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Escherichia coli Capsule Bacteriophages

III. Fragments of Bacteriophage 29

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A glycanase activity, catalyzing the depolymerization of host capsular polysaccharide, is associated with *Escherichia coli* capsule bacteriophage no. 29, a small virus with an isometric head, carrying a base plate with a set of spikes. The bacteriophage particles were disrupted by mild acid treatment (5 to 8 min at pH 3.5 and 37 C), and the enzymatically active fragments were isolated and subjected to sodium dodecyl sulfate-gel electrophoresis as well as to electron microscopy. Of the at least nine different polypeptide chains found in the complete virion, three (of 57,000 ± 3,000, 29,500 ± 2,000, and 13,500 ± 1,000 daltons) were detected in detached base plates. They had the appearance of six-pointed stars of about 14 nm in outer diameter, with a central hole or prop, carrying six (or, possibly, a multiple thereof) spikes. Two sizes of polypeptide chains (57,000 and 29,500) were found in pure spikes, cylindrical particles of about 14.5 to 15 nm in length and 5 nm in diameter, and one (57,000) in — still capsule depolymerizing — spike subunits of roughly 5 nm in diameter. Phage 29 spike preparations, homogeneous in analytical ultracentrifugation and immunoelectrophoresis, were found to have a molecular weight of 245,000, as determined from the sedimentation equilibrium, and to contain equimolar amounts of the two polypeptides, probably three copies of each per organelle. The amino acid analysis of the isolated spikes revealed that aspartic acid, alanine, serine, and glycine are their dominant constituents; no amino sugars or other carbohydrates were detected in the preparations.

Escherichia coli capsule bacteriophages (20–24) are specific for the thick (about 400 nm, as seen in India ink preparation) heteropolysaccharide capsules of their host organisms, the chemical structure of which has been elucidated in some detail in a number of cases (24). The phages adsorb to neither acapsular host mutants nor, with the exception of a few specific cross-reactions, to related strains with capsules of different serotype and chemical structure. Morphologically, most of these viruses belong to Bradley group C (3) and carry a set of spikes, linked (probably always through a base plate) to an isometric head. Obviously, the spikes enable these bacteriophages to recognize the appropriate host polysaccharide and to carry out at least the very first phase of virus-host interaction. In many (possibly in all) *E. coli* capsule phage systems, this first phase includes a degradation of the host capsular glycan by the action of "bacteriophage-borne" enzymes. The cleavage of glycosidic (by glycanases) and of *O*-acetyl linkages (by esterases) have hitherto been recognized.

In pursuit of a more detailed investigation of the molecular basis of virus-host surface interaction in these model systems, we have isolated enzymatically active fragments of *E. coli* capsule bacteriophage 29 after disruption of the virus particles by mild denaturation. The aim was to substantiate the morphological localization of the capsule-degrading activity, to develop an isolation procedure for the enzymatically active phage organelles, and to gain some preliminary insight into their molecular weight, polypeptide chain, and amino acid composition.

Phage 29 was selected for the following reasons. (i) It is a small virus of the simple and common structure described above (24). (ii) The acidic capsular polysaccharide of its host, *E. coli* Bi161/42 (O9:K29[A]:H^-, the serological test strain for the *E. coli* K29 antigen) has been analyzed (14). (More recent evidence [F. Fehmel, Dr.rer.nat. thesis, University of Freiburg, Germany, 1972], however, indicates that the structure proposed by L. B. Nhan et al. [14] for the *E. coli* K29 capsular polysaccharide is partially erroneous and has to be reinvestigated.)

(iii) A capsule-depolymerizing activity has been found to be associated with the purified virus particles (22, 23). (Until it has been established if the enzyme is a hydrolase or a lyase, it is denoted by the less accurate terms "[host] capsule depolymerase" or "glycanase.") (iv) Phage 29 forms plaques surrounded by halos that are due to a phage-induced capsule depolymerase ("free depolymerase 29"), probably consisting of free phage spikes. Preliminary evidence has shown that the depolymerase particles have about the same dimensions as have these organelles in situ, and that they also catalyze the cleavage of glucosidic bonds in the capsular glycan (23).

In an accompanying paper by W. Bessler et al. (1), the isolation of free depolymerase 29 and its comparison with phage 29 spikes is described in detail.

MATERIALS AND METHODS

Many of the techniques, especially the electron optical equipment and methods, as well as all the media, have been described previously (24).

Bacteriophage and host bacteria. *E. coli* capsule bacteriophage no. 29 and its host, *E. coli* Bi161/42 (O9:K29[A]:H$^-$, the serological test strain for the *E. coli* K29 antigen) (24) were used exclusively.

Propagation and purification of phage particles. An Eschweiler (Kiel, Germany) "Kleinfermenter Kiel" was employed for the preparation of phage 29 lysates: 14 liters of P medium was inoculated with *E. coli* Bi161/42 and grown at 37 C under strong aeration (7 liters/min) and with stirring (400 to 500 rpm) to an optical density (OD) of 0.5 at 660 nm (1-cm cell), keeping a constant pH of 7.2. The culture then contained about 2×10^8 colony-forming organisms per ml and was inoculated with 2 to 5 PFU per bacterium. About 20 min later lysis occurred. The incubation was continued until about 1.5 h after infection, and bacterial debris and secondary growth were removed by centrifugation (20 min) at $5,000 \times g$. The clarified lysates obtained generally contained 2×10^{10} to 5×10^{10} PFU and 40 to 60 host capsule depolymerase (enzymes, glycanase) units (DU) per ml. From the lysates, the phage particles were concentrated by polyethylene glycol precipitation (31), or, alternatively, by negative pressure dialysis, and then purified by repeated isopycnic centrifugation. Thus, 0.5 M NaCl and 10% polyethylene glycol 6,000 (wt/vol) (Fluka no. 8/260) were added to the clarified lysates and the mixture was stored at 4 C for 48 h or more. Continuous flow centrifugation at 17,000 to $20,000 \times g$ and 12 to 20 liters/h then sedimented about 95% of the plaque-forming activity. Portions (1 volume) of the sediment, taken up in phosphate-buffered saline (PBS) to give titers of about 10^{13} PFU/ml, or, alternatively, of concentrated lysate obtained by negative pressure dialysis and recentrifugation (10 min at $5,000 \times g$), were placed on linear CsCl gradients (3 volumes) in Spinco SW25.2 or SW27 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 from $\rho \cong 1.15$ to 1.65 g/ml. After centrifugation for 2 h at $50,000 \times g$, the phage bands at $\rho \cong 1.48$ g/ml were withdrawn with a syringe and dialyzed against PBS or another suitable buffer (see below). Most (70 to 90%) of the PFU in the crude lysate were generally recovered. In most cases, the isopycnic centrifugation was repeated once. If stored at 4 C over chloroform and with 0.05% (wt/vol) sodium azide added, the suspensions kept both their plaque-forming and their depolymerase activity at least for several months.

