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DEGRADATION OF BACTERIAL SURFACE CARBOHYDRATES BY VIRUS-ASSOCIATED ENZYMES

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Abstract - Virus-associated glycanases acting on bacterial capsular polysaccharides or on the O-specific side chains of cell wall lipopolysaccharides, carboxylic ester and amide hydrolases removing O- and N-acetyl residues from these glycans, as well as lyases for bacterial exopolysaccharides are known to occur on bacteriophages for different bacterial species, notably for many belonging to the family of Enterobacteriaceae. A review of the current knowledge on this group of enzymes is presented, covering the following aspects: (i) Isolation and selection of appropriate bacteriophages, (ii) purification of bacteriophage particles and of the virus organelles ("spikes" or thick "fibers") carrying the active center(s), (iii) electron microscopy of the viruses, (iv) molecular properties of the virus organelles, (v) enzymology (pH dependence, kinetics, substrate specificity etc.) of the reactions as catalyzed by complete bacteriophage particles, (vi) applications of the viral glycanases, which allow the preparative isolation of oligosaccharides consisting of one and more repeating units of the host surface heteropolysaccharides.

INTRODUCTION

At the onset of the infective process, bacteriophages may be confronted with a glycocalix surrounding the host bacterial cell. In the case of encapsulated bacteria, the host exopolysaccharide layer often has a thickness of several hundred nm, which corresponds to nearly ten times the diameter of a virus of the most common morphological type. Therefore, many (if not all) bacteriophages capable of infecting encapsulated host cells carry exopolysaccharide degrading enzymatic activities, the active centers of which are part of the viral tail "spikes" or "fibers". As shown by Bayer et al. (1), such phages can be visualized under the electron microscope on their way from the outer surface of the bacterial capsule to the cell wall underneath. With one noticeable difference, the same holds for viruses infecting non-encapsulated bacteria: In these cases, the phages only have to cope with the much thinner layer of outer carbohydrates on the cell wall - such as the glycan moieties in the lipopolysaccharides of gram-negative bacteria - and therefore, many viruses are able of infecting these cells without degradation of the glycocalix. In consequence, the incidence of host surface carbohydrate-degrading enzymatic activities is lower among bacteriophages for non-encapsulated cells than among viruses for encapsulated bacterial species.

Obviously, this group of viral enzymes - which have been called "penetrases" - poses many interesting virological and biochemical problems. Examples are the quest for the *in situ* conformation of the bacterial exopolysaccharides and for the mechanism of viral translocation through the bacterial glycocalix, or the search for the sites on the bacterial cell walls (the "second receptors") where, after penetration, the viruses eject their nucleic acid content. In

this lecture, however, we shall concentrate on a description of the methods for the isolation of virus-associated penetrases and on some applications of these enzymes in carbohydrate chemistry and biochemistry. There is no time either, to include the bacteriophage-coded murolytic enzymes which are produced in infected bacteria - often without incorporation into the virus particles - and which mainly help the phages to escape from the host cells.

SCOPE OF ENZYMES

The bacteriophage-associated enzymes which degrade bacterial outer surface carbohydrates may be classified according to the type of reaction they catalyze, and according to the genus of the respective bacterial host:

Most viral penetrases hitherto described are hydrolases, but a few lyases have also been found. The hydrolases are either glycanases (glycoside hydrolases; EC 3.2.1) depolymerizing bacterial capsular or exopolysaccharides (CPS) (see, for instance, Ref. 2-5) or the O-specific side chains of cell wall lipopolysaccharides (LPS) (Ref. 6-12) - or they are "deacetylases" (carboxylic ester or amide hydrolases; EC 3.1.1 or 3.5.1) which cleave off acetyl substituents occuring on these two groups of bacterial surface carbohydrates (Ref. 13-15). The two known bacteriophage-associated lyases (carbon-oxygen lyases; EC 4.2.99) act on capsular (exo-)glycans and produce oligosaccharides terminating in unsaturated glycuronic acids (Ref. 16, 17).

To date, virus-associated enzymes of these types have mainly been studies using phages for bacteria belonging to the family of Enterobacteriaceae, and within this family to the genera Escherichia coli (see, for instance, Ref. 1, 2, 9, 18), Klebsiella (Ref. 3-5) Salmonella (Ref. 6-8, 10-15), Shigella (Ref. 19), and Proteus (Ref. 18, 20). However, some penetrases of phages for other gram-negative organisms, such as Pseudomonadaceae (Ref. 21, 22), Rhizobiaceae (Ref. 23, 24), and Azotobacteriaceae (Ref. 17, 25, 26), as well as for a few gram-positive bacteria, all Streptococcus spp. (Lactobacillaceae) (Ref. 16, 27) have also been described.

It should be stressed that the apparently limited occurence of these phage enzymes throughout the bacterial realm is only due to a lack of investigations. Viruses carrying host surface carbohydrate degrading activities can certainly be found for many more bacterial genera, if they are looked for. The same probably also holds for the limited range of reactions catalyzed by the viral penetrases hitherto known. For instance, bacteriophages infecting thickly encapsulated Klebsiella spp. have been described, which neither depolymerize nor deacetylate the host exopolysaccharide (Ref. 28), and which thus possibly catalyze an as yet unidentified type of reaction. Also, the thickness of the cell wall of many gram-positive bacteria indicates that virus-associated teichoidases may be looked for. Finally, bacterial outer surface constituents other than carbohydrates may also be acted upon. Hongo and Yoshimoto (29), for instance, found a phage-induced depolymerase active on the γ-polyglutamate capsules of Bacillus spp.

ISOLATION AND SELECTION OF APPROPRIATE BACTERIOPHAGES

Obviously, bacteriophages may be found in the natural habitat of their host bacteria, for instance, in the case of Enterobacteriaceae, in human or animal faeces or in sewage. In our laboratory, we generally proceed as follows for the isolation of Klebsiella and E.coli phages (Ref. 30): Large samples of sewage are mixed with concentrated nutrient broth and then with an actively growing culture of the bacteria for which a virus shall be isolated. After incubation, a dilution series of the concentrated and sterilized enrichment culture is placed on nutrient agar plates seeded with the bacteria and incubated again. The plates are then inspected for bacteriophage plaques and single virus colonies are purified by repeated isolation. In this manner, bacteriophages for non-encapsulated Enterobacteriaceae are practically always found, while viruses for encapsulated bacteria are more rare (Ref. 31). For some strains, for instance E.coli K235 with a colominic acid capsule, phages were immediately isolated, while for others, like Klebsiella strains with the serotype K4 capsule, many samples of sewage had to be tested before a virus was found.

Selection of bacteriophages with host exopolysaccharide depolymerase activities (glycanases and lyases)

Next, one is confronted with the problem of selecting viruses with host surface carbohydrate degrading activities amongst such isolates. This is very easy in the case of depolymerases acting on exopolysaccharides, because phages with a characteristic plaque morphology always carry such enzymes (Ref. 5, 17, 32, 33): The plaques proper of these viruses are surrounded by large halos, in which the bacterial growth is decapsulated. As we shall see, the halos are due to the overproduction of free, enzymatically active "spikes" or "fibers" in addition to complete virus particles. The free organelles diffuse out from the plaque center and depolymerize the capsules in the surrounding bacterial lawn.

