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Endo-*N*-Acetylneuraminidase Associated with Bacteriophage Particles

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A bacteriophage (ϕ1.2) has been isolated for *Escherichia coli* K235 (O1:K1:H$^-$). ϕ1.2 is specific for the host capsular polysaccharide (colominic acid). The phage forms plaques with acapsular halos and thus carries a glycanase activity for colominic acid, a homopolymer of α(2→8)-linked *N*-acetylneuraminic acid (NeuNAc) residues. Upon incubation with purified ϕ1.2 particles, a solution of K1 polysaccharide loses viscosity and consumes increasing amounts of periodate. Also, by gel filtration, the production of colominic oligosaccharides (down to a size of two to three NeuNAc residues) can be demonstrated. No NeuNAc monomers, however, are formed. The capsules of *E. coli* strains with the K92 antigen, which consists of NeuNAc residues linked by alternating α(2→8) and α(2→9) bonds, are also depolymerized by the ϕ1.2 enzyme. Under the electron microscope, phage ϕ1.2 is seen to belong to Bradley's morphology group C (D. E. Bradley, Bacteriol. Rev. **31**:230–314, 1967); it has an isometric head, carrying a baseplate with six spikes. By analogy to other virus particles with host capsule depolymerase activity, it is probable that the ϕ1.2 endo-*N*-acetylneuraminidase activity is associated with these spikes.

Bacteriophages infecting encapsulated bacteria often carry host capsule-degrading enzymatic activities. Phage-associated glycanases active on *Klebsiella* and *Escherichia coli* exopolysaccharides have been well characterized (13, 14, 21, 24, 29, 34, 35). These enzymatic activities are generally associated with the spikes (or thick fibers) of the viruses (5, 6, 28, 30) and help them to penetrate the outermost layer of the host cell (4).

During the biosynthesis of progeny virus in these systems, an excess of free spikes (capsule depolymerase) is often formed. This leads to a characteristic plaque morphology; i.e., the plaque proper is surrounded by a large conspicuous halo in which the bacterial growth is decapsulated (transparent and O agglutinable) and which continues to extend long after the medium is exhausted (5, 6, 32). For this reason, it is easy to select bacteriophages carrying host capsule depolymerase activities amongst new virus isolates for encapsulated bacteria.

The *E. coli* capsular polysaccharide of serotype K1 (colominic acid) consists of α(2→8)-linked residues of *N*-acetylneuraminic acid (NeuNAc), which may or may not be *O*-acetylated (3, 7, 23, 27, 37). We have isolated a bacteriophage which forms plaques with halos on *E. coli* K235, the classical strain producing colominic acid (3), because such a virus could be expected to carry an *N*-acetylneuraminidase with special properties. In contrast to the many known neuraminidases of bacterial, animal, or animal virus origin (10, 11), a bacteriophage-associated neuraminidase should degrade colominic acid by an endo mechanism (13, 14, 24, 35) and should be easy to prepare (as whole virus) in comparatively large amounts (28, 30).

Bacteriophages specific for the *E. coli* K1 antigen have been isolated before (16, 25), but they were not characterized with respect to their possible neuraminidase activity.

MATERIALS AND METHODS

Media, volatile buffer, and general techniques. With the exception of the large-scale propagation of virus (see below), which was carried out in P medium (33), Merck standard I medium was used throughout. The volatile buffer contained 0.05 M ammonium carbonate and 0.1 M ammonium acetate and was adjusted to pH 7.2 with 1 M aqueous acetic acid. The general techniques described by Adams (1) and Kauffmann (18) were used for handling the bacteriophage and for the bacterial serology, respectively.

Bacteria. *E. coli* K235 (O1:K1:H$^-$; L+O), originally described by Barry and Goebel as producing colominic acid (3), was used as the host for bacteriophage ϕ1.2. This strain, as well as the others employed for the host range experiments and listed in Table 1, was kindly supplied by Frits Ørskov, World Health Organization International Escherichia Center, Statens Seruminstitut, Copenhagen, Denmark. All strains were routinely checked for K agglutination and O inagglutinability.