Determination of capsule depolymerase activity. The capsule depolymerase activity of whole phages or phage fragments was determined by incubating (3 h at 37 C) 0.02-ml portions of serial 1:2 sample dilutions in broth on a lawn of heat-killed (90 min at 60 C) host bacteria ("spot test") as described previously (23), or, much more accurately, from the liberation of reducing power. For the latter purpose, K29 polysaccharide was isolated from *E. coli* Bi161/42 by the phenol-water-cetyltrimethylammonium bromide procedure (14), and 200 μg of the glycan in 90 μl of PBS was incubated (60 min at 37 C) with 10 μl of a suitable test sample dilution (also in PBS). The reducing sugars liberated were then determined by the method of Park and Johnson (15) with glucose as a standard. Under these conditions, the reducing equivalents liberated were found to be proportional to the amount of depolymerase up to about 0.3 DU (in 10 μl).

On the basis of this estimation procedure, the DU was redefined (compare reference 23) as that amount which, under the above conditions and within 1 min, liberates the equivalent of 1 nmol of glucose in reducing power. The amount of enzyme (in 0.02 ml of broth) just sufficient to cause visible decapsulation and *O*-agglutinability in the (inaccurate) spot test corresponded to roughly 0.05 DU, and 1 DU was found to be associated with 7.5×10^9 PFU of purified phage 29 particles.

Techniques of phage fragmentation. (i) Dimethyl sulfoxide. In this method (compare reference 7), under cooling with ice, dimethyl sulfoxide was slowly added to suspensions of purified phage 29 particles in PBS (for instance, 1.5×10^{12} PFU; i.e., 200 DU per ml) until concentrations between 25 and 75% (vol/vol) were reached. The mixtures were kept at 0 C and sequential samples (taken after 3, 30, and 300 min) were slowly diluted with ice-cold broth and assayed for residual plaque-forming and depolymerase activity.

(ii) Acid. In this method (compare references 4 and 27), phage suspensions in 0.2 M aqueous glycine (adjusted to pH 7 with HCl) were brought to 37 C, and 0.01 or 0.2 N HCl, respectively, was then added until pH 5, 4.5, 4, 3.5, 3, 2.5, or 2 was reached. Incubation was continued, and sequential samples were neutralized with 0.04 or 0.4 aqueous Tris and assayed as above.

(iii) Alkali and/or urea. For treatment with alkali and/or urea (compare references 8 and 27), 1 N

NaOH was added to phage suspensions in the pH 7 glycine-hydrochloride buffer at 40 C, until pH 9.5 or 11 was reached. Solid urea, to concentrations of 0, 2, 4, or 8 M, was then dissolved in samples of the neutral and alkaline virus suspensions. Incubation was continued, and sequential samples, neutralized with 1 N HCl where necessary, were assayed.

For electron microscopy, 0.003 M Mg^{2+} and pancreatic DNase I (10 to 20 μg/ml) were added to the neutralized (or dialyzed in the case of dimethyl sulfoxide treatment) samples, and the mixtures were incubated at 37 C for 2 to 4 h. They were then dialyzed against 1% (wt/vol) ammonium acetate of pH 7.0.

Isolation of phage 29 fragments. Generally, batches of about 2×10^{14} PFU of purified virus particles in 0.2 M glycine-hydrochloride of pH 7.0 (50 ml) were processed as follows.

(i) Disruption. After prewarming at 37 C, 0.2 M HCl was added dropwise under gentle stirring until pH 3.5 was reached. Stirring and warming were continued for 5 to 8 min, and the mixture was then neutralized with 0.4 M aqueous Tris. DNase I (20 μg/ml) and 0.003 M Mg^{2+} were added and the DNA was digested by incubation at 37 C for 2 to 3 h. The solution obtained was concentrated to about 2.5 ml by negative pressure dialysis.

(ii) Zonal centrifugation. Portions (200 to 250 μl) of the mixture of phage fragments were placed on 10 to 30% (wt/vol) linear sucrose gradients (18 ml in 0.1 M Tris-hydrochloride of pH 7.0) in Spinco SW27 swinging-bucket rotor tubes. After centrifugation for 13 h at 26,000 rpm (94,000 × *g*), 500-μl fractions were withdrawn from the top of the gradients, and their depolymerase activity and absorption at 280 nm was determined. The slower-sedimenting, enzymatically active fractions (generally no. 4 to 20; compare Fig. 3) were pooled, concentrated by negative pressure dialysis, and dialyzed against equilibration buffer (see below); the final volume was 3 to 5 ml. To increase yields, the faster-sedimenting heads (fractions >30, after dialysis against PBS and concentration) were again subjected to zonal centrifugation.

(iii) Ion exchange chromatography. The solution obtained was applied to a DEAE Sephadex A25 column (length, 8 cm; area, 1.1 cm^2), equilibrated with 0.02 M Tris-hydrochloride of pH 7.0. After thorough washing with the same buffer, the enzymatically active phage fragments were eluted by adding 0.6 M Tris-hydrochloride buffer of the same pH with a linear gradient mixing device. The fractions (2.5 ml) containing spikes or spike subunits (see Fig. 4) were pooled and concentrated to about 0.02% (wt/vol) protein, by using an Amicon model 8MC Diaflo ultrafiltration set equipped with a P30 membrane.

For electron microscopy, fractions were generally dialyzed against 1% ammonium acetate.