Selection of bacteriophages with glycanase activities for the host cell wall lipopolysaccharide

The selection of bacteriophages with glycanase activities for the O-specific side chains of Enterobacteriaceae cell wall LPSs is more difficult, because - due to the much thinner glycocalix of these hosts - the viruses do not exhibit the characteristic plaque morphology and also because, as we have seen, they are comparatively rare amongst the phages growing on non-encapsulated cells. However, it has generally been observed (Ref. 6, 7, 9, 12, 18) that viruses which are specific for the LPS side chains, i.e. phages which grow on these "smooth" hosts but not on "rough" host mutants lacking these side chains, often carry such enzymes. It is therefore possible to preselect for O-specific bacteriophages amongst new isolates, and to test these for LPS hydrolases with a good chance of finding viruses of the desired type. In practice, one may proceed as follows (Ref. 12, 18): A rough bacterial mutant is first isolated, for instance from aged broth cultures of the host; the bacterial mutants can be recognized due to the "rough" appearance of their colonies and due to their agglutinability in salt solutions. The phage mixture obtained from sewage is then absorbed with large amounts of host mutant bacteria, non-adsorbing viruses are plated, on host and host mutant, and O-specific phages are selected. The virus particles are finally purified by the methods to be described later, incubated with isolated host cell wall LPS, and the mixture is tested for an increase in reducing power. A more elegant method for this last part of the selection procedure is used in the laboratory of Lindberg (12): The O-specific phages to be tested are incubated with bacteria, the LPS of which has been radiolabelled in vivo. After centrifugation, the supernatants are monitored for radioactivity.

Selection of bacteriophages with "deacetylase" activities

No methods for the selection of bacteriophages catalyzing the hydrolysis of acetyl substituents on bacterial CPS or LPS have been developed. That is, all phage enzymes of this type have been detected by incubation of the isolated substrates with virus particles and subsequent acetyl analyses of the former (Ref. 13, 14, 34, 35). It may be worth noting, however, that the known viruses with "deacetylase" activities are also specific for the respective host surface glycans, i.e. they are either capsule- or O-specific, respectively.

PURIFICATION OF BACTERIOPHAGE PARTICLES AND BACTERIOPHAGE ENZYMES

Propagation of bacteriophages

Larger amounts of phages may be obtained by the well-known "agar overlayer" technique of Adams (36). Unfortunately, this method does not work with all bacteriophages, and it is often difficult to reproduce. Therefore, we generally propagate our viruses in liquid culture, using a dialyzable medium (Ref. 30): If the cell density is followed by optical density measurements, the host cultures can be infected at the appropriate time and with the appropriate amount of phage to ensure that lysis (liberation of progeny phage) occurs just before the medium is exhausted. By this procedure, 0.1 to 10 l (or even larger) batches of virus suspensions with 10^{12} to 10^{14} plaque-forming units (PFU) per l are reproducibly obtained.

Purification of virus particles

After low speed centrifugation, the bacteriophages are precipitated from the

lysates by addition of 0.5 M NaCl and polyethylene glycol 6000 (generally 10 wt%) and storage over night at 4° C. They are then collected by another low speed centrifugation and purified by isopycnic ultracentrifugation through a CsCl density gradient from ρ=1.15 to 1.65 g/ml (Ref. 37, 38). If at least 10^{11} PFU are applied to the gradients, the phage bands between ρ=1.4 and ρ=1.5 g/ml are clearly visible; they are collected and dialyzed against a suitable buffer of about pH 7. The enzymatically active virus particles thus isolated are homogenous, as evidenced by electron microscopy, analytical ultracentrifugation, and immunoelectrophoresis with a lysate antiserum (Ref. 39). Near to 10^{13} PFU of pure bacteriophages are often obtained from 1 l of lysate, and the phage suspensions can generally be stored at 4° C over chloroform for several months without loss of infectivity or enzymatic activity.

Purification of enzymatically active virus organelles

Both the purified complete virus particles and the isolated phage organelles harboring the enzymatically active center(s) can be used as catalysts. The latter may be obtained from three different sources, employing conventional protein purification methods including ion exchange and gel permeation chromatography. One source is the free, that is not phage-associated organelles occuring in the lysates besides intact virus particles: The latter are selectively sedimented by ultracentrifugation or by precipitation with polyethylene glycol, and the free virus fragments are isolated from the supernatants (Ref. 7, 25, 32, 33, 40-44). Another source is disintegrated virus particles. For instance by treatment with mild acid or with EDTA, the bacteriophages may be disrupted without substantial loss of enzymatic activity, and the virus debris can then be fractionated (Ref. 15, 37, 45, 46). A third source is bacteria infected with phage mutants (conditionally lethal mutants, non-permissive conditions) inducing the formation of viral enzyme, but not of intact virus. The infected cells are disrupted and their contents fractionated (Ref. 47-50). Depending on the type of virus-associated enzyme isolated, the fractions may be tested by incubation on dead or outgrown lawns of host bacteria (determination of CPS depolymerase activities) (Ref. 32, 33), by incubation with bacterial cells radiolabelled in the LPS (test for LPS glycanase activities) (Ref. 7, 49), or by incubation with acetylated substrate and determination of the acetic acid liberated (test for "deacetylase" activities) (Ref. 34, 35).

MORPHOLOGY OF VIRUSES AND MOLECULAR PROPERTIES OF ENZYMATICALLY ACTIVE VIRUS ORGANELLES

Electron microscopy of bacteriophages with host surface carbohydrate-degrading enzymatic activities

Many bacteriophages carrying such activities have been visualized by electron microscopy (see, for instance, Ref. 1, 5, 12, 15, 30, 32, 37, 45, 50-54). Figure 1 shows examples of the results which may be summarized as follows: The majority of these viruses - for instance 36 of 50 Klebsiella phages with capsule depolymerase activities (Ref. 5) and 5 of the 7 S.typhi Vi bacteriophages with "deacetylase" activities (Ref. 53) - were found to belong to Bradley's (55) morphology group C. They have a head of 45 to 65 nm in diameter, carrying a base plate with the "spikes" attached to it. The base plates were often seen to have a hexagonal shape with one or two enzymatically active organelles at each corner, that is six or twelve "spikes" in total. In some cases, additional structural elements of these viruses could be demonstrated, such as a short cone-shaped tail protruding from the head through the center of the base plate.

In addition to these group C phages, a minority of group B and a few group A phages were also found, for instance 11 and 3, respectively, amongst 50 Klebsiella bacteriophages carrying CPS depolymerases. The group B phages have a long flexible tail, and the group A phages have a long stiff tail furnished with a contractile sheath. Also in the group A and group B viruses, the organelles carrying the carbohydrate hydrolase or lyase activities could be distinguished as "spikes" or thick "fibers" attached to the distal end of the tail.

