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Substrate Specificity of Two Bacteriophage-Associated Endo-*N*-Acetylneuraminidases

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For *Escherichia coli* Bos12 (O16:K92:H$^-$), a bacteriophage (ϕ92) has been isolated which carries a depolymerase active on the K92 capsular polysaccharide. As seen under the electron microscope, ϕ92 belongs to Bradley's morphology group A and is different from the phage ϕ1.2 previously described (Kwiatkowski et al., J. Virol. **43**:697–704, 1982), which grows on *E. coli* K235 (O1:K1:H$^-$), depolymerizes colominic acid, and belongs to morphology group C. The specificity of the ϕ1.2- and ϕ92-associated endo-*N*-acetylneuraminidases has been studied with respect to the following substrates (all alkali treated, and where NeuNAc represents *N*-acetylneuraminic acid): (i) [-α-NeuNAc-(2 → 8)-]$_n$ (colominic acid), (ii) [-α-NeuNAc-(2 → 8)-α-NeuNAc-(2 → 9)-]$_n$ (*E. coli* K92 polysaccharide), and (iii) [-α-NeuNAc-(2 → 9)-]$_n$ (*Neisseria meningitidis* type C capsular polysaccharide). The increase in periodate consumption of these glycans upon incubation with purified ϕ1.2 or ϕ92 particles was measured, and the split products obtained from all substrates after exhaustive degradation were analyzed by gel chromatography. It was found that the *Neisseria* polysaccharide is not appreciably affected by either virus enzyme and that ϕ1.2 only depolymerizes a small fraction of the K92 glycan. Colominic acid, however, is completely degraded by both agents, ϕ92 yielding smaller fragments (one to six NeuNAc residues) than ϕ1.2 (two to seven). Phage ϕ92 additionally depolymerizes the K92 glycan, essentially to oligosaccharides of two, four, and six residues. The size distribution of these K92 oligosaccharides indicates that the ϕ92 enzyme predominantly cleaves the α(2 → 8) linkages in this polymer.

Bacteriophages infecting encapsulated bacteria often have glycanase activities associated with their tail spikes, which degrade the host capsular polysaccharides (3, 18, 27, 32; H. Geyer, K. Himmelspach, B. Kwiatkowski, S. Schlecht, and S. Stirm, Pure Appl. Chem., in press). This also holds for the *Escherichia coli* bacteriophage ϕ1.2 recently described (17), the depolymerase of which acts on the *E. coli* serotype K1 capsular polysaccharide (colominic acid), a homopolymer of α(2 → 8)-linked *N*-acetylneuraminic acid (NeuNAc) residues (2, 20, 23, 35).

To gain more insight into bacterioviral endo-*N*-acetylneuraminidases, we have now isolated a new bacteriophage (ϕ92) for *E. coli* Bos12 (O16:K92:H$^-$) which depolymerizes the *E. coli* K92 polysaccharide. This glycan is also a polymer of NeuNAc, but with alternating α(2 → 8) and α(2 → 9) linkages (9). In addition, we have investigated the substrate specificity of the ϕ1.2 and ϕ92 enzymes with respect to colominic acid, the *E. coli* K92 glycan, and the *Neisseria meningitidis* type C capsular polysaccharide which consists of NeuNAc residues linked by α(2 → 9) bonds only (5, 15).

MATERIALS AND METHODS

Most of the materials and methods and notably also phage ϕ1.2 and all bacteria used in this study have been described recently (17).

Bacteriophage ϕ92. The virus was isolated from sewage with *E. coli* Bos12 (O16:K92:H$^-$) as a prospective host. It was selected because of its plaque morphology (4, 17, 27), i.e., because it forms plaques with halos in which the bacterial growth is O16 agglutinable.

Polysaccharide substrates. The *E. coli* K92 polysaccharide was obtained from *E. coli* Bos12. The crude glycan was isolated according to Gotschlich et al. (11); it was extracted with phenol and further purified (9, 10, 12). The final products contained 77 to 91% (wt/wt) sialic acid as determined with resorcinol-hydrochloric acid-copper sulfate (8, 29, 34), 0.2% protein as analyzed with Coomassie brilliant blue (6) (with bovine serum albumin as a standard), and 2% other carbohydrates as estimated with anthrone-sulfuric acid (14) (with glucose as a standard).