Sodium dodecyl sulfate-polyacrylamide (SDS-PAA) gel electrophoresis. One volume (e.g., 15 μl) of "desintegration mixture" (4% [wt/vol] SDS, 30% sucrose, 2.5% dithiothreitol, 0.01 M Tris, and 0.001 EDTA in water; pH 8) was added to two volumes (e.g., 30 μl) of phage suspensions (about 6×10^{12} PFU/ml; i.e., 0.15% protein), or to solutions of enzymatically active virus fragments (around 0.03% protein) in "electrophoresis buffer" (9). The mixtures (final concentrations: 2% SDS, and 0.8% dithiothreitol) were heated for 1 min in a boiling water bath. Following the detailed description given by G. Fairbanks et al. (9, 13) the electrophoretic molecular sieving was carried out in SDS-containing gels (total acrylamide concentration 5.81%, cross-linked with 3.6% *N,N'*-methylene bisacrylamide), using Pyronin Y as a tracking dye and Coomassie blue R250 for staining. Phosphorylase a from rabbit muscle (94,000 daltons, Fluka), lipid-free bovine serum albumin (68,000 daltons, Behring), ovalbumin (43,000 daltons, Fluka), chymotrypsinogen A from bovine pancreas (25,000 daltons, Boehringer), and myoglobin from horse heart (17,500 daltons, Fluka) were used for calibration (29, 30). The gels were scanned at 620 nm in a model 240 Gilford spectrophotometer with a model 2410-5 synchronous scanner.

Immunoelectrophoresis. The immunoelectrophoretic homogeneity controls of whole-phage and detached-spike preparations were carried out as follows. Crude phage 29 lysate was concentrated 1:1,000 by negative pressure dialysis and emulsified with an equal volume of complete Freund adjuvant (Difco). Four rabbits were injected with 1.5 ml of the emulsion, 0.1 ml into each of the foot pads and the rest subcutaneously into the legs. Six weeks later, the same dose was applied again, and the animals were bled by cardiac puncture after another three weeks. The sera obtained were analyzed for the depolymerase neutralization titer (compare reference 26), by incubating (1 h at 37 C) a 1:2 dilution series in broth with equal volumes of 10 DU (crude spikes) per ml, and then by testing for residual depolymerase activity in the spot test. The serum, the highest dilution of which (1:32,000) was found barely sufficient to neutralize the depolymerase, was selected for further use. The immunoelectrophoreses (10) were run (60 or 90 min at 6 V/cm) in a Gelman instrument, using gels of 1% (wt/vol) agarose in a 0.02 M sodium barbiturate buffer of pH 8.6, containing 0.7 mM calcium lactate, and applying 10 to 40 μg of protein to the antigen holes. After electrophoresis, the serum troughs were filled with undiluted serum, and the precipitation arcs were developed by storage for 24 to 48 h at room temperature in a moist chamber, washed, and in most cases stained with amido black.

Analytical ultracentrifugation and estimation of physical parameters. All analytical ultracentrifugations were carried out in a Spinco model E centrifuge equipped with Schlieren as well as with ultraviolet optics and a scanner, and with the An-H-Ti rotor. The sedimentation coefficient ($s^0_{20,w}$) of detached phage 29 spikes (0.3% [wt/vol] and less, in a 0.1 M Tris-hydrochloride buffer of pH 7.0) was obtained by extrapolating $s_{c,\,solv.}$ to infinite dilution and standard conditions (18). The partial specific volume of the organelles was calculated from their amino acid composition (5), and their molecular weight was determined from the partial specific volume and the sedimentation equilibrium (32). Solutions containing

0.042% protein or less in PBS were equilibrated, and the apparent molecular weights were extrapolated to infinite dilution. The density of the solvent was determined by the method of H. Stabinger et al. (19), with the apparatus furnished by Parr (Graz, Austria). The isoelectric point of the spikes, finally, was estimated by isoelectric focusing (25), by using the LKB equipment and following the instructions of the manufacturers.

Chemical analyses of detached spikes. The dry weight of isolated phage organelles was determined as follows. A sample (about 100 μg) in PBS, and the same amount (200 μl) of solvent, were each placed on a disk of asbestos and dried at 70 C in vacuo (5 mm of Hg) over granular phosphorus pentoxide until constant weight was reached (about 2 h). The difference was weighed with a Cahn electric balance (accuracy about ± 15 μg) (B. Kickhöfen and R. Warth, manuscript in preparation). The protein content of phage or phage-fragment samples was estimated by the Folin technique with bovine serum albumin as a standard; in the case of pure spike fractions (as well as throughout Table 1), the values were multiplied with a correction factor (0.80) obtained from the dry weight determination. A Beckman model 120 B analyzer was used for the amino acid analyses after hydrolyzing purified spikes with 6 N HCl at 110 C for 24 h. Cystine-cysteine was determined as cysteic acid (17). For the roughly semiquantitative amino acid analysis of the single-spike polypeptides, a total of 350 μg of protein was separated on 20 SDS-PAA gels (see above). The Coomassie-stained bands were then cut from the gels, minced, and hydrolyzed for 24 h after addition of norleucine as an internal standard. The polyacrylic acid, which is insoluble in 6 N HCl, was filtered off, and the supernatant was analyzed as usual. The carbohydrate content of detached phage 29 organelles was determined by the indole-sulfuric acid method (12), by using glucose as a standard.

RESULTS

Disruption of phage 29 particles. The virus preparations were first checked for homogeneity. As purified by repeated isopycnic centrifugation, they appeared free of contaminants in immunoelectrophoresis and analytical ultracentrifugation, as well as under the electron microscope (see Fig. 5a).

By using experimental conditions similar to those described previously for the fragmentation of other bacteriophages (e.g., 4, 7, 8, 27), phage 29 particles were subjected to treatment with dimethyl sulfoxide, acid, or alkali and/or urea. Assuming that a large drop in plaque-forming ability, concomitant with no or a moderate drop in depolymerase activity, should signal a disruption of the viruses without serious destruction of their enzymatically active organelles, phage 29 particles were exposed to increasingly severe treatment with each of these denaturing agents, and the loss of PFU and DU followed. Some representative results are summarized in Fig. 1. It can be seen that, generally, denaturation of phage 29 caused an equally or even more rapid loss of its glycanase as compared to its plaque-forming activity. Mild acid treatment, however, constituted an exception. Storage of the viruses at pH values between about 3.5 and 4 at 37 C reciprocally caused their infectivity to drop much faster than their ability to depolymerize host capsular polysaccharide (Fig. 1A). Electron optical inspection of the phage fragments obtained by the mild acid treatment thus defined (and subsequent DNase digestion) generally showed mainly empty heads, often with some spikes still linked to them, and detached spikes (Fig. 2). Commonly, very few free base plates, with some or all spikes still attached to them, were also seen. On one occasion, however, a single phage 29 batch yielded considerably larger amounts of free base plates. Attempts to regulate the dissociation in this direction, i.e., to detach the base plates specifically and reproducibly, were unsuccessful.