Bacteriophage. *E. coli* capsule bacteriophage ϕ1.2

TABLE 1. Host range of *E. coli* capsule bacteriophage φ1.2

E. coli strain	Serotype	Composition of capsule[a]	Plating efficiency[b]	Rate of adsorption[c] (ml/min)	Capsule depolymerization[d]
K235 (host)	O1:K1:H$^-$	→8) -α-NeuNAc*p*- (2→[e]	1	5.2×10^{-9}	+
F11119/41	O16:K1:H$^-$	Same as above[e]	5×10^{-4}	4×10^{-9}	+
C375	O132:K1:H$^-$	Same as above[f]	10^{-1}	2.5×10^{-9}	+
U5/41 (D698)	O1:K1:H7	Same as above[g]	1	2.3×10^{-9}	+
U9/41	O2:K1:H4	Probably the same[h]	~1	3×10^{-9}	+
H61	O45:K1:H10	Probably the same[h]	~1	2.3×10^{-9}	+
U9/41-K1$^-$	O2:K1$^-$:H4	No capsule, acapsular mutant of U9/41[i]	$<10^{-9}$	$<10^{-10}$	−
Bos12	O16:K92:H$^-$	→8)-α-NeuNAc*p*- (2 → 9) -α-NeuNAc*p*- (2→[j]	$<10^{-9}$	2.7×10^{-9}	+
6181/66	O73:K92:H34	Probably the same[k]	$<10^{-9}$	2×10^{-9}	+
Bi316/42	O9:K9:H12	Gal, HexN, NeuNAc[l]	$<10^{-9}$	$<10^{-10}$	−
Su3684/41	O56:K$^+$:H$^-$	NeuNAc[m]	$<10^{-9}$	$<10^{-10}$	−

[a] Abbreviations: Gal, galactose; HexN, unidentified hexosamine; NeuNA*cp*, *N*-acetylneuraminic acid (pyranoside).

[b] Relative to that on *E. coli* K235.

[c] In Merck standard I broth at 37°C.

[d] Depolymerization of capsule, as determined by the incubation of about 2×10^{10} PFU of purified phage on outgrown lawns of bacteria (for details, see the text).

[e] Colominic acid, not *O*-acetylated (27).

[f] Colominic acid, partially *O*-acetylated at positions 7 and 9 (27).

[g] Colominic acid, exhibiting variation between *O*-acetylated and non-*O*-acetylated forms (27).

[h] Chemically not investigated K antigen, which is serologically identical with, or closely related to, that of *E. coli* U5/41, the test strain for the K1 antigen.

[i] Acapsular mutant of U9/41, selected for formation of transparent colonies and for O16 agglutinability.

[j] See Egan et al. (12).

[k] Carrying a K antigen serologically identical to that of Bos12.

[l] See MacLennan et al. (22).

[m] Surface polysaccharides containing NeuNAc (26).

was isolated from Giessen sewage (cf. 33) with *E. coli* K235 as a prospective host, and it was selected because of its plaque morphology (5, 6, 29). The virus was purified by seven consecutive single plaque isolations, and phage stocks with about 10^9 PFU per ml were obtained by inoculation of liquid host cultures from single plaques.

Bacterial antisera. A *Neisseria meningitidis* group B antiserum, purchased from the Institut Pasteur, Paris, France, served as an *E. coli* K1 antiserum (27), and a K9 antiserum was obtained by immunization of a rabbit with Formalin-treated *E. coli* Bi316/42. An *E. coli* K92 antiserum was not available.

An *E. coli* O1aO1b antiserum was purchased from Difco Laboratories, Detroit, Mich. For O2 and O9 antisera, rabbits were injected with boiled cultures of *E. coli* U9/41 and Bi316/42, respectively. O16, O45, O56, O73, and O132 antisera, analogously prepared against the respective serological test strains of *E. coli*, were kindly supplied by V. Schäfer, Hygiene-Institut der Universität, Frankfurt, Federal Republic of Germany (FRG).