The *N. meningitidis* capsular polysaccharides of

serogroups C, W-135, and Y were a gift from H. J. Jennings, National Research Council of Canada, Ottawa. A large sample of the type C glycan was also kindly supplied by Behringwerke AG, Marburg, Federal Republic of Germany.

Before use, all polysaccharide substrates were subjected to mild alkali treatment and passed over a Bio-Gel P-30 column to separate off low-molecular-weight components (17).

Large-scale propagation and purification of phage ϕ92 particles. With some modifications, the preparation of ϕ92 particles was carried out as previously described for ϕ1.2 (17). *E. coli* Bos12 was equally grown in P medium, but to an optical density of 1.0 at 660 nm (4.5×10^8 colony-forming organisms per ml), and then infected with ϕ92 to a multiplicity of infection of 0.1. About 1 h later, lysis occurred. After roughly another 2 h, the optical density rose again. The supernatant then obtained by centrifugation of the lysate generally had a titer around 6×10^{10} PFU per ml.

For precipitation of the viruses, solid NaCl (to 0.5 M) and polyethylene glycol 6,000 at a concentration of 10% (wt/vol) were employed, and the ϕ92 particles were collected by low-speed centrifugation after storage at 4°C for one night only.

In isopycnic centrifugation, ϕ92 banded at $\rho = 1.48$ g/ml. The final yield was about 4×10^{13} PFU of purified virions from 1 liter of lysate.

Determination of periodate consumption. The periodate consumption (1, 13) of the polysaccharide substrates and of the oligosaccharides obtained from them was determined as previously described (17), except for the following simplification. The solutions of the polysaccharides or of oligosaccharides produced after phage action and heat inactivation (all in volatile buffer and 15 mM with respect to NeuNAc residues) were not lyophilized and dried but were directly mixed with the 1 M sodium acetate buffer and with the sodium metaperiodate solution.

RESULTS

Isolation and host range of *E. coli* bacteriophage ϕ92. With *E. coli* Bos12 (O16:K92:H^-) as a prospective host, phage ϕ92 was isolated from sewage. It was selected because it forms plaques with halos in which the bacterial growth is O agglutinable. This plaque morphology is characteristic for viruses carrying host capsule depolymerase activities (4, 17, 27). The host range of ϕ92 was tested with a set of *E. coli* strains, the surface polysaccharides of which all contain NeuNAc (Table 1).

Electron microscopy of phage ϕ92. The results of electron optical visualization of ϕ92 are shown in Fig. 1. For comparison, a micrograph of phage ϕ1.2 (17) is included.

It can be seen that ϕ92 has an isometric head of about 78 nm in diameter and a tail of around 110 nm in length. This tail is furnished with a contractile sheath of about 100 nm in length and 22 nm in thickness; at its distal end it carries curly spikes of roughly 21 nm in length.

ϕ92 thus belongs to Bradley's morphology group A (7) and is clearly distinct from group C phage ϕ1.2 (Fig. 1c) (17).

Increase in periodate consumption of polysaccharide substrates after incubation with purified ϕ1.2 or ϕ92 particles. To test the substrate specificity of the host capsule depolymerases associated with ϕ1.2 and ϕ92, a set of three NeuNAc polymers was used: colominic acid, [-α-NeuNAc-(2 → 8)-]$_n$; *E. coli* K92, [-α-NeuNAc-(2 → 8)-α-NeuNAc-(2 → 9)-]$_n$; and *N. meningitidis* type C polysaccharide, [-α-

TABLE 1. Host range of *E. coli* bacteriophage ϕ92

E. coli strain	Serotype	Sugar composition of capsule[a]	Plating efficiency[b]	Rate of adsorption[c] (ml/min)	Capsule depolymerization[d]
Bos12 (host)	O16:K92:H^-	NeuNAc[e]	1	1.4×10^{-9}	+
6181/66	O73:K92:H^-	Probably the same[f]	0.1	1.2×10^{-9}	+
K235	O1:K1:H^-	NeuNAc[e]	0.5	2.6×10^{-9}	+
F11119/41	O16:K1:H^-	NeuNAc[e]	0.25	2.0×10^{-9}	+
C375	O132:K1:H^-	NeuNAc[g]	1	0.7×10^{-9}	+
U5/41 (D698)	O1:K1:H7	NeuNAc[h]	0.75	ND	+
Bi316/42	O9:K9:H12	Gal, HexN, NeuNAc[i]	$<10^{-9}$	$<10^{-10}$	−
Su3684/41	O56:K^+:H^-	NeuNAc[j]	$<10^{-9}$	$<10^{-10}$	−