Isolation, visualization, and SDS-PAA gel electrophoresis of phage 29 fragments with host capsule depolymerase activity. From the mixture of phage fragments, obtained by exposure to pH 3.5 at 37 C for 5 to 8 min, the empty heads (still carrying some spikes and a considerable portion of the total depolymerase activity; see Fig. 2 and Table 1) were essentially separated off by zonal centrifugation through a sucrose gradient (Fig. 3, Table 1). Electron optical inspection of the slowly sedimenting, enzymatically active fractions showed large amounts of free virus spikes. Besides these, however, some residual empty phage heads, some DNA fragments, and very few single base plates were generally seen. On zonal centrifugation of the one batch which had decayed differently (see above), large amounts of free base plates were additionally found in this fraction. The crude spikes were further purified by ion exchange chromatography (Fig. 4 and Table 1). The residual empty heads and some morphologically undefined and enzymatically inactive particles did not adsorb to the column under the conditions used; they were not further investigated. On application of a linear molarity gradient, two fractions with depolymerase activity were eluted. Electron optical visualization of these fractions (Fig. 5) showed that the main peak generally contained free spikes only, and very few, if any, single base plates. In the case of the one phage batch which had decayed

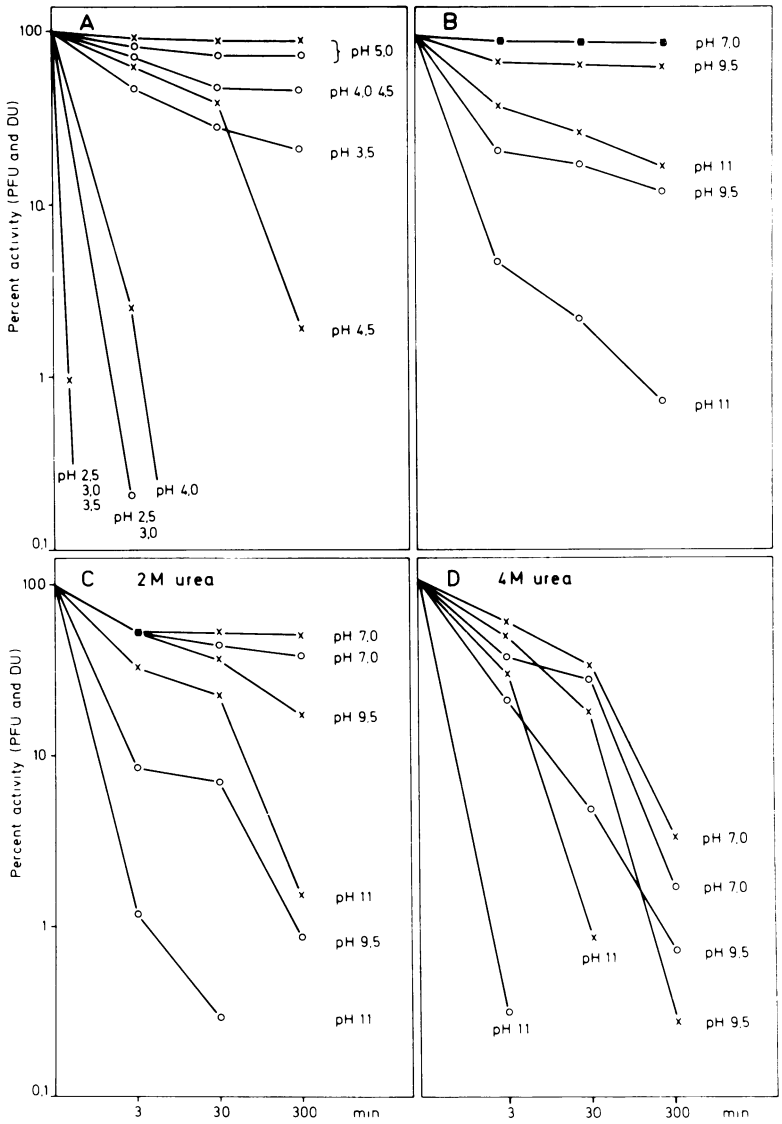

FIG. 1. *Loss of plaque-forming* (×) *and of host capsule depolymerase* (○) *activity of phage 29 particles under the influence of different denaturing conditions. Panels: A, acid at 37 C; B, alkali at 40 C; C, 2 M urea at different pH values and 40 C; D, 4 M urea at different pH values and 40 C.*

in a different manner (see above), considerable amounts of base plates were found in this fraction. The spikes were seen to be roughly cylindrical particles of around 14.5 to 15 nm in length and 5 nm in diameter. The base plates had the shape of six-pointed stars (about 14 nm in outer diameter) with a central hole or prop, carrying six (or, perhaps a multiple thereof) spikes, possibly linked to each of the six points. Some minute particles with a diameter of roughly 5 nm and occasionally showing a central point were seen in the very small second ion exchange fraction with depolymerase activity. After disintegration by heating for 1 min to 100 C in a buffer containing 2% SDS and 0.8% dithiothreitol, whole-phage 29 particles and the different enzymatically active virus fragments were subjected to SDS-PAA gel electrophoresis. The results are summarized in Fig. 6 and Table 2. It can be seen that of the (at least) nine

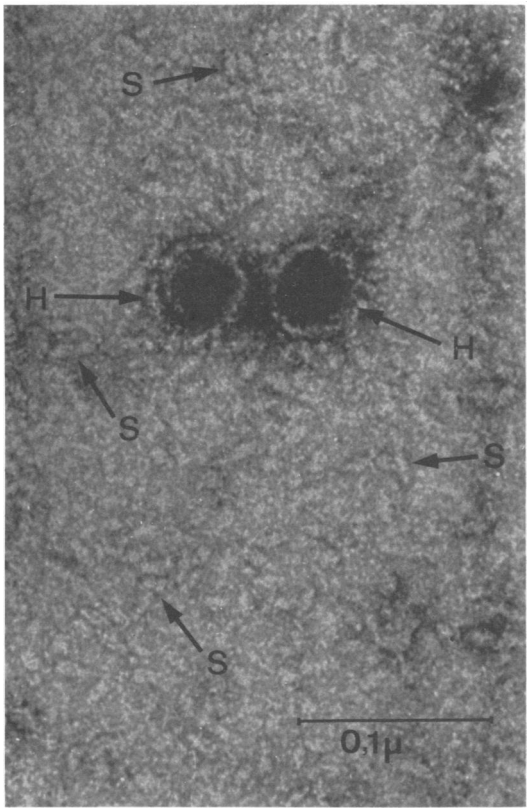

FIG. 2. *Electron micrograph of disrupted phage 29 particles. Purified virions were exposed to pH 3.5 at 37 C for 5 to 8 min, and the fragments obtained were digested with DNase and dialyzed. The micrograph shows particles negatively stained with uranyl acetate and formate, ×250,000. The arrows point to empty virus heads (H) with some spikes still linked to them, and to detached spikes (S).*