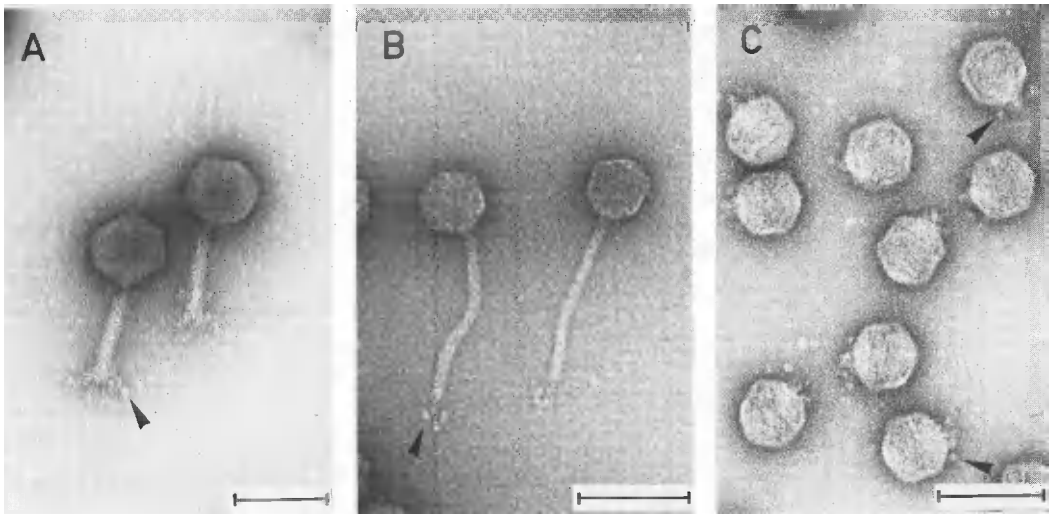


Fig. 1. Electron microscopy of bacteriophages with host surface carbohydrate degrading enzymatic activities. A, Escherichia coli bacteriophage ϕ92, belonging to Bradley's morphology group A and carrying a depolymerase for the E.coli serotype K92 capsular polysaccharide. B, Klebsiella bacteriophage ϕ28, belonging to group B and carrying a depolymerase for the Klebsiella K28 exopolysaccharide. C, E.coli bacteriophage ϕ1.2, belonging to group C and carrying a colominic acid-depolymerizing enzyme. Negatively stained with uranyl acetate. Arrows point to enzymatically active tail "spikes" or "fibers". Marker bars: 100 nm. By courtesy of Dr. B. Boschek, Institut für Virologie am Klinikum der Universität, D-6300 Giessen, German Federal Republic.

Molecular properties of enzymatically active virus organelles

As we have seen, enzymatically active phage organelles have been isolated by three different approaches. When visualized under the electron microscope, oblong or drop-shaped particles were seen in such preparations, and were between 4 and 7.5 nm in thickness and 11 to 25 nm in length. Molecular weight determinations, especially by analytical ultracentrifugation and from the gel elution volumes, yielded values of between 140000 and 404000, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that these enzymes may consist of polypeptides of one or of two size classes. Especially well studied viral spikes are those of Klebsiella bacteriophage ϕ11 (Ref. 32, 46), which catalyze the hydrolysis of the Klebsiella serotype K11 CPS and those of phage P22, which hydrolyze the O-specific side chains in S.typhimurium LPS (Ref. 47-50). Each of the six corners of the group C phage ϕ11 base plate carries a pair of drop-shaped "spikes" of 5 to 6.8 nm in thickness and 12.5 nm in length. They were shown to have a molecular weight of 155000 and to consist of one polypeptide each of 62500 and 94000 daltons, respectively. P22 also belongs to morphology group C, and its "spikes" have been reported to have a thickness of 7.5 nm, a length of 25 nm, and a molecular weight of 173000 (Ref. 50). They were found to consist of polypeptides of one size only, with 76000-77000 daltons (Ref. 47, 50). In the case of Klebsiella phage ϕ11 and of Aerobacter aerogenes phage K-2 (Ref. 43, 44) - carrying a host CPS depolymerase consisting of 31000 and 70000 dalton polypeptides -, enzymatically active enzyme subunits comprising of only one polypeptide size class (62500 or 70000 daltons, respectively) were also obtained (see also Ref. 37). In several instances, component analyses of enzymatically active phage organelles were performed. No rare amino acids or glycoprotein sugars were found. In the case of the P22 "spike" protein, an N- and C-terminal amino acid analysis as well as a separation of tryptic peptides has additionally been reported (Ref. 48).

ENZYMOLOGY OF BACTERIOPHAGE-CATALYZED REACTIONS

Upon incubation with purified phage particles (or isolated phage organelles), the carbohydrate substrates - cell wall LPS, extracted from the bacteria by the phenol/water procedure (Ref. 56), or CPS precipitated from such extracts with cetyltrimethylammonium bromide (Ref. 57)- are either depolymerized or deacetylated. Some examples (Ref. 18, 34, 58) are summarized in Fig. 2. In the case of the S.typhi Vi bacteriophage III enzyme (Fig. 2C) acetylated pectic acid was used as a substrate, because it is more easily accessible and equally well deacetylated as the natural substrate, Vi polysaccharide (see below).