Colominic acid and NeuNAc. Colominic acid (extracted from *E. coli* O16:K1 HNM) was purchased from Sigma Chemical Co., St. Louis, Mo. Before use, 300-mg samples were dissolved in 120 ml of ice-cold 0.1 M aqueous NaOH and stored for 45 to 60 min in the cold (9). They were then neutralized with cold 2 M aqueous HCl, dialyzed against water, and lyophilized. Portions (100 mg each) of the product were passed over a Bio-Gel P-30 column (see below), using the volatile buffer. The fractions appearing in the void volume were collected and lyophilized. NeuNAc was obtained from Serva, Heidelberg, FRG.

Large-scale propagation and purification of phage particles. One-liter batches of P medium were inoculated with *E. coli* K235, and the bacteria were grown at 37°C under strong aeration to an optical density of 1.5 at 660 nm, keeping a constant pH of 7.2. At this stage, the culture contained about 7×10^8 colony-forming organisms per ml and was inoculated with phage to a multiplicity of infection of 0.01. About 30 min later, lysis occurred. After the addition of some silicon antifoam, the incubation was continued until the optical density rose again (another 30 to 90 min). Bacterial debris and secondary growth were then sedimented by centrifugation (20 min at 5,000 × *g*). The supernatant generally had a titer around 3×10^{10} PFU per ml.

For precipitation of the viruses with polyethylene glycol (38), solid NaCl (to 0.5 M) and polyethylene glycol 6,000 (Fluka, Buchs, Switzerland; to 2.5%, wt/vol) were dissolved in the clarified lysate, and the

mixture was stored at 4°C overnight. After another low-speed centrifugation, more polyethylene glycol 6,000 (to a total of 9%) was added, and the mixture was stored at 4°C for at least 18 h again. A third low-speed centrifugation then sedimented most of the PFU which were recovered in a small volume by decanting the supernatant.

For isopycnic centrifugation, 1- to 3-ml portions of the concentrated phage suspensions thus obtained (about 2×10^{12} PFU per ml) were placed on linear CsCl gradients in Beckman SW41 swinging-bucket rotor tubes. The gradients, in a 0.1 M Tris-hydrochloride buffer of pH 7.5 containing 0.5% (wt/vol) NaCl and 0.1% NH_4Cl, ranged in density from 1.15 to 1.65 g/ml. After centrifugation for 90 min at 90,000 × *g*, the phage band at a density of 1.47 g/ml was withdrawn with a syringe and dialyzed against volatile buffer in the cold. About 10^{13} PFU of purified virus particles were thus obtained from 1 liter of lysate. If stored at 4°C over chloroform, the suspensions kept both their plaque-forming and their deploymerase activity (see below) for at least 1 month.

Determination of capsule depolymerization. Broth agar plates were seeded with the different strains of *E. coli* listed in Table 1 and incubated overnight. One drop of a suspension of purified virus (4.5×10^{11} PFU per ml of broth) was then placed on the outgrown lawns of bacteria, and the plates were incubated at 37°C overnight again. They were finally inspected for capsule depolymerization (6), i.e., for formation of transparent and O-agglutinable spots.

Incubation of colominic acid with phage particles. A 7-ml portion of volatile buffer, containing 4.5 mg of colominic acid (about 15 mM with respect to NeuNAc residues) and 7.5×10^{11} PFU of purified φ1.2 particles per ml, was incubated at 37°C, and the viscosity was followed by running the reaction in a Cannon-Fenske viscosimeter with a capillary with a 0.30-mm inside diameter (Schott, Hofheim, FRG) and measuring the efflux time at intervals. For the determination of the increase in periodate consumption, 0.4-ml samples were withdrawn after 20, 40, 60, and 90 min and after 2, 3, 5, 8, and 24 h, heated in a boiling water bath for 3 min, cooled, lyophilized, and stored in vacuo over P_2O_5 and KOH. The samples were taken up in 1.2 ml of a 1 M sodium acetate buffer of pH 4.0, and 1.2 ml of 3 mM sodium metaperiodate in the same buffer was added. The mixtures were stored at room temperature in the dark, and after 6 and 24 h, 0.1-ml portions were analyzed for residual periodate with the ferrous complex of 2,4,6-tri-2-pyridyl-*s*-triazine, as detailed by Avigad (2). A sample taken at zero time, colominic acid incubated with boiled phage for 6 h, and a solution of colominic acid alone served as controls and consumed, within experimental error, the same minor amount of periodate (reference absorbance at 593 nm). The periodate consumption after 6 and 24 h in the dark was generally the same.