different polypeptide chains present in whole virions, three (namely, P.5, P.7, and P.9, of about 57,000, 29,500, and 13,500 daltons, respectively) were found in the base plate-rich fraction obtained from the one batch that had decayed in an unusual way, two (namely, P. 5 and P.7) in the pure spike preparations generally obtained, and one (namely, P.5) in the enzymatically active minute particles in the second ion exchange fraction.

Homogeneity, physical parameters, and chemical analyses of purified phage 29 spikes. With the exception of the one batch that had decayed in a different manner (see above), no or very few base plates were generally seen in the spike fractions obtained after ion exchange chromatography (Fig. 5c). The homogeneity of these preparations was further checked by analytical ultracentrifugation and immunoelectrophoresis (Fig. 7). The molecular weight of the purified organelles, also as calculated from the sedimentation equilibrium (Fig. 8), and some other physical parameters are summarized in Table 3. The amino acid analysis of the organelles is recorded in Table 4; no amino sugars or rare amino acids were detected. The single-spike constituent polypeptide chains (P.5 and P.7; see Fig. 6 and Table 2) were also analyzed. Although these analyses were carried out directly on hydrolysates of gel slices obtained by SDS-PAA gel electrophoresis (see Materials and Methods) and can thus be considered roughly semiquantitative only, their accuracy was sufficient for the following conclusions. (i) Phage 29 spikes contain polypeptides P.5 and P.7 in equimolar proportion; a molar ratio $\cong 1$ was calculated from the sums of the amino acid residues found in either of the two gel bands, and from the molecular weights of P.5 and P.7. (ii) With the exception of arginine (4.1 mol% in P.5, versus 2.1 in P.7) and valine (5.5 mol% in P.5, versus 2.1 in P.7), P.5 and P.7 do not exhibit larger differences in amino acid composition. The determination of carbohydrates in isolated spikes, by using the indole-sulfuric acid method (12) with glucose as a standard, yielded values below 1% and the ultraviolet spectrum of the organelles showed that they were essentially free of nucleic acids ($OD_{280}/OD_{260} = 1.62$).

DISCUSSION

As stated in the introduction, it was the aim of this investigation to achieve a morphological localization of the host capsule depolymerase within the virions of *E. coli* capsule bacteriophage no. 29, to develop an isolation procedure for the enzymatically active phage organelles, to determine their molecular weight, and to analyze their polypeptide and amino acid composition.

The results presented in Fig. 5 and 6 and in Table 2 justify the following conclusion about the structure of phage 29 and the localization of the viral enzyme. (i) The phage carries a base plate linked in an unknown manner to its isometric head of about 45 nm in diameter. This base plate has (when detached) the shape of a six-pointed star of about 14 nm in outer diameter with a central hole or prop; in spite of this complex morphology and comparatively large size, it appears to consist of small polypeptides of one size only (P.9 with 13,500 ± 1,000

TABLE 1. *Isolation of spikes from disrupted bacteriophage 29 particles*

Determinations	Whole phage 29 particles[a]	Disrupted phage 29 particles[b]	Slowly sedimenting phage 29 fragments[c]	Phage 29 spikes after ion exchange[d]
Total volume (ml)	50	2.5	4	2
Total PFU	3×10^{14}	10^9	-	-
Total DU[e]	4×10^4	1.5×10^4	7×10^3	6×10^3 [d]
Total protein[f] (mg)	75	72	5.5	0.4
Percent yield of DU[e]	100	37.5	17.5	15[d]
DU[e] per mg of protein[f]	535	208	1.3×10^3	1.5×10^4
Enrichment factor[g]		1	6.25	72

[a] Purified by repeated isopycnic centrifugation.

[b] Disrupted by exposure to pH 3.5 at 37 C for 5 to 8 min, and digested with DNase (see Fig. 2).

[c] Slowly sedimenting phage fragments with capsule depolymerase activity, obtained by zonal centrifugation of disrupted particles. The yield after one repetition of the centrifugation of the faster sedimenting phage heads is given (see Fig. 3).

[d] Major depolymerase peak obtained by ion exchange chromatography of the slowly sedimenting phage fragments (see Fig. 4). The minor peak, consisting of enzymatically active spike subunits (compare Fig. 5), contained about 12.5 DU (0.3% of the depolymerase activity originally associated with intact phage 29 particles).

[e] Host capsule depolymerase units, as defined in Materials and Methods.

[f] Determined by the Folin technique with bovine serum albumin as a standard, and corrected for the chromogenicity of phage 29 spikes throughout (see Materials and Methods).

[g] Based on the capsule depolymerase activity of disrupted phage particles.

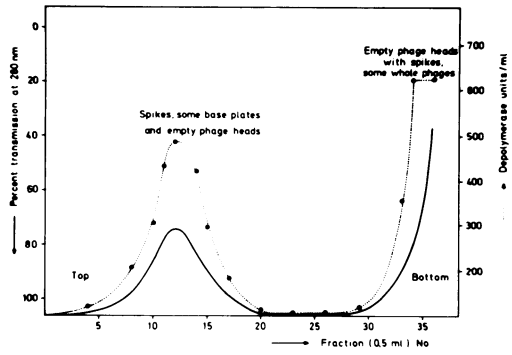

FIG. 3. *Zonal centrifugation of phage 29 fragments. The virus fragments, obtained by acid treatment of phage 29 particles (see Fig. 2), were placed on a 10 to 30% (wt/vol) linear sucrose gradient in a Spinco SW27 swinging-bucket rotor tube and centrifuged for 13 h at 26,000 rpm (94,000 × g). Portions (500 μl) were then withdrawn, and the host capsule depolymerase activity (●) and transmission at 280 nm (—) were determined. The peak fractions were also dialyzed and inspected under the electron microscope.*