[a] Abbreviations: Gal, galactose; HexN, hexosamine; NeuNAc, *N*-acetylneuraminic acid.
[b] Relative to that on *E. coli* Bos12.
[c] In Merck standard I broth at 37°C. ND, Not determined.
[d] Depolymerization of capsule, as determined by incubation of about 10^{10} PFU of purified ϕ92 particles on outgrown lawns of bacteria; for details see Kwiatkowski et al. (17).
[e] Not *O*-acetylated (9, 23).
[f] Carrying a K antigen serologically identical to that of Bos12.
[g] Partially *O*-acetylated at positions 7 and 9 (23).
[h] Exhibiting variation between *O*-acetylated and not *O*-acetylated forms (23).
[i] See MacLennan et al. (19).
[j] Surface polysaccharides containing NeuNAc (22).

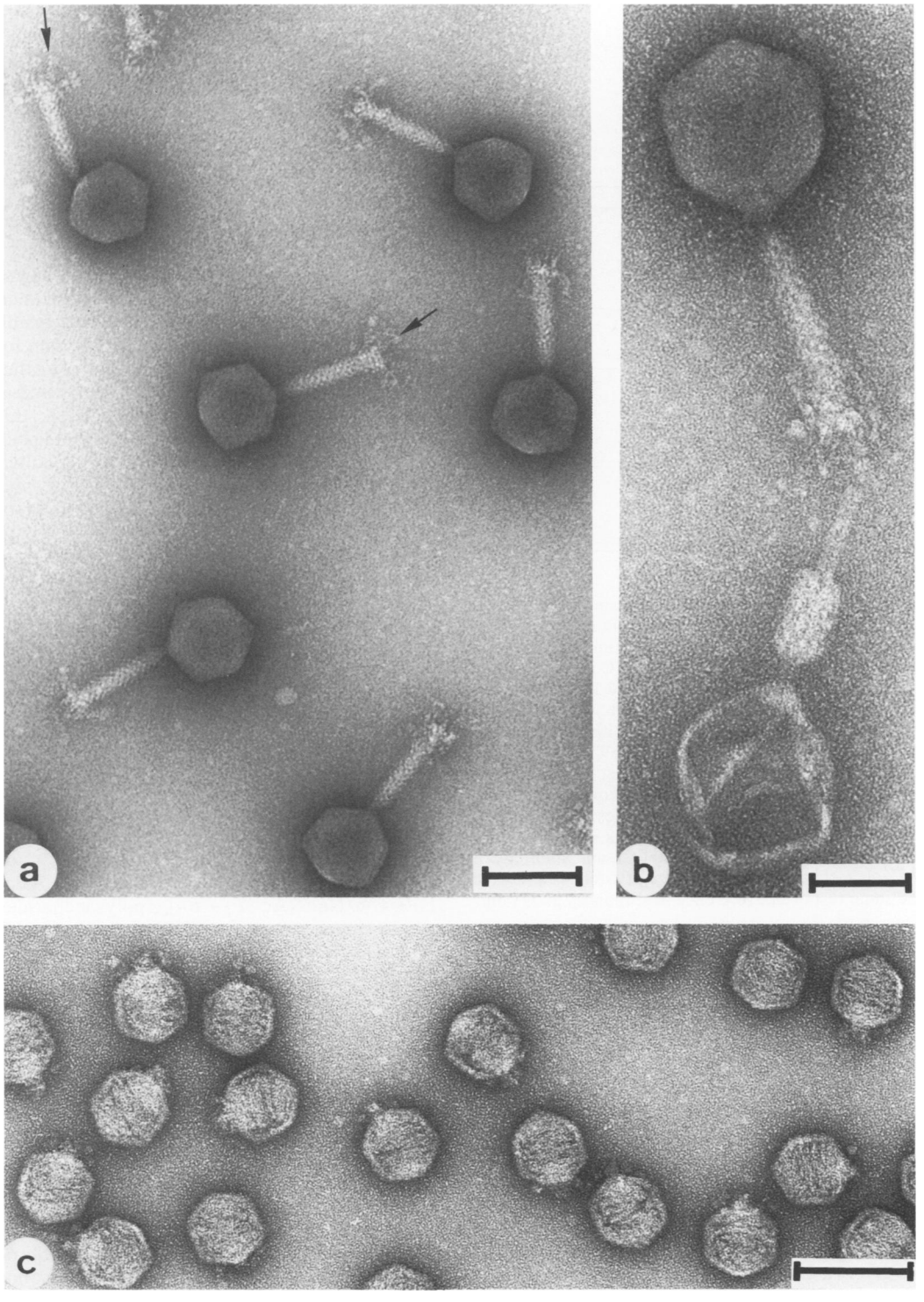

FIG. 1. Electron microscopy of *E. coli* bacteriophages carrying endo-*N*-acetylneuraminidase activities. (a and b) Phage ϕ92 (×140,000 and ×280,000, respectively). (c) Phage ϕ1.2 (×165,000). All specimens were stained with uranyl acetate. Marker bars: 100 nm (a and c) or 50 nm (b). Arrows point to ϕ92 tail appendages (curly spikes).

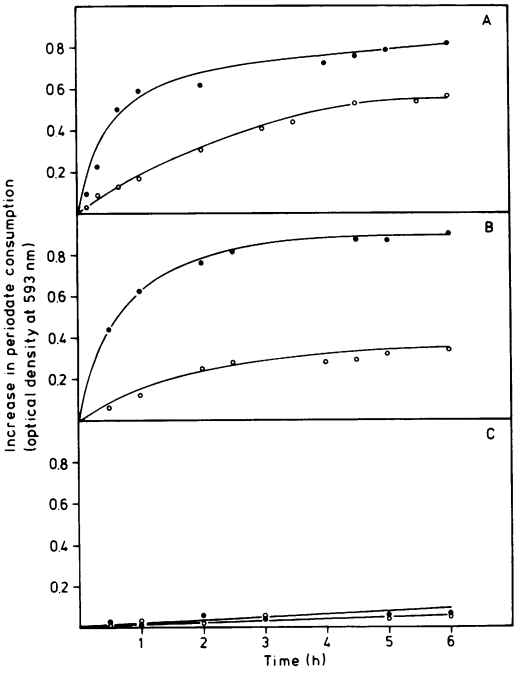

FIG. 2. Increase in periodate consumption of polysaccharide substrates after incubation with purified phage particles. Solutions of colominic acid (A), *E. coli* K92 (B), or *N. meningitidis* type C polysaccharide (C) in 0.05 M ammonium carbonate–0.1 M ammonium acetate buffer of pH 7.2 (all 15 mM with respect to NeuNAc residues) were incubated with purified particles of phage ϕ1.2 (○) or of phage ϕ92 (●) (5×10^{11} PFU per ml) at 37°C. At intervals, aliquots were withdrawn and tested for periodate consumption (6 h of exposure to the oxidant). Samples taken at time zero of phage action, polysaccharides incubated for 6 h with boiled phage (3 min at 100°C), and solutions of the polysaccharides alone served as controls and gave the same respective reference absorbances at 593 nm.

NeuNAc-(2 → 9)-]$_n$. Since the K1 and K92 polysaccharides of the respective host strains are not *O*-acetylated (9, 23), and since bacteriophage-associated glycanases may be specific for acetylated or non-acetylated substrates (31), all glycans were subjected to mild alkali treatment before use.

In a first approach, the increase in periodate consumption of the polysaccharides after incubation with purified virus particles was determined, using the general conditions previously established (17, 21, 27, 33). The consumption after 6 h of exposure to periodate was measured, because probably due to the stereochemistry of the glycol groups involved (13, 24), the oxidation of the *E. coli* K92 and *N. meningitidis* polymers proceeds very slowly and is still practically inapparent after this time, while the consumption of free NeuNAc is almost completed after 6 h.