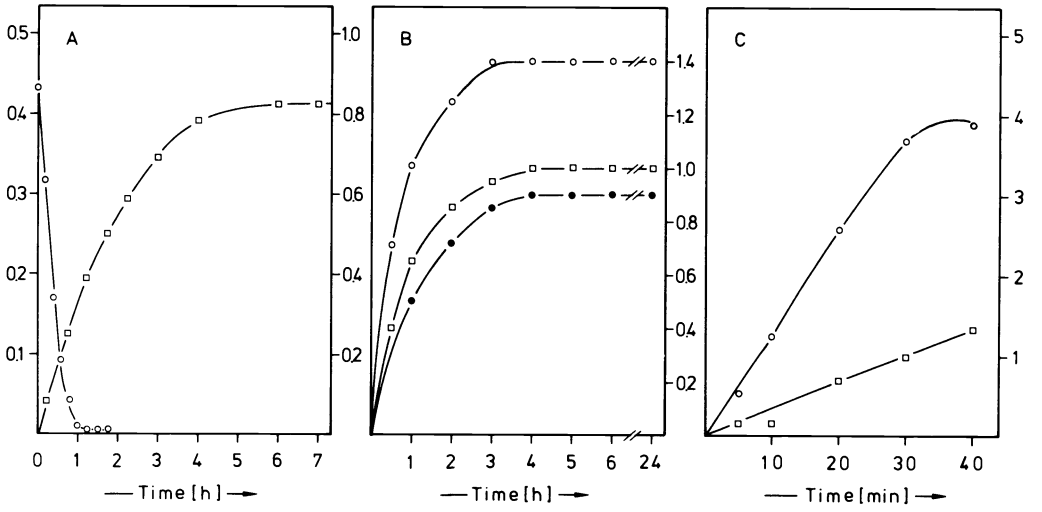


Fig. 2. Incubation of bacterial surface carbohydrates with purified bacteriophage particles: Course of reactions. A, loss of viscosity ($[\eta/\eta_o]$ - 1; o-o-o-, left scale) and increase in reducing power (glucose equivalents [µmoles/ml]; □-□-□-, right scale) upon incubation of Klebsiella serotype K13 capsular polysaccharide (2 mg/ml) with phage ϕ13 (10^{10} PFU/ml) in physiological, phosphate-buffered saline of pH 7 at 37° C. B, increase in reducing power (scale as in A) upon incubation of the respective host cell wall lipopolysaccharides (2 mg/ml) with Proteus mirabilis phage "otto" (o-o-o-), or Escherichia coli phages ϕ144 (□-□-□-) or ϕ7 (●-●-●-) ($2x10^{10}$ to $4x10^{10}$ PFU per ml, other conditions as in A) (with permission of J.Gen.Virol.). C, liberation of acetic acid (µmoles/ml, as determined by gas-liquid chromatography) upon incubation of acetylated pectic acid (6 mg/ml) with Salmonella typhi Vi phage III ($2x10^{11}$: o-o-o-, or $5x10^{10}$ PFU/ml: □-□-□-) in a 67 mM phosphate buffer of pH 7.8 at 37° C.

pH dependence, ion requirements, and temperature denaturation

All bacteriophage enzymes so far examined in our group, exhibited optima between pH 7 and pH 8 (Ref. 3, 34, 59, 60). Since none of the agents showed any dependence on specific ions, the reactions may conveniently be run in phosphate or volatile buffers - for instance in a 0.05 M ammonium carbonate/0.1 M ammonium acetate buffer - of this pH range. In the cases studied, enzyme denaturation was found to occur between 40 and 60° C; therefore, we generally use 37° C.

Reaction kinetics

In Fig. 3, some kinetic data obtained with the Klebsiella bacteriophage ϕ13 glycanase are shown (Ref. 59). With an excess of substrate, a linear

relationship between virus concentration (Fig. 2A), or between reaction time (Fig. 2B) and the amount of reducing sugar liberated is observed, and Michaelis-Menten (Fig. 2C) or Lineweaver-Burk plots (Fig. 2D) can thus be obtained with the complete bacteriophages as catalysts. In the case of the ϕ13 enzyme, a K_m of 170 μM (in terms of Klebsiella K13 CPS repeating units) and a V_{max} of 140 nmoles of glucose equivalents liberated per min by 10^{10} PFU were determined in this manner. As seen in Fig. 2C and D, the reaction velocity decreases again at substrate concentrations above about 1 mM. This is not due to the increase in viscosity of the polysaccharide solutions, because higher viscosity caused by addition of an inert agent (such as glycerol) does not have this effect. The reaction is, however, inhibited by its products (Fig. 2E).

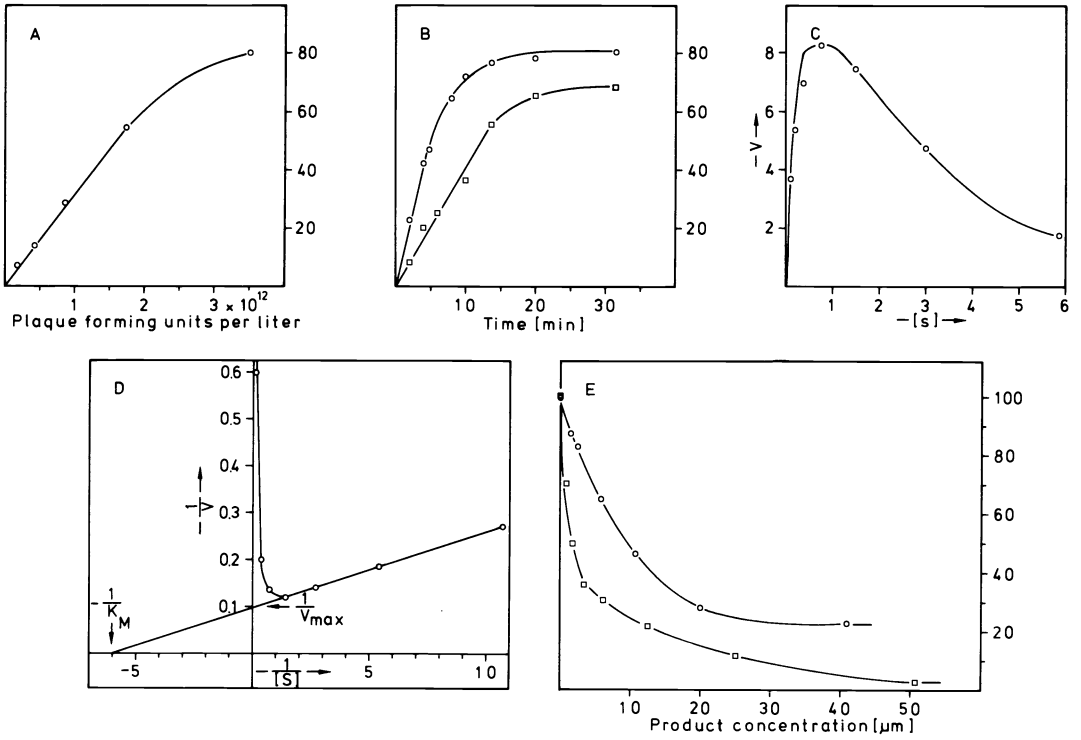


Fig. 3. Kinetics of depolymerization of Klebsiella serotype K13 capsular polysaccharide by particles of bacteriophage ϕ13 at pH 7.8 and 37° C (Ref. 59; with permission of Elsevier/ North Holland Press B.V.). A, a 1.36 mM (in repeating units) solution of the glycan was incubated for 7 min with different amounts of virus, and the reducing sugars liberated (μM glucose equivalents) were determined. B, liberation of reducing sugars by 1.8×10^{12} (o-o-o-) or by 7×10^{11} plaque forming units (PFU) x l^{-1} (□-□-□-) as in A, but as a function of time. C, plot of reaction velocity as catalyzed by 7×10^{11} PFU x l^{-1} (V, μmoles of glucose equivalents x l^{-1} x min^{-1}) versus substrate concentration ([S], mmoles repeating unit x l^{-1}). D, double-reciprocal plot of C. E, percent activity after preincubation (15 min at 37° C) of virus with reaction products: o-o-o-, K13 pentasaccharide (one repeating unit); □-□-□-, K13 decasaccharide (two repeating units). Other conditions as in A (7×10^{11} PFU x l^{-1}).