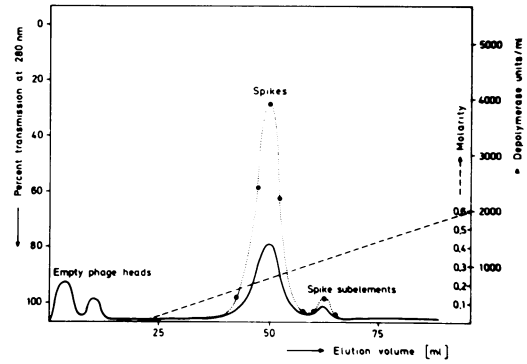

FIG. 4. *Ion exchange chromatography of phage 29 fragments. The slowly sedimenting phage 29 fragments, obtained by zonal centrifugation (see Fig. 3), were adsorbed to a DEAE Sephadex column (8 cm in length; 1.1 cm² in area) from 0.02 M Tris-hydrochloride buffer of pH 7.0, and eluted (9 ml/h) with a linear molarity gradient (- -) of the same buffer. The host capsule depolymerase activity (●) of the fractions (2.5 ml) and the transmission at 280 nm (—) are given. The peak fractions were also dialyzed and inspected under the electron microscope (see Fig. 5).*

daltons). (ii) Six (or perhaps a multiple thereof) spikes are attached to the base plate, possibly at each of its six points. Both on the intact virion and when detached, these spikes appear to be of roughly cylindrical shape with around 14.5 to 15 nm in length and 5 nm in diameter. They contain (at least) two different polypeptide chains of 57,000 ± 3,000 and 29,000 ± 2,000 daltons (P.5 and P.7, respectively). (iii) The active center of the host capsule-depolymerizing glycanase is part of the spike structure, and within these organelles, part of a possibly doughnut-shaped sub-element containing polypeptide P.5 (57,000 ± 3,000 daltons) only and

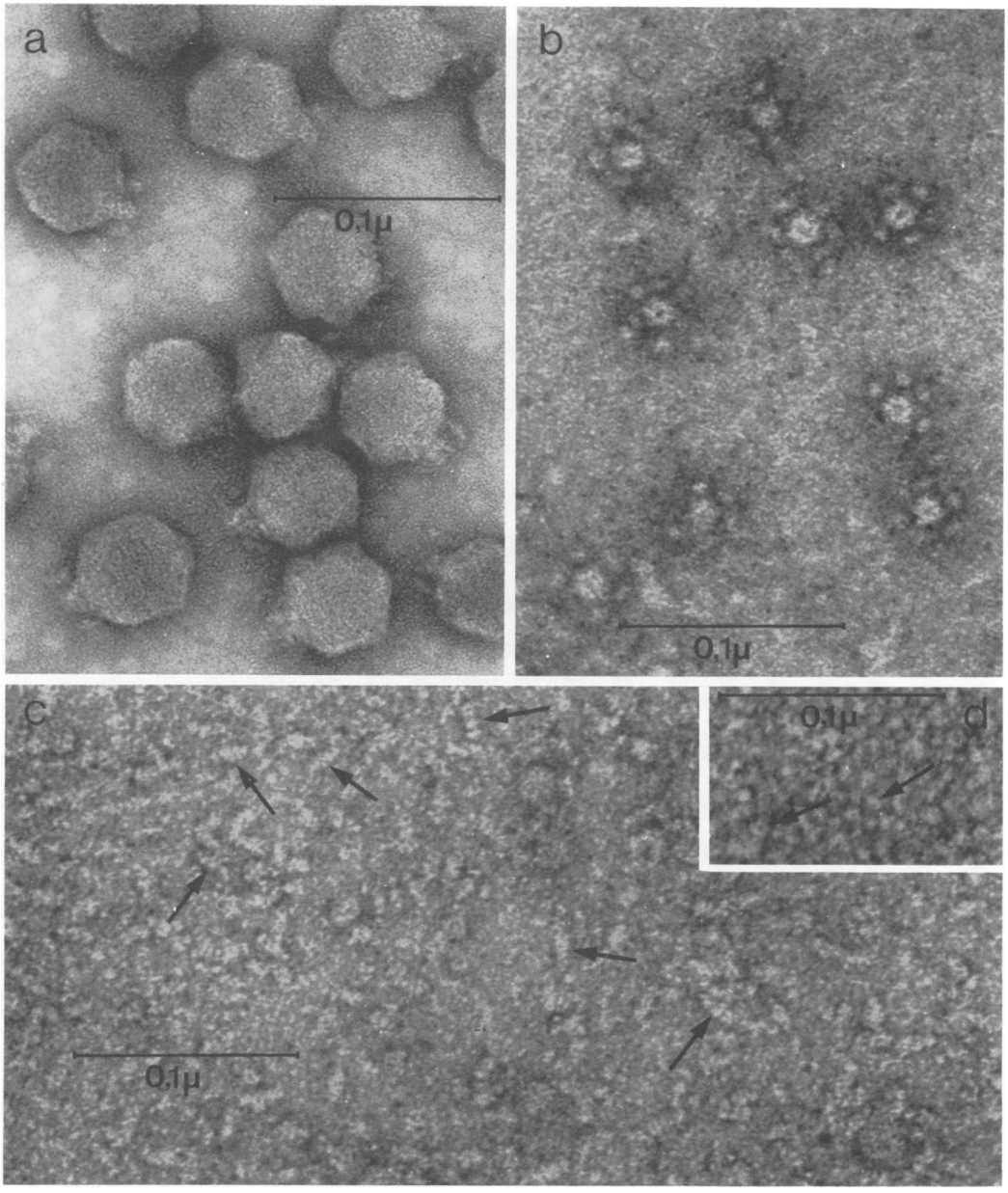

FIG. 5. *Electron microscopy of phage 29 and of its fragments with host capsule depolymerase activity. (a) E. coli capsule bacteriophage no. 29. (b) Fraction containing large amounts of base plates with some or all of the spikes still linked to them. (c) Fraction containing detached spikes only. (d) Phage 29 spike sub-elements still exhibiting depolymerase activity. All specimens were negatively stained with uranyl acetate and formate, ×300,000.*

