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Bacteriophage ϕ6: a Lipid-Containing Virus of *Pseudomonas phaseolicola*[1]

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The purification and properties of a lipid-containing bacteriophage, ϕ6, are described. The phage contains a lipid envelope which is probably essential for infection. Infectivity of ϕ6 was lost in the presence of organic solvents, sodium deoxycholate, and phospholipase A. The fatty acid composition of the phage lipid was similar to that of the *Pseudomonas phaseolicola* host cells. The phage was composed of about 25% lipid, 13% RNA, and 62% protein. The buoyant density of ϕ6 was 1.27 g/ml in cesium chloride. The morphology of ϕ6 was unusual; it had a polyhedral head of about 60 nm surrounded by a membranous, compressible envelope which appeared to assume an elongated configuration upon attachment to pili. The adsorption rate constant was 3.3×10^{-10} ml/min in a semi-synthetic medium and 3.8×10^{-10} ml/min in a nutrient broth-yeast extract medium. The latent period was shorter in the former medium (80–115 min compared with 120–160 min), and the average burst size was larger (250–400 compared with 125–150). The eclipse period coincided with the latent period.

Bacteriophage ϕ6 was isolated during an investigation of bacteriophages of phytopathogenic pseudomonads. It was extremely sensitive to organic solvents and a detergent, which suggested that it might contain lipid. Phage PM2 (4) is the only lipid-containing bacteriophage previously described. The present paper describes the isolation and purification of phage ϕ6. In addition, electron microscopy of the phage and some of its biological and biochemical properties are presented.

MATERIALS AND METHODS

Media. A nutrient broth-yeast medium (NBY; reference 15) was used for the initial experiments. Subsequently, a semi-synthetic medium (SSM) was also used. SSM medium contains: 0.2 g of $MgSO_4 \cdot 7H_2O$; 6.0 g of Na_2HPO_4; and 3.0 g of KH_2PO_4; dissolved in 850 ml of distilled water and autoclaved separately from 50 ml of 10% (wt/vol) glucose and 100 ml of 20% (wt/vol) acid-hydrolyzed casein (Nutritional Biochemicals Corp.). The casein hydrolysate solution was filtered through Whatman no. 1 paper for clarification before autoclaving. Both acid and pancreatic hydrolysates of casein (Difco) were tested in later experiments; the phage was more stable in media containing these hydrolysates than with the Nutritional Biochemical product.

Isolation. The host strain *Pseudomonas phaseolicola* HB10Y was used for enrichment culture with *P. phaseolicola*-infested bean straw as previously described (16), except that a sample of the presumed lysate was filtered through a Millipore, 0.45-μm membrane filter.

The phage formed semi-clear plaques about 2 mm in diameter, surrounded by a halo, on the host strain HB10Y after 24 h of incubation at 24 ± 2 C. Plaque-picking and initial purification procedures were as previously described (16).

Growth and purification of ϕ6. Phage lysates were prepared in 7.0-liter quantities of SSM in a bench-top 14-liter fermentor (New Brunswick Scientific Co., Inc.). The air flow rate was 16 liters/min, and the mixing was at 500 rpm and 26 C. Host cells were grown for 20 h at 24 ± 2 C and subcultured to an initial optical density (OD) of 0.05 (10^8 colony-forming units [CFU]/ml) at 640 nm. When the cells had grown to an OD of 0.09 to 0.12, phage at a phage-bacterium ratio of 1:4 to 1:6 was added. From 0.5 to 1.0 ml of an antifoam agent (Union Carbide SAG 4130) was added at the start of lysate production. Considerable clarification occurred after an additional 7 h of incubation. High-titer lysates of about 0.5×10^{11} to 4×10^{11} PFU/ml for ϕ6 were routinely obtained. Such high-titer lysates could also be obtained by increasing the phage-bacterium ratio to 5:1 and shortening the incubation period to 2 h and 40 min when the host cells were previously grown to an OD of 0.5.

Phage lysates were stored at 4 C for 24 to 72 h, warmed to 26 C, and treated with 1 μg of DNase (Worthington Biochemical Corp.) per ml in the presence of 10^{-3} M $MgSO_4 \cdot 7H_2O$. After 3 h of treatment, the lysates were centrifuged at 16,000 × *g* for 10 min to remove bacterial debris. The supernatant fluid was

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stored overnight at 4 C and then concentrated with 10% (wt/vol) polyethyleneglycol 6000 powder (PEG, Union Carbide) for 3 to 4 h at 4 C in the presence of 0.5 M NaCl, by using the method of Yamamoto et al. (17). The PEG-treated phage was pelleted by centrifugation at 16,000 × *g* for 10 min, and the pellet was resuspended in cold 0.01 M potassium phosphate (pH 7.1) containing 10^{-3} M $MgSO_4 \cdot 7H_2O$ (buffer A) to a volume of 1% of the initial lysate, i.e., 60 to 70 ml.