A similar study was carried out with S.typhi Vi bacteriophage III "deacetylase" and Vi polysaccharide or acetylated pectic acid as substrates (Ref. 34). In this case, a K_m of 10 to 10.5 mM (in acetyl residues) and a V_{max} of 12 to 15 nmoles of acetic acid liberated per min by 10^{10} PFU (at pH 7.8 and 37° C) were found. Also in this system, an inhibitory effect of one reaction product, the partially deacetylated polysaccharide, has been observed.

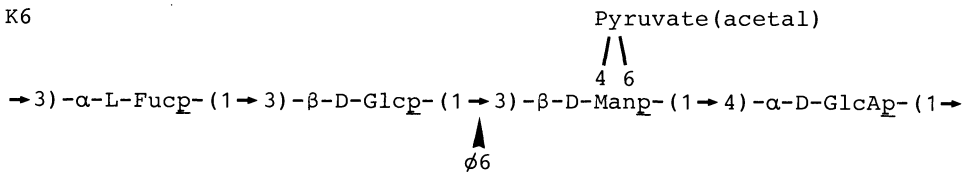
Substrate specificity

The substrate specificity of these enzymes has been studied, for instance, in the case of several Klebsiella phage-associated CPS glycanases (Ref. 3, 4, 5, 11, 59, 61, 62), for some Salmonella phage associated LPS glycanases (Ref. 10 -12, 47, 51, 63), and for the "deacetylase" of S.typhi Vi bacteriophage III (Ref. 34).

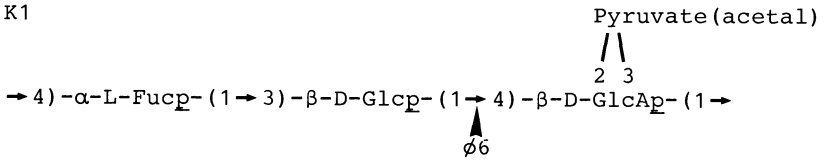
Host capsule glycanases. In an extensive study (Ref. 5), 55 different Klebsiella bacteriophages were tested for their enzymatic action on 74 different (acidic) Klebsiella capsular polysaccharides (serotypes K1-K72, K74, and K80). All glycans were mildly alkali-treated before use to exclude the possible influence (Ref. 61) of O-acetyl substituents on the enzyme specificities observed. The reducing sugars in all reaction products (oligosaccharides) were identified, mostly by reduction with sodium borohydride, hydrolysis, reaction with hydroxylamine/acetic anhydride, and gas-liquid chromatography of the resulting mixture of one alditol acetate and several aldonitrils. Since the primary structures of the majority of these Klebsiella CPS were known, about 50 glycanase cleavage sites could thus be identified. The results may be summarized as follows: (i) The Klebsiella virus-associated CPS glycanases were found to be very specific, 33 cross-reacting with none, 18 with one, two with two, and one each with 3 or 4 of the 73 heterologous polysaccharides, i.e. those from Klebsiella strains other than the respective phage host. (ii) In most cases where one polysaccharide was acted upon by several phage enzymes, the same bonds were split by the different agents. (iii) Most often, the polysaccharide chains were hydrolyzed on either side of the negative charges (glycuronic acid or pyruvate acetal carboxylate groups), but reducing glycuronic acids were never produced. (iv) In most cases, the reducing end sugars formed were substituted at position 3. (v) Most often, β-glycosidic linkages were hydrolyzed.

The glycanase, associated with Klebsiella bacteriophage ϕ6 was found to depolymerize the CPSs of serotypes K6 (homologous glycan), K1 and K57, and the phage ϕ13 enzyme was found to act upon the K2, K22 and K37 glycans in addition to the K13 polysaccharide (the K22 and K37 CPSs contain an unsaturated glycuronic acid, or 4-O-[(S)-1-carboxyethyl]-D-glucuronic acid, respectively [Ref. 64, 65]):

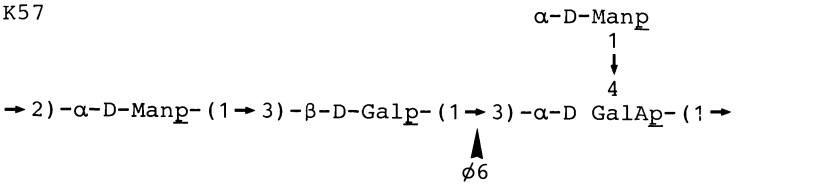
K6

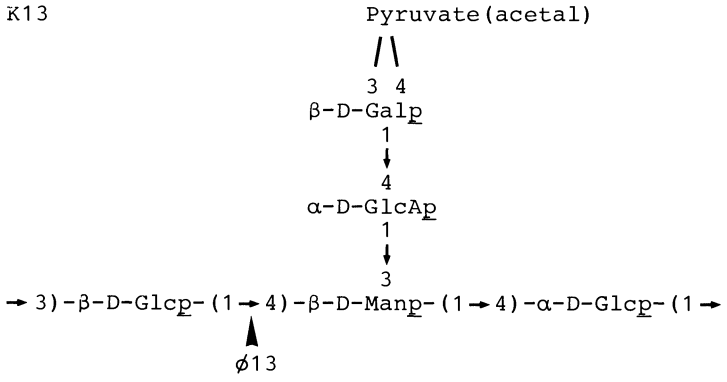


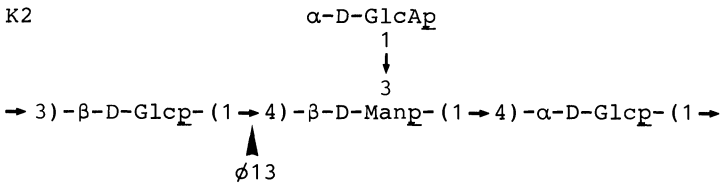
K1

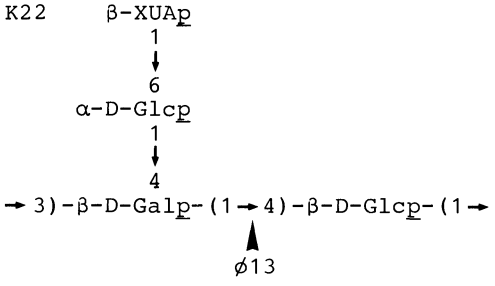


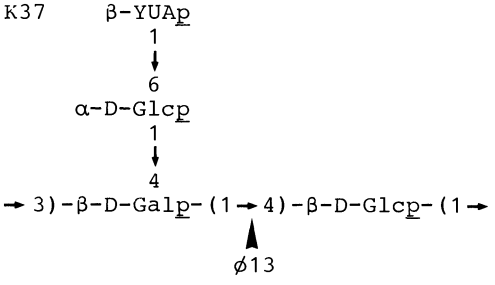
K57









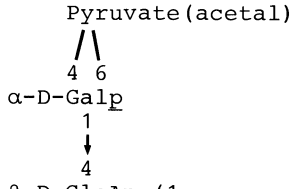


The substrate specificities of the ϕ6 and ϕ13 glycanases were further studied with polysaccharide derivatives (Ref. 59, 62): Especially, both enzymes - as well as others of this type (Ref. 3, 60) - were found to no longer act on their substrates, if the carboxylate groups in these glycans had been reduced to primary hydroxyls (Ref. 66). These findings, together with the homologies of the respective cross-reacting substrates, suggest that the ϕ6 and ϕ13 glycanases both recognize single functional groups (i.e. not complete sugar monomers) distributed around the susceptible glycosidic oxygens within the glycan repeating units. In acidic CPSs, one of these functional groups generally appears to be a carboxylate residue. Others may be hydroxyl groups, because the equatorial OH on C-2 and the primary one on C-6 of the reducing sugar liberated by the ϕ6 glycanase are conserved in all three substrates of this enzyme; and the same holds for the equatorial hydroxyl group on C-2 and the primary one on C-6 of the sugar residues to the left and to the right, respectively, of the ϕ13 cleavage sites. As evidenced by the location of the carboxylate residues in the ϕ13 substrates, the functional groups recognized

by phage glycanases may occur not only on chain, but also on branch sugars of the substrates.