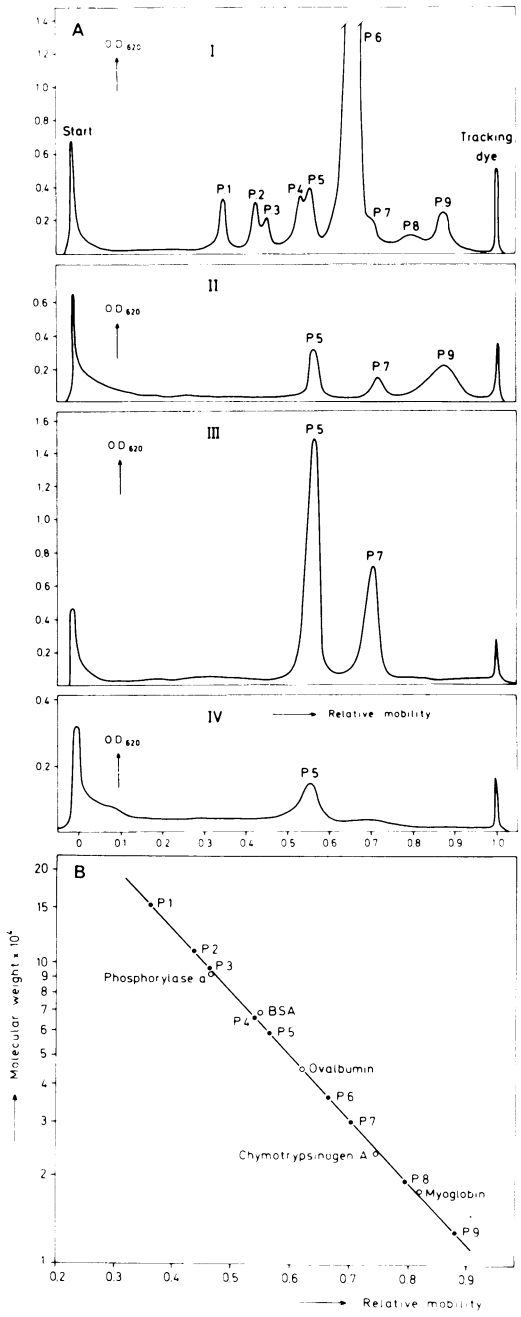

FIG. 6. *SDS-PAA gel electrophoresis of phage 29 and its fragments with host capsule depolymerase activity (compare Fig. 5). The samples were disintegrated by heating for 1 min to 100 C in a buffer containing 2% (wt/vol) SDS and 0.8% dithiothreitol, and the electrophoretic molecular sieving was carried out in SDS-containing gels (total acrylamide concentration, 5.81%, cross-linked with 3.6% N,N'-methylene bisacrylamide). Panel A: Densitometric scans of the Coomassie blue-stained gels showing polypeptide patterns of (I) whole phage 29 virions (about 50 μg of protein, 2 × 10¹¹ PFU); (II) a fraction containing large amounts of detached base plates with some or all of the spikes still linked to them, as well as free spikes (8 μg); (III) pure spikes (15 μg); and (IV) spike subunits still exhibiting depolymerase activity (5 μg). Panel B: Molecular weight calibration curve, showing the relative mobilities of (i) the molecular weight markers (○) phosphorylase a (94,000), bovine serum albumin (BSA; 68,000), ovalbumin (43,000), chymotrypsinogen A (25,000), and myoglobin (17,500) and of (ii) the phage polypeptide chains P.1 to P.9 (●) plotted against the logarithm of molecular weight. For further details see Materials and Methods.*

TABLE 2. *SDS-PAA gel electrophoresis of E. coli capsule bacteriophage 29 particles and of their fragments with host capsule depolymerase activity*[a]

Polypeptides in whole phage 29 virions			Polypeptides in phage 29 fragments		
Designation	Relative mobility	Mol wt[b]	Fraction rich in base plates	Spikes	Spike subelements
P.1	0.358	150,000			
P.2	0.434	108,000			
P.3	0.459	95,000			
P.4	0.540	65,000			
P.5	0.563	59,000[b]	P.5	P.5	P.5
P.6[c]	0.665	36,000			
P.7	0.706	30,000[b]	P.7	P.7	
P.8	0.798	19,000			
P.9	0.877	13,000[b]	P.9		

[a] Compare Fig. 5 and 6.

[b] The results of one experiment are given. Repeated determinations of the molecular weights yielded values generally varying by less than 8%. In the case of P.5, P.7, and P.9, molecular weights of 57,000 ± 3,000, 29,500 ± 2,000, and 13,500 ± 1,000, respectively, were always obtained.

[c] Polypeptide P.6 is the main constituent of phage 29 virions, and thus probably the major head polypeptide.

having a diameter of roughly 5 nm. The localization of this sub-element within the spike is unknown. However, it can be speculated that, for effective contact with the host capsule during the infective process, it should be located at or near to the distal tip of the organelle.

By using zonal centrifugation and ion exchange chromatography (Fig. 3 and 4, Table 1), phage 29 spikes can be isolated from whole virions after their disruption by mild acid treatment. One milligram of purified organelles is obtained from 5 × 10^{14} to 10^{15} PFU in this manner. In terms of enzyme units, the yield

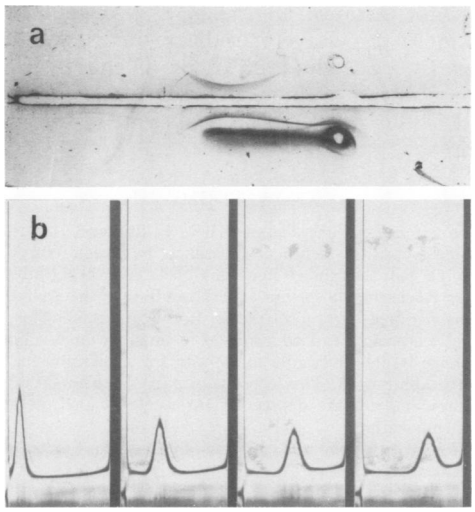

FIG. 7. *Homogeneity of purified phage 29 spikes. Panel a: For immunoelectrophoresis, 4 μl 0.1 M Tris-hydrochloride buffer of pH 7.0, containing isolated phage 29 spikes (about 10 μg, top), or the mixture of virus fragments obtained by acid treatment (about 40 μg, bottom) were applied to the antigen holes. After electrophoresis (90 min at 6 V/cm), the serum trough was filled with undiluted rabbit serum against crude concentrated lysate. The slide was stored for 48 h at room temperature, and the precipitation arcs were then washed and stained with amido black. Panel b: Analytical ultracentrifugation; the figure shows four photographs of the sedimentation of phage 29 spikes (0.3% [wt/vol] in 0.1 M Tris-hydrochloride buffer of pH 7.0) at 44,000 rpm (141,000 × g). The pictures were taken 2 (left), 18, 30, and 42 (right) min after start of the run; $s_{20,c} = 9.8 \times 10^{-13}$ S.*

