology and of correctly positioned branches with hexuronic acid residues are insufficient criteria for the recognition of phage-13 glycanase substrates. As seen from the data in Tables I and IV, the native or partially debranched *Klebsiella* type-25 glycan, *i.e.*, with substituted or with (partially) unsubstituted GlcA branches at the same positions as in type-22 and type-37, is resistant, although it complies with both requirements. Moreover, this situation cannot be due to a difference in substitution by *O*-acetyl groups[42], since mildly alkali-treated[26,30] (*i.e.*, deacetylated) polysaccharides were used throughout.

The chain dihexoside homology found in the four phage-13 glycanase substrates also occurs in hyaluronic acid (*cf.* Table IV), chondroitin, and several other polysaccharides whose conformations have been studied extensively[43-45]. The unproven assumption that the torsional angles φ and ψ[43] around the oxygen bonds of the susceptible glycosidic linkages in the *Klebsiella* glycans are the same as those accepted for these other polysaccharides, as well as tentative model building (especially of the side chains), led to the following more-detailed, but largely hypothetical, concept of sufficient structural requirements for phage-13 glycanase substrates. Hydrolysis of heteropolysaccharides occurs when the repeating units contain (*a*) a chain 3*eq*,1*eq* → 4*eq*,1*eq* dihexopyranoside sequence (see Fig. 5), with a hydroxyl and a hydroxymethyl group protruding equatorially at positions 2 and 5′ from one face (roughly inverted positions of the two $^4C_1$ chairs), and on both sides of the susceptible linkage, and (*b*) a branch carboxyl-group which can assume a distance of ~7–9 Å from the central glycosidic oxygen. The latter requirement would exclude the *Klebsiella* type-25 (and, possibly, type-47[40]) and the *Pneumococcus* type-III capsular polysaccharides, as well as hyaluronic acid (Tables I and IV).

It is evident that the general approach chosen for the elucidation of substrate specificity in bacteriophage-borne glycanases yields sufficient, but perhaps not necessary, criteria. However, analysis of further enzymic cross-reactions for other heteropolysaccharides (especially neutral ones[7,8,46], the substrate criteria of which may be simpler) can be expected to lead to more precise criteria. For instance, in the light of the present results, it already appears possible that the previous findings with the *Klebsiella* phage-11 glycanase[6], which hydrolyzes glycans with → 3)-β-D-Glc*p*-(1→ 3)-β-D-GlcA*p*-(1 → 3)-α-D-Gal*p*-(1 → chain-sequences to yield reducing Glc, may have to be decreased to a requirement for 3*eq*,1*eq* → 3*eq*,1*eq* sequences with a smaller distance between the carboxyl group and the susceptible glycosidic oxygen.

ACKNOWLEDGMENTS

We thank Professor Horst Sund (Fachbereich Biologie, Universität Konstanz) for generous and expert enzymological advice, Professor Horst Friebolin (Institut für Organische Chemie, Universität Heidelberg) for the p.m.r. spectrum of the *Klebsiella* type-2 repeating-unit tetrasaccharide, Hannelore Thoma for excellent technical assistance, and the Max Planck-Gesellschaft and the Fonds der Chemischen Industrie for support.