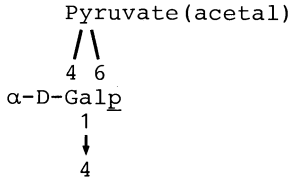
It should also be stressed that the sterical arrangement, i.e. the relative distances of the substrate functional groups interacting with the enzyme protein seem to be decisive. This is, for instance, apparent from the finding (Ref. 62) that the Klebsiella serotype K11 and K21 polysaccharides - before or after (partial) removal of the branches by Smith degradation [Ref. 67], or by partial acid hydrolysis [Ref. 68], respectively) - are resistant to the ϕ6 glycanase, although their repeating units contain the functional groups just discussed:

K11



→ 3)-α-D-Galp-(1→3)-β-D-Glcp-(1→3)-β-D-GlcAp-(1→

K21



→ 2)-α-D-Manp-(1→3)-β-D-Galp-(1→3)-α-D-GlcAp-(1→3)-α-D-Manp-(1→

It is tempting to postulate that the conformations of the K11 and K21 polysaccharides must differ from those of the ϕ6 substrates in such a way that the recognition group distances are crucially altered.

Finally, it should be mentioned, that the viral CPS glycanases probably interact with more than one substrate recognition site. This is suggested by the observation (Ref. 3, 5, 58-60, 62, 69-71) that the velocity of phage-catalized hydrolysis generally decreases with the size of the substrates: Substrate fragments of two and three repeating units were often found to be almost resistant to the enzymes, but they may, as we have seen, act as reaction inhibitors.

Glycanases acting on the O-specific side chains of host cell wall lipopolysaccharides. Substrate specificity studies of such enzymes (Ref. 11) have, for instance, been carried out with Salmonella newington bacteriophage ε^{34} (Ref. 10) and with 16 different S.typhimurium phages, notably P22 (Ref. 12, 47, 51, 63), using cell wall LPSs from different Salmonella spp., as well as chemically modified host LPS and other polysaccharides exhibiting structural similarities to the homologous substrate. In these systems, however, the glycanase recognition sites could generally not be narrowed down to functional groups, mainly because the cross-reacting LPS subtrates identified all had the same chain sugars. This holds especially for the extensively studied glycanase activity associated with the "spikes" of phage P22: All Salmonella lipopolysaccharides found to be acted upon by this enzyme comprise the chain trisaccharide →2)-α-D-Manp-(1→4)-α-L-Rhap-(1→3)-α-D-Galp-(1→ , substituted at position 3 of the mannose by a 3,6-dideoxyhexose (3,6-dideoxy-D-ribo-, D-xylo-, or -D-arabino-hexose in Salmonella serogroups A, B or D, respectively). However, some of these results are in good agreement with those on the substrate specificities of viral CPS glycanases just described: (i) The LPS glycanases were also found to be highly specific. For instance, the P22 enzyme is sensitive to a substitution of the chain galactose residue by α-D-glucose; if this substitution occurs at position 6, the polymer is rendered inert to the enzyme, if it occurs at position 4, the glycan is an even better substrate than the unsubstituted one. Also, the P22 hydrolase does not accept LPS O-specific side chains in which the chain α-galactose is linked to position 6 instead of 2 of the ensuing mannose. (ii) The studies of Lindberg et al. (Ref. 12, 51, 63) strinkingly demonstrate that

different viral enzymes acting on the same substrate generally cleave the same linkages: All 16 S.typhimurium phages, including P22, were found to cleave the chain rhamnose linkages. (iii) Also with these enzymes, functional groups of branch sugars may be part of the recognition sites. The P22 enzyme, and those of some, but not all of the other S.typhimurium phages, did not cleave an LPS, the 3,6-dideoxyhexose branches of which had been removed by partial acid hydrolysis.

"Deacetylase" of S.typhi Vi bacteriophage III. The action of Vi phage III particles on a variety of acetylated substrates has been tested (Ref. 34); the results are summarized in Table 1:

TABLE 1. Action of the Vi bacteriophage III "deacetylase" on various substrates

Substrate[a]				Enzyme susceptibility	
Monomer	D.P.[b]	Substituents	D.S.[c]	K_m[d]	V_{max}[e]
2-amino-2-deoxy-α-D-galacturonic acid[f]	500[g]	N- and O-acetyl	0.6[f]	10	12
α-D-galacturonic acid	150[h]	O-acetyl	1.1	10.5	15
	150[h]	O-monofluoro-acetyl	0.5	-[i]	-
	150[h]	O-propionyl	1.1	-	-
	3[j]	O-acetyl	2.1	4.5	1.5
	2[j]	O-acetyl	2.2	+[k]	+
	1[j]	O-acetyl	2.9	+	+
α-L-guluronic acid[l]	20	O-acetyl	1.1	10	1.2
β-D-mannuronic acid[l]	15-20	O-acetyl	0.8	-	-

[a](1→4)-linkages only. [b]Degree of polymerization. [c]Degree of substitution. [d]mM acyl residues; at pH 7.8 and 37° C. [e]nmoles of acid liberated per min by 10^{10} plaque-forming units. [f]Vi polysaccharide, natural substrate, as extracted from Citrobacter Ci23, the host organism used. [g]According to Heyns and Kiessling (72). [h]Pectic acid. [i]No enzyme action. [j]Tri- or disaccharide, or methyl glycoside, respectively. [k]Enzyme susceptible substrate; kinetic parameters not determined. [l]From alginic acid (Ref. 73).

As we have already pointed out, acetylated pectic acid was found to be an even better substrate for this enzyme than the homologous one, Vi polysaccharide. O-acetyl residues were also removed from smaller substrates down to the methyl glycoside of 2,3,6-tri-O-acetyl-α-D-galacturonic acid, but O-monofluoroacetyl and O-propionyl residues were not acted upon. The finding that the Vi phage III "deacetylase" also accepted acetylated α-L-guluronan - in which the sugar units are diaxially linked as in pectic acid -, but not acetylated β-D-mannuronan - in which they are diequatorially linked (Ref. 74, 75) -, suggested that also this viral enzyme is especially sensitive to conformational alterations of its substrates. An additional finding resulted from proton magnetic resonance spectroscopy of acetylated pectic acid before and after phage action: The enzyme was not found to be specific for O-acetyl residues at positions 2 or 3 of the α-D-galacturonic acid units.