The results obtained in this manner with the two phages and three polysaccharides are summarized in Fig. 2. It can be seen that colominic acid is depolymerized by both ϕ1.2 and ϕ92, but that the latter yields somewhat smaller oligosaccharides; that the same holds for the K92 polysaccharide with a more pronounced difference in the effect of the two phages; and that the periodate consumption of the *N. meningitidis* type C glycan does not increase in the presence of either virus.

Gel chromatography of polysaccharide degradation products. All three substrates were incubated with ϕ1.2 or ϕ92 under the same conditions as in Fig. 2, but with the reactions run for 24 h. After this time, no appreciable further depolymerization was expected. For the degradation of colominic acid by phage ϕ1.2, this follows from the results previously published (17), and for the other reactions, it was concluded from the plateau values of periodate consumption reached already after about 4 to 6 h of phage action (Fig. 2).

The products of exhaustive polysaccharide degradation were first subjected to chromatography through Bio-Gel P-30 as shown in Fig. 3. It can be seen (i) that colominic acid is completely depolymerized by both phages and that the ϕ92 products again appear to be somewhat smaller than the ϕ1.2 oligosaccharides, (ii) that the *E. coli* K92 polysaccharide is only partially degraded by ϕ1.2, whereas it is completely converted to split products by ϕ92, and (iii) that the gel chromatographic behavior of the *N. meningitidis* type C glycan is not appreciably altered by either phage. With the same approach, the *N. meningitidis* type W-135, [-α-Gal-(1 → 4)-α-NeuNAc-(2 → 6)-]$_n$, and type Y, [-α-Glc-(1 → 4)-α-NeuNAc-(2 → 6)-]$_n$, polysaccharides (where Gal is galactose and Glc is glucose) (15) were also found to be resistant to the action of both viral enzymes (data not shown).

For further characterization, the ϕ1.2 and ϕ92 degradation products of colominic acid and K92 polysaccharide were pooled as indicated in Fig. 3 and additionally separated over a column of Bio-Gel P-4 (−400 mesh), calibrated with oligosaccharides of the isomaltose series (Fig. 4). The $[(V_e - V_{BSA})/(V_{Glc} - V_{BSA})]^{1/3}$ (where V_e, V_{BSA}, and V_{Glc} are the elution volumes for the products, bovine serum albumin, and glucose, respectively) values ($\approx K_d^{1/3}$) of the product peaks were also plotted against (molecular weight)$^{0.555}$ (Laboratory Manual on Gel Chromatography, Bio-Rad Laboratories, Richmond, Calif., 1975) (Fig. 5). The straight line obtained in this manner for saccharides P1 (free NeuNAc) to P7 and P2′ to P6′ indicates that they consist of one to seven NeuNAc residues. For P2 and P3 this conclusion is confirmed by the finding (Fig.