Gel chromatography of colominic oligosaccharides. Colominic acid (4.5 mg/ml) and particles of phage φ1.2 (4.5×10^{11} PFU per ml) were incubated at 37°C for 24 h, as described above, in the presence of some chloroform to ensure sterility. After evaporation of the chloroform, a 1-ml sample was placed on a column (2 by 100 cm) of Bio-Gel P-30 (50 to 100 mesh; Bio-Rad Laboratories, Richmond, Calif.) and eluted at 16 ml/h. Fractions (3.75 ml each) were collected, and 1-ml portions were tested with resorcinol-hydrochloric acid-copper sulfate (9, 36).

The fractions containing the colominic oligosaccharides (see Fig. 2a; from 2.4 mg of polymer) were pooled, lyophilized, and further separated at 1.1 ml/h through a column (1 by 170 cm) of Bio-Gel P-4 (−400 mesh). Fractions of 20 drops were collected and analyzed as described above.

All gel chromatography was carried out in the cold. Both columns were first tested with bovine serum albumin (see Fig. 2, V_0), glucose (see Fig. 2, V_e), colominic acid, and NeuNAc. In addition, the P-4 column was calibrated with oligosaccharides of the isomaltose series (two to eight glucose residues), kindly supplied by K. Himmelspach, Max Planck-Institut für Immunbiologie, Freiburg, FRG.

Electron microscopy. Small volumes of virus suspensions in volatile buffer (1.5×10^{12} PFU per ml) were placed on specimen grids coated with carbon films, negatively stained with 2% aqueous uranyl acetate, and observed with a Zeiss (Oberkochen, FRG) EM-10CR instrument (equipped with ion getter pumps).

For disruption, φ1.2 particles (7×10^{11} PFU per ml of M-9 buffer) were treated (6 h at 37°C) with 75 mM EDTA, and then with DNase, as previously detailed for Vi bacteriophage III (19); under these conditions, φ1.2 lost over 99% of its infectivity. The disrupted virions were dialyzed against volatile buffer and stained and visualized as described above.

RESULTS

Isolation and host range of *Escherichia coli* capsule bacteriophage φ1.2 and substrate specificity of the virus-associated capsule depolymerase. With *E. coli* K235 (O1:K1:H$^-$), the strain originally described to produce colominic acid (3), as a prospective host, bacteriophage φ1.2 was isolated from sewage. The virus was selected because of its plaque morphology, i.e., the formation of acapsular (transparent and O-agglutinable) halos around the plaque proper (5, 6, 32).

As seen in Table 1, φ1.2 adsorbs with comparable rates to the six *E. coli* K1 strains tested, irrespective of their O serotype and of the differences in *O*-acetylation of their colominic acid capsules (27). All of these K1 antigens are substrates for the φ1.2-associated capsule depolymerase, as evidenced by the production of haloed plaques on all six strains and by the formation of transparent and O-agglutinable spots after incubation of outgrown bacterial lawns with suspensions of purified virus (Table 1, last column). Like the other *E. coli* capsule (K) bacteriophages previously described (33), φ1.2 does not adsorb to acapsular mutants of these strains (e.g., to *E. coli* U9/41-K1$^-$), and, vice versa, φ1.2-resistant host mutants are generally O agglutinable (data not shown).

Table 1 also gives evidence that φ1.2 additionally adsorbs to and depolymerizes *E. coli* K92 capsules. In the K92 systems, however, adsorp-

tion is reversible (data not shown), and no virus multiplication occurs. Two further strains, the surfaces of which also contain NeuNAc, were found to be adsorption resistant and depolymerization resistant.