APPLICATIONS

The bacteriophage-associated glycanases, active on bacterial capsular (exo-) polysaccharides or on the O-specific side chains of cell wall lipopolysaccharides may be used for various purposes, because they generally allow the preparative isolation of oligosaccharide fragments (one and more repeating units) of these glycans. In our laboratory, mixtures containing several mg of substrate per ml of volatile buffer, and around 10^{10} (i.e. 10^9 to 10^{11}) PFU of purified virus particles per mg of glycan, are incubated at 37° C and about pH 7. For optimal depolymerization, the reactions are run for 24 h; after this time, further hydrolysis generally proceeds very slowly. Shorter reaction times yield larger fragments, since all these enzymes act by an endo mechanism. After evaporation of the buffer, the mixtures of oligosaccharides obtained may be separated by chromatographic procedures, such as ion exchange chromatography (for acidic oligosaccharides), gel filtration, or high performance liquid chromatography (Ref. 3, 12, 16, 18, 51, 58-60, 62-64, 69-71, 76). The bacteriophage contamination of the products may generally be neglected, because it is very small - 10^{10} PFU of a group C virus correspond to roughly 10 µg of protein and nucleic acid (Ref. 15) -, and since the phages are eluted far from the oligosaccharides. For critical product separations, however, it is advisable to remove the viruses by a gel filtration step prior to oligosaccharide chromatography. Two examples of viral glycanase product separations are shown in Fig. 4.

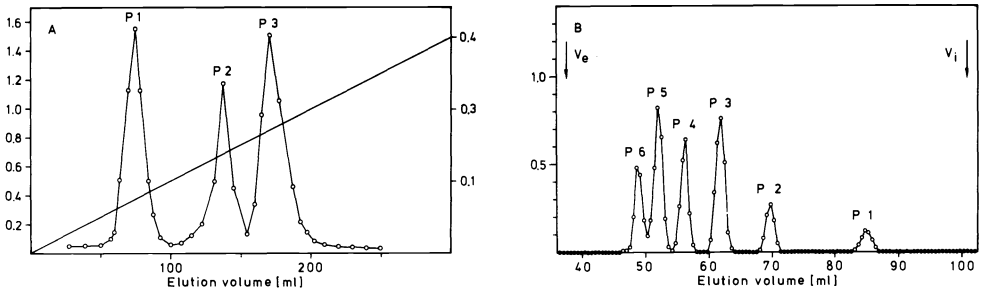


Fig. 4. Separation of bacteriophage glycanase products. A, Klebsiella serotype K2 capsular polysaccharide (for structure see text; 7.5 mg/ml) was incubated with particles of Klebsiella phage ϕ2 (7.5×10^{10} PFU/ml) at 37° C and pH7 for 24 h (exhaustive degradation). The products were adsorbed to DEAE Sephadex A25 from a 0.05 M Tris/HCl buffer of pH 7.2, eluted with a linear NaCl gradient (——) in the same buffer, and detected with carbazole-sulfuric acid (extinction at 535 nm; o-o-o-) (Ref. 70; with permission of Springer Verlag). B, Colominic acid (homopolymer of α[2→8]-linked N-acetylneuraminic acid residues; 4.5 mg/ml) was incubated with particles of Escherichia coli phage ϕ92 (5×10^{11} PFU/ml) for 24 h. The products were separated through Biogel P-4 and detected with resorcinol-HCl-$CuSO_4$ (extinction at 580 nm: o-o-o). Left scales: extinction, right scale in A: M NaCl. P1, P2 etc.: oligosaccharides of 1,2 etc. repeating units of substrate. Both polymers were mildly alkali-treated before use.

It should be noted, however, that a complete depolymerization of the substrate to oligosaccharides of one and a few repeating units does not always occur. Several examples have been reported (Ref. 5, 51, 63, 77), in which, from unknown reasons, the viral glycanases either produced such small fragments, but only from a part of the polysaccharide, or in which they completely depolymerized the substrates, but to much larger oligosaccharides.

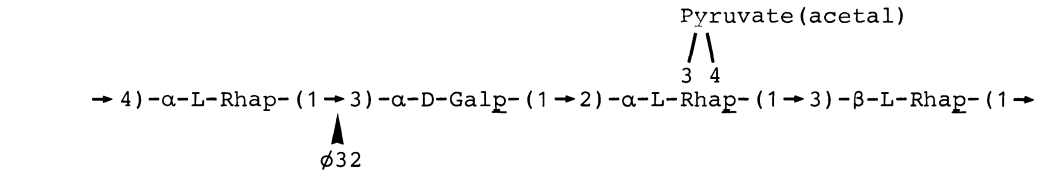
Application of bacteriophage-associated glycanases for the structural analysis of bacterial surface carbohydrates

It is clear that bacteriophage depolymerization may be used as a complement to other methods for the partial degradation of bacterial polysaccharides, such

as partial acid hydrolysis, glycuronic acid or Smith degradation. Some examples of the application of viral enzymes in this context are the structural analyses of the Klebsiella capsular polysaccharides of serotypes K13 (Ref.58), K22 (Ref. 64) and K25 (Ref. 69). The large-scale accessibility of these repeating unit mono- and oligomers is also of advantage for the analyses of bacterial heteroglycans by nuclear magnetic resonance spectroscopy: Much better resolved spectra were obtained with the oligosaccharides than with the polymers (Ref. 71).

Isolation of labile oligosaccharides

With some acid-labile bacterial glycans, bacteriophage degradation may be the only viable method for the isolation of repeating unit oligosaccharides. One example is the Klebsiella serotype K32 CPS, carrying extremely acid-labile 1-carboxyethylidene groups at positions 3 and 4 of rhamnose residues. Only by incubation with particles of bacteriophage ϕ32, which cleaves the repeating units as indicated, could the K32 tetrasaccharide be obtained (Ref. 71):



Further examples are the products of viral glycanase action on bacterial polysaccharides containing N-acetylneuraminic acid. Some of our recent results with these systems are shown in Table 2.