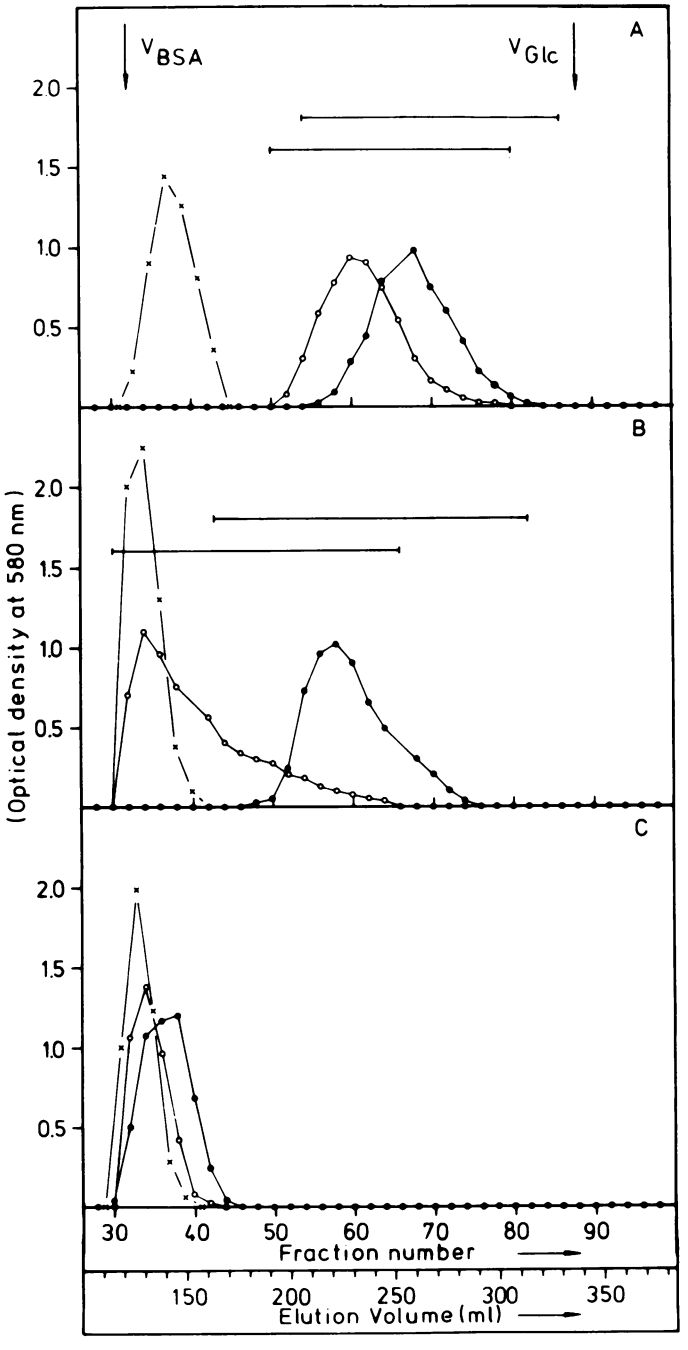

FIG. 3. Bio-Gel P-30 chromatography of polysaccharide degradation products obtained by exhaustive incubation with phage particles. Solutions of colominic acid (A), *E. coli* K92 (B), or *N. meningitidis* type C polysaccharide (C) were incubated with particles of phage φ1.2 or φ92 for 24 h (conditions as in Fig. 2). Portions (2 ml) of the resulting mixtures were chromatographed (16 ml/h) through a column (2 by 100 cm) of Bio-Gel P-30 (50 to 100 mesh). Fractions (3.8 ml) were collected and analyzed for NeuNAc with resorcinol-hydrochloric acid-copper sulfate (8, 29, 34). Symbols: ○, phage φ1.2 products; ●, phage φ92 products. Separately, the column was calibrated with the respective polysaccharides (starting materials, ×). V_{BSA} was determined with bovine serum albumin and V_{Glc} with glucose. Bars indicate fractions pooled for Bio-Gel P-4 chromatography (Fig. 4).