REFERENCES

1 K. TAYLOR, *Biochem. Biophys. Res. Commun.*, 20 (1965) 752–757.
2 B. KWIATKOWSKI, H. BEILHARZ, AND S. STIRM, *J. Gen. Virol.*, 29 (1975) 267–280.
3 M. H. ADAMS AND B. H. PARK, *Virology*, 2 (1956) 719–736.
4 I. W. SUTHERLAND, *Adv. Microb. Physiol.*, 8 (1972) 143–213.
5 E. C. YUREWICZ, M. A. GHALAMBOR, D. H. DUCKWORTH, AND E. C. HEATH, *J. Biol. Chem.*, 246 (1971) 5607–5616.
6 H. THUROW, H. NIEMANN, AND S. STIRM, *Carbohydr. Res.*, 41 (1975) 257–271.
7 K. TAKEDA AND H. UETAKE, *Virology*, 52 (1973) 148–159.
8 D. RIEGER, Y. M. CHOY, G. SCHMIDT, AND S. STIRM, *J. Gen. Virol.*, 34 (1977) 381–385.
9 H. NIEMANN, A. BIRCH-ANDERSEN, E. KJEMS, B. MANSA, AND S. STIRM, *Acta Pathol. Microbiol. Scand. Sect. B*, 84 (1976) 145–153.
10 I. W. DAVIDSON, C. J. LAWSON, AND I. W. SUTHERLAND, *J. Gen. Microbiol.*, 98 (1977) 223–229.
11 W. NIMMICH AND W. MÜNTER, *Z. Allg. Mikrobiol.*, 15 (1975) 127–129.
12 D. RIEGER-HUG AND S. STIRM, unpublished results.
13 K. R. YAMAMOTO, B. M. ALBERTS, R. BENZINGER, L. LAWHORNE, AND G. TREIBER, *Virology*, 40 (1970) 734–744.
14 H. NIEMANN, N. FRANK, AND S. STIRM, *Carbohydr. Res.*, 59 (1977) 165–177.
15 L. C. GAHAN, P. A. SANDFORD, AND H. E. CONRAD, *Biochemistry*, 6 (1967) 2755–2767.
16 H. NIEMANN, H. FRIEBOLIN, AND S. STIRM, unpublished results.
17 B. LINDBERG, B. LINDQVIST, J. LÖNNGREN, AND W. NIMMICH, *Carbohydr. Res.*, 49 (1976) 411–417.
18 B. LINDBERG AND J. LÖNNGREN, personal communication.
19 H. NIEMANN, Dr. rer. nat. Thesis, University of Freiburg, 1976.
20 T. KRZYWY, A. PRZONDO-HESSEK, AND S. ŚLOPEK, *Arch. Immunol. Ther. Exp.*, 19 (1971) 1–14.
21 D. E. BRADLEY, *Bacteriol. Rev.*, 31 (1967) 230–314.
22 B. KWIATKOWSKI, H. NIEMANN, AND S. STIRM, unpublished results.
23 W. BESSLER, E. FREUND-MÖLBERT, H. KNÜFERMANN, C. RUDOLPH, H. THUROW, AND S. STIRM, *Virology*, 56 (1973) 134–151.
24 C. RUDOLPH, E. FREUND-MÖLBERT, AND S. STIRM, *Virology*, 64 (1975) 236–246.
25 S. SCHLECHT AND O. WESTPHAL, *Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg., Abt. I: Orig. Reihe A*, 200 (1966) 241–259.
26 H. THUROW, Y. M. CHOY, N. FRANK, H. NIEMANN, AND S. STIRM, *Carbohydr. Res.*, 41 (1975) 241–255.
27 F. KAUFFMANN, *The Bacteriology of* Enterobacteriaceae, Munksgaard, Copenhagen, 1966.
28 M. ADAMS, *Bacteriophages*, Interscience, New York, 1959.
29 W. NIMMICH, *Z. Med. Mikrobiol. Immunol.*, 154 (1968) 117–131.
30 K. JANN, B. JANN, F. ØRSKOV, I. ØRSKOV, AND O. WESTPHAL, *Biochem. Z.*, 342 (1965) 1–22.
31 H. NIEMANN, B. KWIATKOWSKI, U. WESTPHAL, AND S. STIRM, *J. Bacteriol.*, 130 (1977) 366–374.
32 R. L. TAYLOR, J. E. SHIVELY, AND H. E. CONRAD, *Methods Carbohydr. Chem.*, 7 (1976) 149–151.
33 T. BITTER AND H. M. MUIR, *Anal. Biochem.*, 4 (1962) 330–334.
34 J. T. PARK AND M. J. JOHNSON, *J. Biol. Chem.*, 181 (1949) 149–151.
35 B. KICKHÖFEN AND O. WESTPHAL, *Z. Naturforsch. Teil B*, 7 (1952) 655–659.
36 H. THUROW, H. NIEMANN, C. RUDOLPH, AND S. STIRM, *Virology*, 58 (1974) 306–309.
37 M. J. HOW, J. S. BRIMACOMBE, AND M. STACEY, *Adv. Carbohydr. Chem.*, 19 (1964) 303–358.
38 Y. M. CHOY AND G. G. S. DUTTON, *Can. J. Chem.*, 51 (1973) 3015–3020.
39 B. LINDBERG, K. SAMUELSSON, AND W. NIMMICH, *Carbohydr. Res.*, 30 (1973) 63–70.
40 H. BJÖRNDAL, B. LINDBERG, J. LÖNNGREN, K.-G. ROSELL, AND W. NIMMICH, *Carbohydr. Res.*, 27 (1973) 373–378.
41 H. E. CONRAD, J. R. BAMBURG, J. D. EPLEY, AND T. J. KINDT, *Biochemistry*, 5 (1966) 2808–2817.
42 I. W. SUTHERLAND, *J. Gen. Microbiol.*, 94 (1976) 211–216.
43 D. A. REES, *J. Chem. Soc., B*, (1969) 217–226.
44 D. A. REES, *MTP Int. Rev. Sci., Org. Chem. Ser. 1*, 7 (1973) 251–283.
45 E. D. T. ATKINS, D. H. ISAAC, I. A. NIEDUSZYNSKI, C. F. PHELPS, AND J. K. SHEEHAN, *Polymer*, 15 (1974) 263–271.
46 S. IWASHITA AND S. KANEGASAKI, *Eur. J. Biochem.*, 65 (1976) 87–94.