After overnight storage at 4 C, the phage was purified by centrifugation at 70,000 × *g* for 75 min in a 10 to 35% (wt/vol) linear sucrose gradient column (3) equilibrated with buffer A. Gradients were scanned with a UV photometric analyzer (Instrumentation Specialties Co.), and the fractions were collected. After it had been determined that the peak absorbing material corresponded to the peak infectious titer, only the peak material was collected from the gradients. Further purification of ϕ6 was accomplished by an additional 6 to 7 h of centrifugation at 40,000 × *g* on a 30 to 60% (wt/vol) linear sucrose gradient column. Again, a correspondence between UV-absorbing material and infectivity occurred. Sucrose was removed by overnight dialysis against buffer A. The phage was stored at −20 or −70 C.

Alternatively, purification was accomplished by mixing 1 to 2 ml of PEG-concentrated material with 30% (wt/vol) CsCl in 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0, and centrifuging to equilibrium in a fixed-angle rotor at 100,000 × *g* for 15 h at 4 C. Only one infectious zone appeared in the CsCl gradients, corresponding to the UV-absorbing material. The phage was collected by means of a syringe with a bent needle, diluted at least 1:5 with buffer A, and centrifuged at 70,000 × *g* for 2 h at 4 C to pellet the phage and dilute out the cesium chloride. Pellets were resuspended in buffer A to the desired volume and then stored as above. All of the experiments reported were conducted with phage purified on sucrose gradients unless otherwise indicated.

Delays in processing of ϕ6 after PEG treatment of even 24 to 72 h resulted in aggregation or disintegration of the virus as seen by an increase of noninfectious light-scattering zones on the sucrose gradients. However, crude lysates could be stored at 4 C for several days or frozen at −20 C for months without a detectable loss of infectivity.

Adsorption rates and one-step growth experiments. The adsorption rate and one-step growth experiments were determined in NBY and SSM at 24 C essentially as described by Adams (1). To 0.9 ml of cells at 0.5×10^8 to 2×10^8 CFU/ml, 0.1 ml of ϕ6 at 10^7 to 2×10^7 PFU/ml was added. Samples were diluted 1:10 at intervals into ϕ6 antiserum for 10 min, diluted further, and plated to determine the number of infected bacteria. Antiserum at the final dilution had no effect on plaque formation. The antiserum was prepared as previously described (16).

In one-step growth experiments, phage was added to the bacteria at a ratio of 0.005 to 0.02 and shaken briefly by hand. After 20 min, 0.2 ml of the phage-bacterium mixture was added to 1.8 ml of ϕ6 antiserum; this mixture was diluted after 10 min into the respective growth medium. Further dilutions were made in the same media to obtain volumes of 10 ml. The flasks were shaken on a rotary shaker at 350 rpm at 24 ± 2 C, except as noted elsewhere. Samples were removed at appropriate intervals and plated.

The eclipse period was determined in one-step growth experiments performed under stationary conditions in SSM. The cells were lysed with a mixture containing 200 μg of lysozyme (Sigma Chemical Co.) per ml, 0.0025 M EDTA, 0.05 M NaCl, and 0.05 M Tris, pH 7.5 (5). Separate experiments indicated that cell lysis, as determined by phase microscopy, occurred in 3 min or less in this mixture at 0 C and in 5 to 10 min at 25 C. The infectivity of phage ϕ6 was not affected by the lysis mixture under these conditions.

Buoyant density determination. Purified ϕ6 was centrifuged to equilibrium in a Spinco model SW50.1 rotor after layering 0.4 ml of ϕ6 (4×10^{10} PFU) on top of 4.5 ml of a 30.6% (wt/wt) solution of CsCl in water. After 23 h of centrifugation at 150,000 × *g* and 0 C, fractions were collected by needle puncture from the bottom of the tubes. The density at 25 C of five-drop samples was determined by direct weighing of the samples in preweighed, self-filling capillary tubes.