Incubation of colominic acid with particles of phage ϕ1.2. The virus-associated depolymerase was further characterized by incubation of substrate with isolated virions and measurement of the ensuing decrease in viscosity and increase in periodate consumption (Fig. 1). For this purpose, commercial colominic acid was first subjected to mild alkali treatment (9, 17, 31) to saponify putative *O*-acetyl (27) and other ester linkages (15, 20) and then was separated from low-molecular-weight constituents by Bio-Gel P-30 chromatography. The ϕ1.2 particles were purified by consecutive polyethylene glycol precipitation and isopycnic centrifugation (38). The reaction conditions (pH, temperature, concentrations of polysaccharide and phage) were chosen by analogy to those previously found optimal for several other bacteriophage-catalyzed glycan depolymerizations (13, 14, 24, 29, 35).

As seen in Fig. 1, a solution of colominic acid rapidly loses viscosity and consumes increasing amounts of periodate when incubated with ϕ1.2 particles at pH 7.2 and 37°C. Under the conditions chosen, the reaction is complete after 24 h.

Separation of colominic oligosaccharides obtained by phage ϕ1.2-catalyzed depolymerization. The oligosaccharides obtained by exhaustive incubation of saponified, high-molecular-weight colominic acid with ϕ1.2 particles (conditions as in Fig. 1, 24 h of incubation) were separated by Bio-Gel filtration (Fig. 2).

Figure 2a shows that exhaustive phage degradation leaves no starting material and that it produces colominic oligosaccharides, but no monomeric NeuNAc. From Fig. 2b, it is apparent that the colominic oligosaccharides can be separated into six fractions by high-resolution Bio-Gel P-4 chromatography; since standard monomeric NeuNAc and the two smallest colominic oligosaccharide species (P2 and P3) are eluted near oligosaccharides of the isomaltose series which are composed of three, six, and nine glucose units, it appears likely that P2 and P3 consist of two and three NeuNAc residues.

Electron microscopy of bacteriophage ϕ1.2. Intact ϕ1.2 particles, as well as disrupted virions, were negatively stained and visualized in the electron microscope (Fig. 3). For disruption, ϕ1.2 was treated with EDTA, a procedure previously found effective with Vi bacteriophage III (19). Like this virus, ϕ1.2 loses infectivity rapidly in the presence of 75 mM EDTA, and virus fragments, notably empty capsids (Fig. 3b) and a few detached baseplates (Fig. 3c), can be seen in such preparations.

From Fig. 3 it is evident that ϕ1.2 is an isometric virus belonging to Bradley's morphology group C (8), carrying a baseplate with six spikes. It is not impossible that this baseplate is arranged around a central cone-shaped hollow tail (see, for instance, the empty capsid in the upper left corner of Fig. 3c), but this detail cannot be clearly distinguished. The ϕ1.2 head