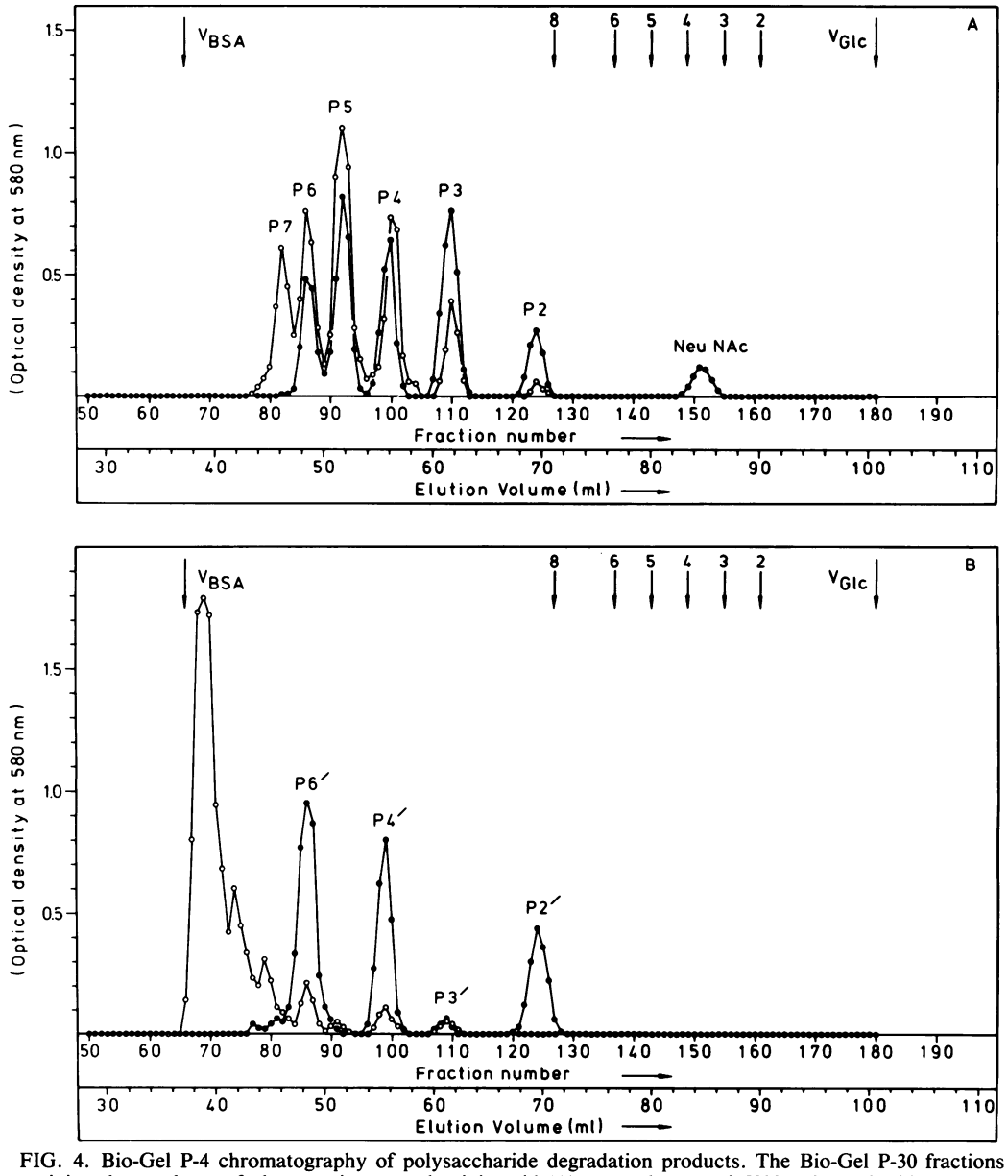

FIG. 4. Bio-Gel P-4 chromatography of polysaccharide degradation products. The Bio-Gel P-30 fractions containing the products of phage action on colominic acid (A) or on the *E. coli* K92 polysaccharide (B) (see bars in Fig. 3) were pooled, lyophilized, taken up in 0.5 ml of volatile buffer, and chromatographed (1.1 ml/h) through a column (1 by 150 cm) of Bio-Gel P-4 (−400 mesh). Fractions of 10 drops were analyzed as in Fig. 3. Symbols: ○, phage ϕ1.2 products; ●, phage ϕ92 products. The column was calibrated with oligosaccharides of the isomaltose series (arrows with numbers, two to eight glucose units). V_{BSA} and V_{Glc} are as in Fig. 3.

4) that they elute near the oligosaccharides of the isomaltose series consisting of six and nine glucose residues, respectively, while free NeuNAc elutes near isomaltotriose.

In conclusion, Fig. 3 and 4 then show the following. (i) Colominic acid is depolymerized by the ϕ1.2 enzyme to oligosaccharides probably consisting of two to seven NeuNAc residues (cf. 17), and by the ϕ92 enzyme to NeuNAc and P2 to P6. (ii) The *E. coli* K92 polysaccharide is only partially degraded by ϕ1.2 to products probably consisting of three, four, six, seven, and more residues, but is completely depolymerized by the ϕ92 neuraminidase to oligosaccharides P2′, P4′, and P6′ (as well as a small amount of P3′).