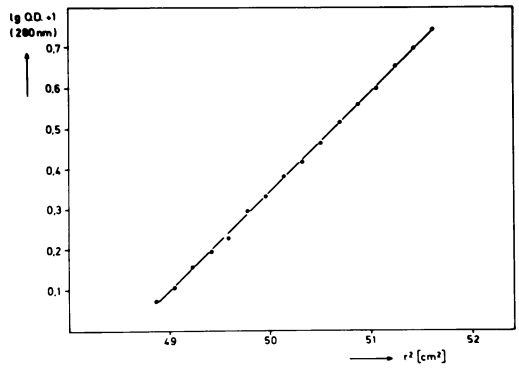

FIG. 8. *Sedimentation equilibrium of purified phage 29 spikes. Detached organelles (0.04% [wt/vol]) were run for 111 h at 6,000 rpm (2,600 × g) in the An-H-Ti rotor of a Spinco model E analytical ultracentrifuge, equipped with ultraviolet optics (280 nm) and a scanner.*

TABLE 3. *Physical parameters of isolated phage 29 spikes*

Parameter	Calculated value
Sedimentation coefficient ($s^0_{20,w}$)	10.3×10^{-13} S
Partial specific volume (V_p)[a]	0.725 cm³/g
Mol wt (avg)	
From V_p and the sedimentation equilibrium[b]	245,000
From the mol wt of the constituent polypeptides (3 × 57,000 plus 3 × 29,500)[c]	259,500
From V_p and the electron optical dimensions[d]	241,500
Isoelectric point	4.4

[a] Calculated from the amino acid analysis (Table 4; [5]).

[b] See Fig. 8.

[c] Since P.5 and P.7 (Fig. 6 and Table 2) occur in equimolar amounts in the organelles (see text), only a combination of three copies each of P.5 (3 × 57,000) and of P.7 (3 × 29,500) gives a molecular weight near to the value determined from V_p and the sedimentation equilibrium (see also Discussion).

[d] Assuming cylinders of 14.8 nm in length and 5 nm in diameter.

amounts to roughly 15%, the loss being mainly due to some denaturation during acid disruption and to incomplete detachment of the spikes. In the electron microscope, rare single base plates are seen among the spikes in such preparations. However, their amount, as well as that of other contaminants, is negligible, as evidenced by the sedimentation pattern, the immunoelectropherogram (Fig. 7), the sedimentation equilibrium (linear log of OD versus r^2 plot in Fig. 8), and the SDS-PAA gel electropherogram (only P.5 and P.7; Fig. 6) of the purified organelles.

As estimated from the partial specific volume calculated from the amino acid composition (5), and the sedimentation equilibrium by the reliable method of D. A. Yphantis (32), the molecular weight of detached phage 29 spikes was found to be 245,000 (Table 3). Since the polypeptide chains P.5 and P.7 occur in equimolar amounts in the spikes, it appears that one 245,000 unit contains three copies each of P.5 and P.7 (3 × 57,000 ± 3,000 plus 3 × 29,500 ± 2,000 = 259,000 ± 15,000). Still another molecular weight estimate in the same range is obtained from the electron optical dimensions and the partial specific volume of the organelles (compare reference 28). Assuming cylinders of 14.8 nm in length and 5 nm in diameter (Fig. 5c), 241,500 daltons can be calculated by this

TABLE 4. *Amino acid analysis of phage 29 spikes*[a]

Amino acid	Amino acid residue (g/100 g)[b, c]	mmol of amino acid per g[b, c]	Mol%[c, d]
Lysine	3.4	0.26	3.0
Histidine	1.2	0.09	1.0
Arginine	5.4	0.35	4.0
Cyst(e)ine[e]	1.0	0.10	1.2
Aspartic acid[f]	13.2	1.15	13.4
Threonine	6.7	0.66	7.7
Serine	7.2	0.83	9.6
Glutamic acid[f]	7.6	0.59	6.8
Proline	2.7	0.28	3.2
Glycine	4.6	0.81	9.4
Alanine	7.5	1.06	12.3
Valine	5.8	0.59	6.8
Methionine	0.5	0.04	0.5
Isoleucine	3.7	0.33	3.8
Leucine	7.7	0.68	7.9
Tyrosine	5.2	0.32	3.7
Phenylalanine	5.0	0.34	3.9
Tryptophan[d]		(0.13)[d]	(1.7)[d]

[a] Analyzed after 24 h of hydrolysis only.

[b] Means of two analyses, based on a dry weight determination with an accuracy of about ±15% (see Materials and Methods).

[c] Totals of columns: g/100 g: 88.4 (see footnotes *b*, *d*, and *e*); mmol/g: (8.61 [see footnote *d*]); and mol%: (99.9 [see footnote *d*]).

[d] Tryptophan was not determined. However, for comparison with the amino acid composition of "free depolymerase 29" (free spikes; [1]), 0.13 mmol of tryptophan per g of dry weight was assumed (compare Table 5 in Bessler et al. [1]).

[e] Determined as cysteic acid (17).

[f] Ammonia not determined.

approach. However, because of the large errors for which measurements of object dimensions from negatively stained specimens are notoriously liable (28), the data presented do not rigorously exclude the possibility that the spikes are, in fact, smaller, and that the molecular weight of aggregates is determined from the sedimentation equilibrium (compare reference 2).

The chemical analyses of the enzymatically active phage 29 spikes (see Table 4 and text) show that these organelles do not contain any major constituents besides the common amino acids, especially no amino sugars or other carbohydrates, as do the surface projection of many enveloped animal viruses (6, 16), and that their amino acid composition is very similar to that of other bacteriophage organelles establishing first contact with the host surface (e.g., T4 distal long half fibers [11], φX174 [8] and *Klebsiella* phage 11 spikes [2]). They all contain, for instance, 21 ± 2.5, 17 ± 1, and 21 ± 1.5 mol% respectively, of the acidic components aspartic and glutamic acid, of the hydroxyl group-bearing amino acids serine and threonine, and of the small amino acids glycine and alanine.

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