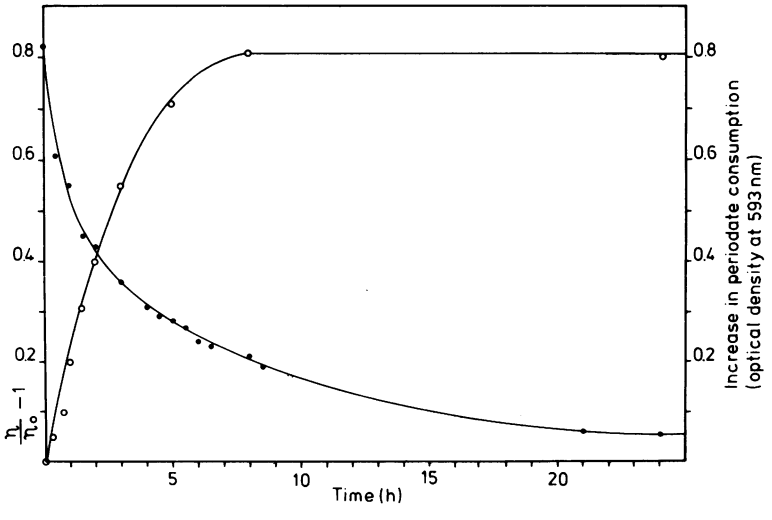

FIG. 1. Depolymerization of colominic acid by particles of *E. coli* capsule bacteriophage ϕ1.2. A solution of colominic acid (4.5 mg/ml) in ammonium acetate-ammonium carbonate buffer of pH 7.2 was incubated at 37°C with purified phage particles (7.5×10^{11} PFU per ml). The loss of viscosity ($\eta/\eta_0 - 1$;●) and increase in periodate consumption (2) (optical density at 593 nm; ○) were determined at different time intervals.