Lipid extraction and analysis. Lipid was extracted from purified phage with a 3:1 (vol/vol) chloroform-methanol solution. A chloroform-methanol extract was also made from an equivalent quantity of phage ϕ91 purified in a manner identical to ϕ6. Phage ϕ91 is a nonlipid-containing phage which also infects HB10Y (Vidaver and Van Etten, unpublished data). An identical chloroform-methanol extract was also made from uninfected HB10Y cells grown to an OD of 0.4 at 640 nm (about 8×10^8 CFU/ml). The cells were harvested by centrifugation, washed three times with buffer A, and frozen before extraction.

Fatty acids in the chloroform-methanol extracts of the phages and cells were converted to methyl esters by the interesterification procedure described by Tulloch, Craig, and Ledingham (14). After methylation, the extracts were spotted onto thin-layer plates containing silica gel G and developed with heptane-ether (9:1 vol/vol). The plates were sprayed with 0.2% rhodamine in 95% ethanol and examined under UV light. Several spots with R_f values similar to those from the HB10Y extract appeared on the thin-layer plates with the extract from ϕ6. No spots were observed with the extract from ϕ91. The region containing the typical methylated fatty acids was removed, extracted with chloroform, evaporated to dryness, and taken up in hexane for analysis by gas chromatography.

Fatty acid analyses were performed on an F and M gas chromatograph (model 402) by use of a hydrogen flame detector. The column consisted of 15% diethyleneglycol adipate polyester on Gas-Chrom P, 60 to 80 mesh, packed in a glass column (4 ft; ca. 121.9 cm) with an internal diameter of 1/8 inch (ca. 0.32 cm). The column temperature was either programmed from 140 to 190 C or run isothermally at 190 C. Hydrogen, helium, and oxygen flow rates per min were 35, 100, and 300 ml, respectively.

Quantitative analyses were done by measuring peak areas on recorder charts with a planimeter. The methyl esters of the fatty acids were identified by comparing the retention times of the extracted acids

with standard fatty acid methyl esters obtained from Applied Science Laboratories.

Lipase sensitivity. Purified ϕ6 (2×10^{11} PFU/ml) was incubated with phospholipase A (Boehringer Mannheim Corp.) at 24 C in the presence of 0.02 M Tris (pH 8.0), 7.5×10^{-3} M $CaCl_2$, and 10^{-3} M $MgCl_2$. Samples were removed at various times and tested for infectivity.

Estimation of nucleic acid, lipid, and protein content. To determine the percentage of lipid in the purified phage, the phage was dried to a constant weight over phosphorus pentoxide, extracted for 3 h at room temperature with chloroform-methanol (3:1 vol/vol), and then centrifuged. The organic-soluble and -insoluble materials were redried to a constant weight. Separate preparations were tested with either orcinol for RNA (11) or indole for DNA (6). Protein was assayed by the procedure of Lowry et al. (8). Bovine serum albumin fraction V, yeast RNA, and calf thymus DNA were used as standards.

Electron microscopy. Both crude and purified lysates were examined with an electron microscope. A sample containing about 5×10^{11} to 10^{12} PFU/ml was applied to a carbon-backed, collodion-coated grid. The droplet was drained with filter paper after 5 to 20 s and then negatively stained with a 3:1 mixture of 2% potassium phosphotungstate and 1% vanadatomolybdate as previously described (16).

RESULTS

Purification of ϕ6. Table 1 shows the various steps of purification and the recovery of ϕ6 at each stage. Good recoveries were obtained, considering the number of manipulations performed and the sensitivity of the phage to certain agents (see below). The PFU and $A_{260\ nm}$ coincided upon centrifugation of ϕ6 from step 6 (Table 1) in 10 to 35% linear sucrose density gradients (Fig. 1A). All of the fractions in the peak region had a specific infectivity of 9×10^{11} to 15×10^{11} PFU/$A_{260\ nm}$. There was a slight shoulder of faster sedimenting infectious material which was probably due to aggregation of the phage; the small shoulder of UV-absorbing material which sedimented more slowly than the phage is probably degraded phage. An additional cycle of centrifugation of the phage taken from the peak material always resulted in the appearance of these small shoulders.