TABLE 2. Action of viral glycanases on bacterial polysaccharides containing N-acetylneuraminic acid

	Escherichia coli bacteriophage:	
	ϕ1.2	ϕ92
Host[a]	K235 (O1:K1:H⁻)	Bos12 (O16:K92:H⁻)
Morphology (Bradley group)	C	A
D.P.[b] of NeuNac oligomers obtained by exhaustive degradation of:		
→8)-α-D-NeuNAcp-(2→[c]	2,3,4,5,6,7[d]	1,2,3,4,5,6
→8)-α-D-NeuNAcp-(2→9)-α-D-NeuNAcp-(2→[e]	±[f]	2,(3),4,6
→9)-α-D-NeuNAcp-(2→[g]	-[h]	-
→4)-α-D-NeuNAcp-(2→4)-α-D-Galp-(1→[i]	-	-
→4)-α-D-NeuNAcp-(2→4)-α-D-Glcp-(1→[j]	-	-

[a]Strain of E.coli (serotype). [b]Degree of polymerization. [c] Colominic acid (E. coli serotype K1 capsular polysaccharide) (Ref. 78-80). [d]Main products underscored. [e]E.coli serotype K92 capsular polysaccharide (Ref. 81). [f]Partial degradation only (see text). [g]Neisseria meningitidis type C CPS (Ref. 82). [h]No degradation. [i]N.meningitidis type W-135 CPS (Ref. 82). [j]N.meningitidis type Y CPS (Ref. 82).

It can be seen that the capsule degrading enzymes of two E.coli bacteriophages have been tested. Colominic acid was found to be completely depolymerized

by both viral glycanases, although the ϕ92 enzyme yielded somewhat smaller fragments (see also Fig. 4) than the one associated with ϕ1.2. The E.coli K92 CPS, in contrast, was only partially acted upon by ϕ1.2, while ϕ92 completely degraded this glycan also to small oligosaccharides. All of the Neisseria meningitidis capsular polysaccharides tested were found to be inert to the action of both phage enzymes, again demonstrating the high specificity of these agents. It is especially interesting that the ϕ92 glycanase mainly produced fragments of 2n repeating units from the K92 glycan. Since ϕ92 also cleaved colominic acid, but not the N.meningitidis type C CPS, these results indicate that ϕ92 might preferentially act upon the α(2→8)-bonds in K92.

Synthesis of artificial antigens

The oligosaccharides obtained by virus degradation of bacterial surface carbohydrates may be coupled to protein carriers. We have tested artificial antigens of this type using Klebsiella pneumoniae B5055 (O1:K2) which is highly pathogenic for mice, and bacteriophage ϕ2 which cleaves the serotype K2 CPS (for structure see above) to oligosaccharides of one, two and three tetrasaccharide repeating units (see Fig. 4). For this purpose, the K2 tetra- (TS), octa- (OS) and dodecasaccharide (DS) were reductively aminated with p-nitrophenylethylamine in the presence of $NaBH_3CN$. After reduction of the nitro groups by catalytic hydrogenation, the oligosaccharide derivatives were diazotized and coupled to hemp seed edestin (for the immunization of rabbits), or to hemocyanin from keyhole limpets (for the immunization of mice). After purification, the conjugates were found to carry 10-50 (edestin) or 600-1700 (hemocyanin) moles of oligosaccharide per mole of protein (Ref. 70).

The artificial antigens were first tested for induction of Klebsiella K2-specific antibodies in rabbits. The animals were immunized with the oligosaccharide-edestin conjugates (together with Friend complete adjuvant) and the sera obtained were, inter alia, tested for agglutination of sheep erythrocytes coated with K2 CPS (passive hemagglutination), or of B5055 bacteria. Passive hemagglutination titers between 1:512 and 1:4096 were obtained for the sera against all three conjugates, comparing well with the titers obtained after immunization with complete B5055 bacteria (between 1:2048 and 1:4096). In addition, the OS- and DS- conjugate antisera were found to agglutinate B5055 cells, while only a slight direct bacterial agglutination was observed with the TS-edestin antiserum. Vice versa the free K2 oligosaccharides were also found to inhibit the precipitation of Klebsiella K2 CPS by antibacterial antisera. Again, K2 OS and DS were better inhibitors (100% inhibition at higher concentrations) than K2 TS (not more than 80% inhibition). It thus appears that a portion of the K2 CPS antibodies recognized that part of the polysaccharide repeating unit which is acted upon by the ϕ2 enzyme, i.e. that only oligosaccharides of two or more repeating units carried all epitopes of the polymer.

Next, mice were immunized with K2 TS-, OS- or DS-hemocyanin and challenged with Klebsiella B5055. The results are shown in Table 3. It can be seen that the K2 OS- and DS-conjugates were found to protect the animals as well as the K2 polysaccharide and almost as well as killed B5055 bacteria. Again, however, the K2 TS-hemocyanin was less effective. In addition to the reason just discussed, this reduced effectiveness of the one repeating unit-determinant may - as suggested by Svenson et al. (83) - also be due to immunodominance of the non-reducing end structure not occuring on the native polysaccharide.

Similar results were obtained in passive immunization of mice with rabbit anti-conjugate antisera. It thus appears that, when coupled to suitable protein carriers, bacterial surface oligosaccharides of two or more repeating units may serve as immunogens, representative of the corresponding bacterial glycans.

These conclusions are in good agreement with the results of Lindberg and coworkers, who used the TS (one repeating unit), OS and DS obtained by P22 cleavage of the O-specific side chain in the LPS of S.typhimurium (see above). Oligosaccharide-phenylisothiocyanate derivatives were prepared and coupled to various protein carriers, notably to bovine serum albumin (Ref. 84). In an extensive series of studies (Ref. 83, 85-87), the immune response against these artificial antigens was investigated in rabbits and mice. Inter alia, the conjugates were found to elicit the formation of Salmonella O4 and O12 (only OS and DS conjugates) antibodies in rabbits, at titers almost as high as those obtained with killed bacteria; and mice could be protected against S.typhimurium infection by active and passive immunization with these antigens.

TABLE 3. Protection of NMRI mice against infection with Klebsiella pneumoniae B5055 by active immunization with homologous killed bacteria, with Klebsiella serotype K2 capsular polysaccharide, or with hemocyanin conjugates of different oligomers of the K2 repeating unit

Vaccine[a]	Dose[b]	Mice dead (of 10) after challenge[c] with the following quantities of bacteria 10^1 10^2 10^3 10^4 10^5 10^6 10^7 10^8	LD_{50}[d]
none[e]		3 6 9 10 10	4.6×10^1
K.pneumoniae B5055[f]	1×10^8	0 0 4 10	1.5×10^7
K2 polysaccharide	1 µg	0 0 10 10	3.6×10^6
K2 tetrasaccharide-hemocyanin	40(10) µg	1 3 9 9	2.1×10^3
K2 octasaccharide-hemocyanin	76(10) µg	0 0 1 3 10 10	1.6×10^6
K2 dodecasaccharide-hemocyanin	68(20) µg	0 0 0 9 10	3.5×10^6
Free K2 dodecasaccharide	20 µg	4 4 10 10 10	5.8×10^1

[a]Immunizations by two injections (intraperitoneally) at an interval of 14 days. [b]Single dose; the values in parentheses represent the amount of oligosaccharide in the conjugates. [c]Infection 14 days after the second immunization. [d]The LD_{50} values represent the final mortality after 15 days. The protective capacity of the carrier protein hemocyanin (single dose 30 µg) was assayed in a separate experiment. The LD_{50} was 8.1×10^1. [e]"Infection control". [f]Acetone-killed bacteria.

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