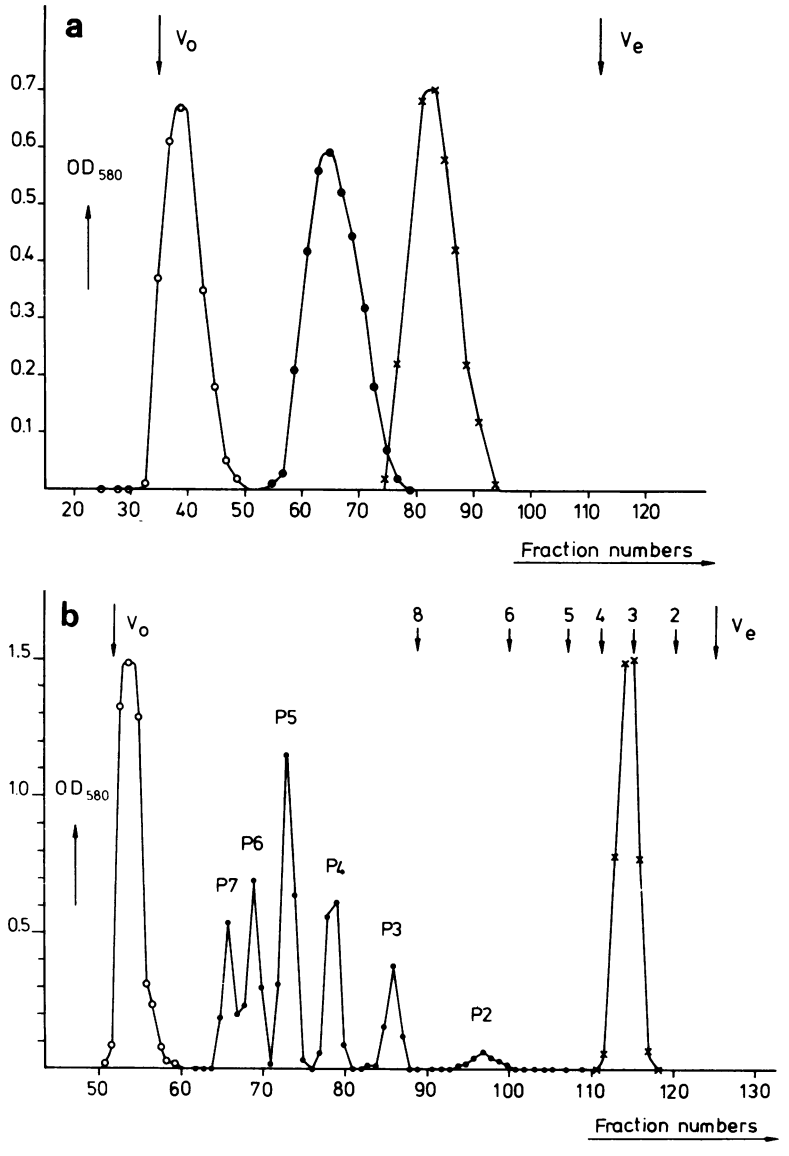

FIG. 2. Gel chromatography of colominic oligosaccharides. (a) Preparative chromatography through Bio-Gel P-30 (50 to 100 mesh). Colominic acid was incubated for 24 h with φ1.2 particles under the same conditions as in Fig. 1; the resulting mixture was directly chromatographed. Separately, colominic acid (starting material) and standard NeuNAc (monomer) were run on the same column. (b) Analytical chromatography through Bio-Gel P-4 (−400 mesh). The mixture of colominic oligosaccharides, as obtained by Bio-Gel P-30 chromatography, was rechromatographed. Again, colominic acid and NeuNAc were separatedly applied. Symbols: ●, φ1.2 degradation products; ○, colominic acid; ×, NeuNAc; as detected with resorcinol-hydrochloric acid-copper sulfate (31, 36) (optical density at 580 nm). V_0 and V_e, elution volumes of bovine serum albumin and glucose, respectively; P2 to P7, colominic oligosaccharide fractions; arrows with numbers, elution volumes of oligosaccharides of the isomaltose series (two to eight glucose units).

has a diameter of about 63 nm, and the drop-shaped spikes (cf. 6) have a length of about 12 nm and a maximal thickness of about 8 nm.

DISCUSSION

As explained above, the bacteriophage described in this study is similar to a large number of bacterial viruses previously investigated, all of which form plaques with acapsular halos on encapsulated *Enterobacteriaceae* and have host capsule depolymerase activities associated with their tail spikes. With this in mind, the results presented justify the following conclusions.