TABLE 1. *Purification of bacteriophage ϕ6*

Steps in purification	Volume (ml)	Titer (PFU/ml)	Total PFU	Recovery (%)
1, Crude lysate.	5,900	5.4×10^{10}	3.2×10^{14}	100
2, After DNase.	5,900	5.5×10^{10}	3.2×10^{14}	100
3, After PEG sedimentation	59	4.3×10^{12}	2.5×10^{14}	78
4, 10 to 35% (wt/vol) sucrose gradient	100	1.2×10^{12}	1.2×10^{14}	38
5, 30 to 60% (wt/vol) sucrose gradient	70	1.3×10^{12}	9.1×10^{13}	28
6, After dialysis.	114	4.1×10^{11}	4.7×10^{13}	15

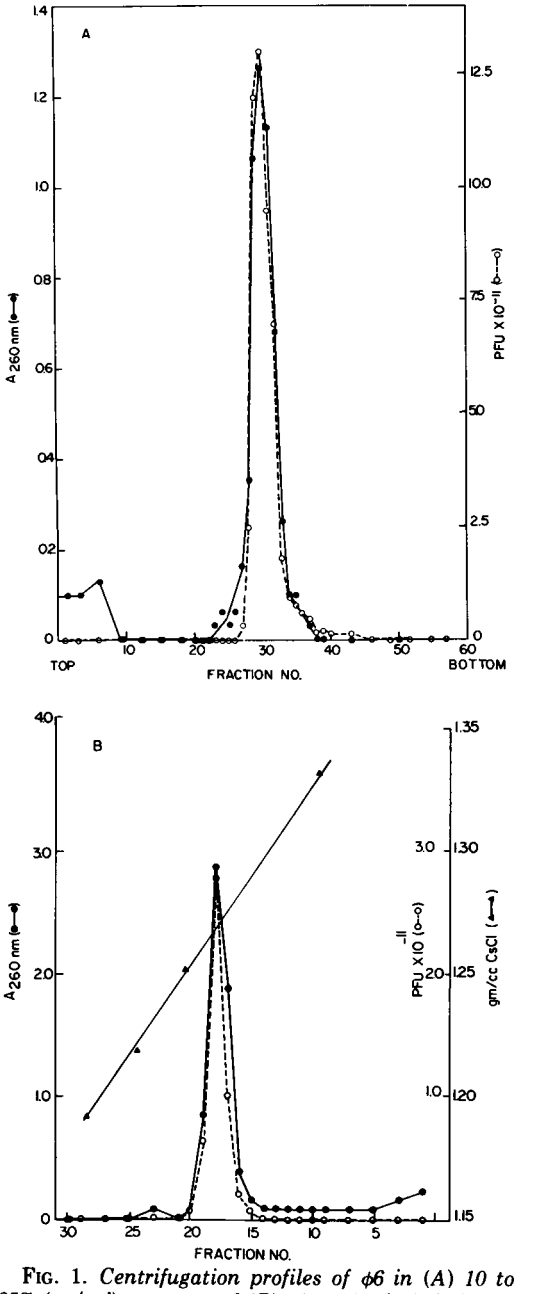

FIG. 1. *Centrifugation profiles of ϕ6 in (A) 10 to 35% (wt/vol) sucrose and (B) 30.6% (wt/wt) CsCl.*

The PFU and $A_{260\ nm}$ also coincided when the phage from step 6 (Table 1) was centrifuged to equilibrium on CsCl gradients (Fig. 1B). How-

ever, there was a marked decrease in infectivity of sucrose-purified phage when it was centrifuged in CsCl; the specific infectivities of all of the fractions in the peak region were 5×10^{10} to 10×10^{10} PFU/$A_{260\ nm}$.

If the PEG-treated lysate was layered directly on CsCl gradients, a single, sharp infectious zone was obtained after centrifugation; the specific activities of fractions from this zone were 3×10^{11} to 4×10^{11} PFU/$A_{260\ nm}$ (not shown). Subjecting the CsCl-purified phage to a second CsCl gradient always resulted in a further decrease in specific infectivity; however, the profile remained the same.

Biological properties of ϕ6. The adsorption rate constant, latent period, and burst size of ϕ6 in two media are reported in Table 2. Although the adsorption rates were similar in both media, SSM was clearly superior for lysate production. Figure 2 illustrates the kinetics of a one-step growth experiment under spontaneous and premature lysis conditions. The results were the same whether samples were diluted into the lysis mixture at 0 C or 25 C. The eclipse and latent periods of ϕ6 were identical; this contrasts with bacteriophage PM2 (5), in which induced lysis appeared to reveal mature particles prior to spontaneous release.