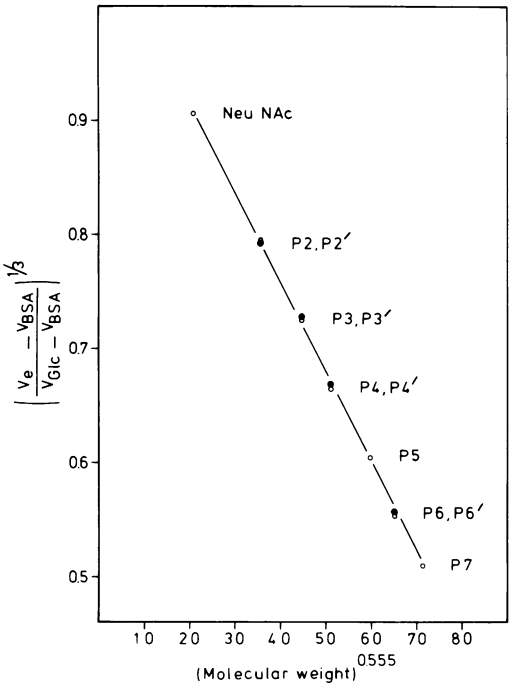

FIG. 5. Molecular weight of NeuNAc oligosaccharides. The $[(V_e - V_{BSA})/(V_{Glc} - V_{BSA})]^{1/3}$ values obtained by Bio-Gel P-4 chromatography of free NeuNAc and NeuNAc oligosaccharides P2 to P7 (○, from colominic acid) and P2′ to P6′ (●, from *E. coli* K92 polysaccharide) (see Fig. 4) were plotted against their (molecular weight)$^{0.555}$, assuming two residues for P2, etc.

DISCUSSION

The host ranges of bacteriophages ϕ1.2 (17) and ϕ92 (Table 1) are, within the set of bacterial strains tested, very similar. The only noticeable difference is that ϕ1.2 adsorbs to but does not multiply on the two strains with the K92 antigen, while ϕ92 both adsorbs to and plates on these bacteria. The electron micrographs (Fig. 1), however, show that the two viruses and also their tail appendages (spikes or curly spikes) have a different morphology. By analogy to several phage-associated host capsule depolymerases previously localized on virus particles (4, 16, 25, 26, 28; Geyer et al., in press), these organelles may be assumed to carry the enzymatic activities, and the ϕ1.2 and ϕ92 neuraminidases could thus be expected to differ also in other respects.

Like the ϕ1.2 enzyme, the ϕ92 neuraminidase apparently acts by an endo mechanism, because the yield of NeuNAc (and $NeuNAc_2$) is always smaller than that of the higher oligomers (Fig. 4).

The substrate specificity of the two viral endo-*N*-acetylneuraminidases (Fig. 2 to 4) differs in two respects. (i) Although colominic acid is completely depolymerized by both enzymes, the ϕ1.2 products are somewhat larger (P5 being the main product) than the ϕ92 products (with a highest and about equal yield of P2, P3, and P4). (ii) The *E. coli* K92 polysaccharide is completely degraded by the ϕ92 enzyme, essentially to products of 2n NeuNAc residues, while ϕ1.2 only partially depolymerizes this substrate. Such partial degradations have been observed in other polysaccharide-phage depolymerase systems, e.g., colanic acid-phage F1 (30). The reason for this reduced accessibility of enzyme-susceptible bonds is obscure but may be due to conformational parameters.

Neither phage enzyme affects the *N. meningitidis* type C glycan (Fig. 2 and 3). As may be expected, this also holds for the *N. meningitidis* types W-135 and Y glycans, which consist of galactose or glucose residues in addition to NeuNAc, and in which the latter is substituted at position 4 (15).

It is interesting that the ϕ92 enzyme depolymerizes the *E. coli* K92 polysaccharide, with alternating α(2 → 8) and α(2 → 9) bonds, essentially to $NeuNAc_2$ (P2′), $NeuNAc_4$ (P4′), and $NeuNAc_6$ (P6′) (Fig. 3 and 4). Since the same enzyme completely degrades colominic acid, with only α(2 → 8) linkages, but does not affect *N. meningitidis* type C glycan, with only α(2 → 9) bonds, this result may be taken to indicate that the P2′ fraction consists mainly of NeuNAc-(2 → 9)-NeuNAc and P4′ and P6′ of α(2 → 8)-linked dimers or trimers, respectively, of P2′. It should be realized, however, that all size determinations of the NeuNAc oligomers are based on their gel chromatographic behavior alone. Since it cannot be taken for granted that these oligosaccharides all exhibit the same conformation irrespective of their size, these molecular weight estimates must be confirmed by other methods.

In summary, it can be concluded that bacteriophage-associated endo-*N*-acetylneuraminidases may exhibit different substrate specificities. It will be interesting to see whether *N. meningitidis* phages can be found which carry yet additional enzymatic activities of this group.

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