Bacteriophage φ1.2 is specific for the *E. coli*

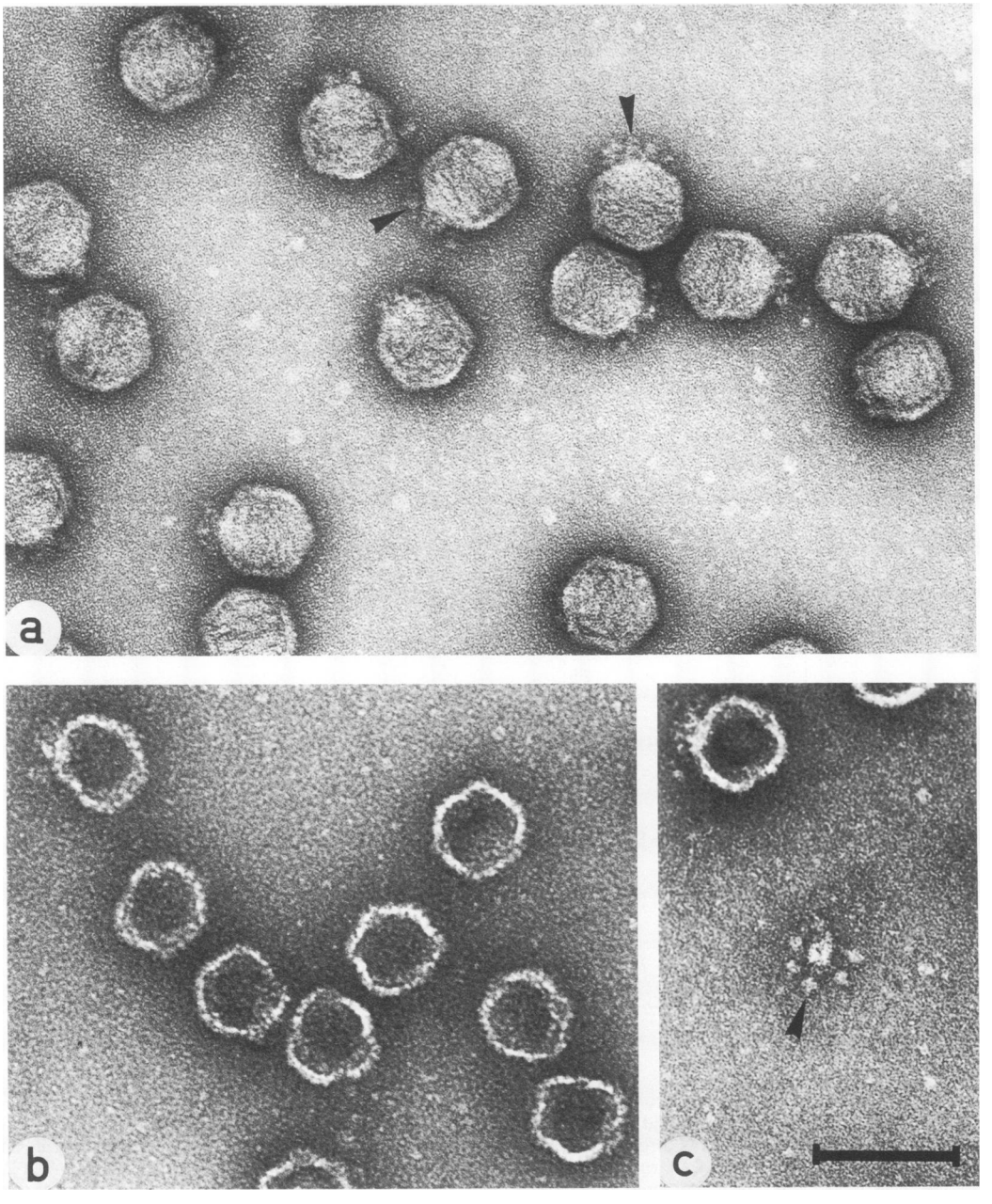

FIG. 3. Electron microscopy of *E. coli* capsule bacteriophage φ1.2. (a) Virus particles; (b) virus particles disrupted by treatment with EDTA and digested with DNase (19); (c) detached baseplate, as occasionally seen in samples of disrupted virus. All specimens were negatively stained with uranyl acetate. ×200,000. Marker bar, 100 nm. Arrows point to virus spikes.

capsular polysaccharide of serotype K1 (colominic acid) in that it adsorbs to and plates on several strains with this K antigen, but not on acapsular (O-agglutinable) mutants thereof and not on two other strains of a different serotype, the surfaces of which also contain NeuNAc. φ1.2 does, however, also adsorb to *E. coli* cells with the K92 antigens, although it does not multiply on the two K92 strains tested (Table 1).

There is a host capsule depolymerase activity associated with the particles of φ1.2, since the phage forms plaques with acapsular halos (5, 6,

29) and purified virions cause a solution of colominic acid to lose viscosity and to consume increasing amounts of periodate (Fig. 1; cf. 13, 14, 24, 29, 35). This depolymerization cannot be due to autohydrolysis, as it occurs at pH 7.2 and is not observed upon incubation with denatured virus or upon incubation of colominic acid alone (see above).

The φ1.2-associated depolymerase is an endo-*N*-acetylneuraminidase since, even after exhaustive digestion, only colominic oligosaccharides (and no NeuNAc) are formed (Fig. 2a).

A series of oligosaccharides of different sizes is produced by φ1.2 degradation of colominic acid. The smallest of these products, P2 and P3, probably consist of two and three NeuNAc residues, respectively, as judged from their gel elution volumes relative to those of oligosaccharide standards (Fig. 2b). The reason for the incomplete degradation of the polymer is obscure. It has, however, also been observed with other phage-associated glycanases (13, 14, 24, 29, 35) and may be related to a decrease in reaction velocity accompanying a decrease in substrate size. It should be noted that the production of relatively small amounts of P2 and P3 also excludes a cleavage of the polysaccharide by an exo mechanism involving removal of two or three NeuNAc residues at a time.

Like the majority of bacterial viruses with host capsule depolymerase activities, phage φ1.2 belongs to Bradley's morphology group C (8) and carries a baseplate with spikes (Fig. 3; cf 5, 6, 19, 28–30). By analogy to the viruses previously studied, it is safe to assume that the φ1.2 endo-*N*-acetylneuraminidase is associated with these spikes and that the halos around the plaques of this phage are also caused by free enzyme (free spikes).

Besides unsubstituted colominic acid, the φ1.2 enzyme also depolymerizes *O*-acetylated derivatives of its substrate, as well as the *E. coli* K92 polysaccharide (Table 1, last column). Experiments are underway to determine the size of the products from these alternative substrates and to establish the relative susceptibility of the α(2→8) and α(2→9) linkages in K92 (12).

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