If the phage lysates were incubated with shaking in one-step growth experiments, it was routinely observed that ϕ6 was unstable in SSM but stable in NBY. However, little or no loss of infectivity occurred in stationary cultures of SSM. The SSM medium was responsible for the inactivation of ϕ6, since ϕ6 prepared from NBY or SSM lysates was unstable to the same degree in SSM. Bovine serum albumin added to SSM at low concentrations partially protected the phage; at high concentrations (100 μg/ml) inactivation was accelerated.

The stability of purified ϕ6, at titers present under one-step growth conditions, was tested under stationary conditions in NBY, SSM, and buffer A at 0 and 24 C. The phage was most stable in NBY and SSM at 0 C. Because of the instability of ϕ6 in SSM at 24 C, fermentor lysates were quickly cooled to 4 C after lysate production; under these conditions no loss of infective units could be detected.

Evidence for a lipid envelope in ϕ6. The sensitivity of ϕ6 to organic solvents and sodium deoxycholate is reported in Table 3. The

TABLE 2. *Biological properties of ϕ6 in SSM and NBY media*

Medium	Adsorption rate constant (10^{-10} ml/min)	Latent period (min)	Avg burst size
SSM[a]	3.3 ± 1.5	80–115	250–400
NBY	3.8 ± 1.5	120–160	125–150

[a] SSM contained acid-hydrolyzed casein from Nutritional Biochemical Co.

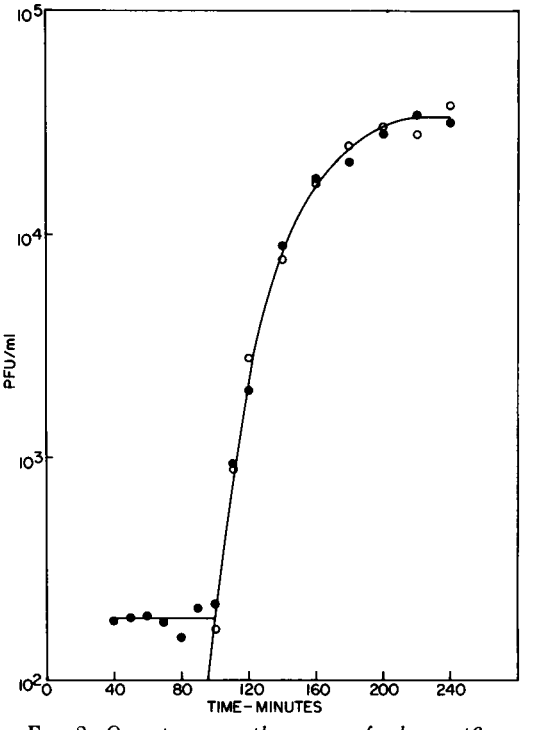

FIG. 2. *One-step growth curve of phage ϕ6 on HB10Y. Incubation was at 24 C in SSM. Symbols: ●, spontaneous lysis; ○, induced lysis with lysozyme.*

TABLE 3. *Inactivation of ϕ6 by chloroform, ethyl ether, toluene, and sodium deoxycholate*

Phage	Treatment[a]	Titer (PFU/ml)
ϕ6	None	3.5×10^{10}
ϕ6	Toluene, 2.5%	1.5×10^{6}
ϕ6	Ethyl ether, 25%	2.1×10^{4}
ϕ6	Sodium deoxycholate, 0.05%	1.6×10^{5}
ϕ6	Sodium deoxycholate, 0.5%	0
ϕ6	Chloroform, 5%	0
ϕ91	None	2.4×10^{8}
ϕ91	Toluene, 2.5%	2.8×10^{8}
ϕ91	Ethyl ether, 25%	1.8×10^{8}
ϕ91	Sodium deoxycholate, 0.05%	2.0×10^{8}
ϕ91	Sodium deoxycholate, 0.5%	1.9×10^{8}
ϕ91	Chloroform, 5%	2.1×10^{8}

[a] Phages ϕ6 and ϕ91 were added to the reagents and mixed with a Vortex mixer for 7 to 8 s. Samples for assay were withdrawn from the aqueous layer after allowing an additional 7 to 8 s for phase separation. Each value given as percent vol/vol.

marked sensitivity of φ6 to all these agents first suggested that the phage might contain lipid. Phage φ91, in contrast, was resistant to these agents. In addition, φ6 lost infectivity upon incubation with phospholipase A (Fig. 3), whereas the enzyme had no effect on φ91. Phospholipase A is a highly purified enzyme and specifically cleaves the beta-fatty acid residue from phospholipids.

A fatty acid analysis of a chloroform-methanol-extractable fraction from φ6 is shown in Table 4. The fatty acid composition of φ6 was similar to that of the host bacterium. Gas chromatographic analysis of the same regions from thin-layer plates of φ91 extracts indicated that less than 5% of the fatty acids attributed to φ6 could be accounted for by general contamination from the host cells.

The lipid content of φ6, as determined by extraction with chloroform-methanol, was estimated at 25% of the total weight. The phage contained 62% protein and about 13% RNA. Identical results were obtained from phage purified on sucrose density gradients or on CsCl gradients. There was no indication of DNA.

The buoyant density of φ6 in CsCl (Fig. 1B) was 1.27 g/ml, which is similar to the lipid-containing phage PM2 (4).

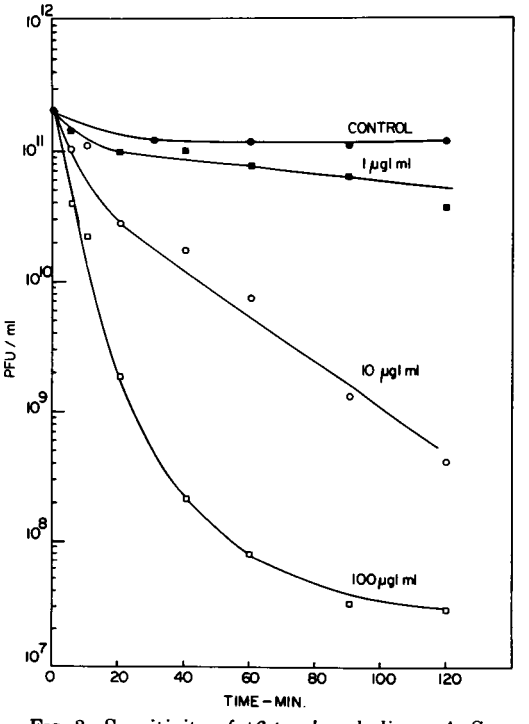

FIG. 3. *Sensitivity of φ6 to phospholipase A. Symbols: ●, no lipase; ■, 1 µg of lipase per ml; ○, 10 µg of lipase per ml; □, 100 µg of lipase per ml.*

TABLE 4. *Fatty acid composition of Pseudomonas phaseolicola and phage φ6*

Carbon skeleton	*P. phaseolicola*	Phage φ6
14:0	0.5[a]	2.3[a]
15:0	0.2	0.4
Unknown A[b]	0.3	0.9
16:0	34.7	33.1
16:1	42.2	40.4
17:0	0.1	Trace
Unknown B[b]	0.6	Trace
Unknown C[b]	0.7	1.0
18:0	0.7	1.0
18:1	20.0	21.0

[a] Values are the percentage of the individual fatty acids in the total fatty acid fraction.

[b] Unknowns A, B, and C gave relative retention values of 15.5, 17.4 and 17.7, respectively.

Electron microscopy demonstrated that φ6 was surrounded by an amorphous, membranous-appearing structure (Fig. 4). The phage head was polyhedral, 60 to 70 nm in diameter. Figure 4A and B show phage attached to pili by the membranous structure. The stage that φ6 becomes associated with the pili is not known, but phage particles were attached to only one or two pili per cell. The amorphous, saclike "tail" could be seen in both crude and purified preparations of φ6 (Fig. 4B-I). Swelling of the head region and a shortening, or disruption, of the tail region was observed after purification; glutaraldehyde fixation increased these effects.

DISCUSSION

The degree to which phage φ6 was purified for the compositional and chemical analyses is difficult to determine because of its extreme sensitivity to many agents commonly employed in virus purification. Furthermore, φ6 probably obtains its "membrane" directly from the bacterial membrane (unpublished data); therefore, the absence of host membrane protein in the purified phage cannot be used as a criterion for purity. However, the following evidence suggests that the phage is reasonably pure. (i) Centrifugation of collected infectious peaks from sucrose or CsCl gradients by rate zonal or equilibrium centrifugation always showed the same type of profile, namely, a sharp peak with a trace of infectious material considered to be an aggregate and noninfectious UV-absorbing material considered to be a degradation product. (ii) The specific infectivity (PFU/$A_{260\ nm}$) was reasonably uniform in fractions obtained from the peak region on both gradients. (iii) The quantitative composition of φ6 was the same regardless of whether the phage was purified on

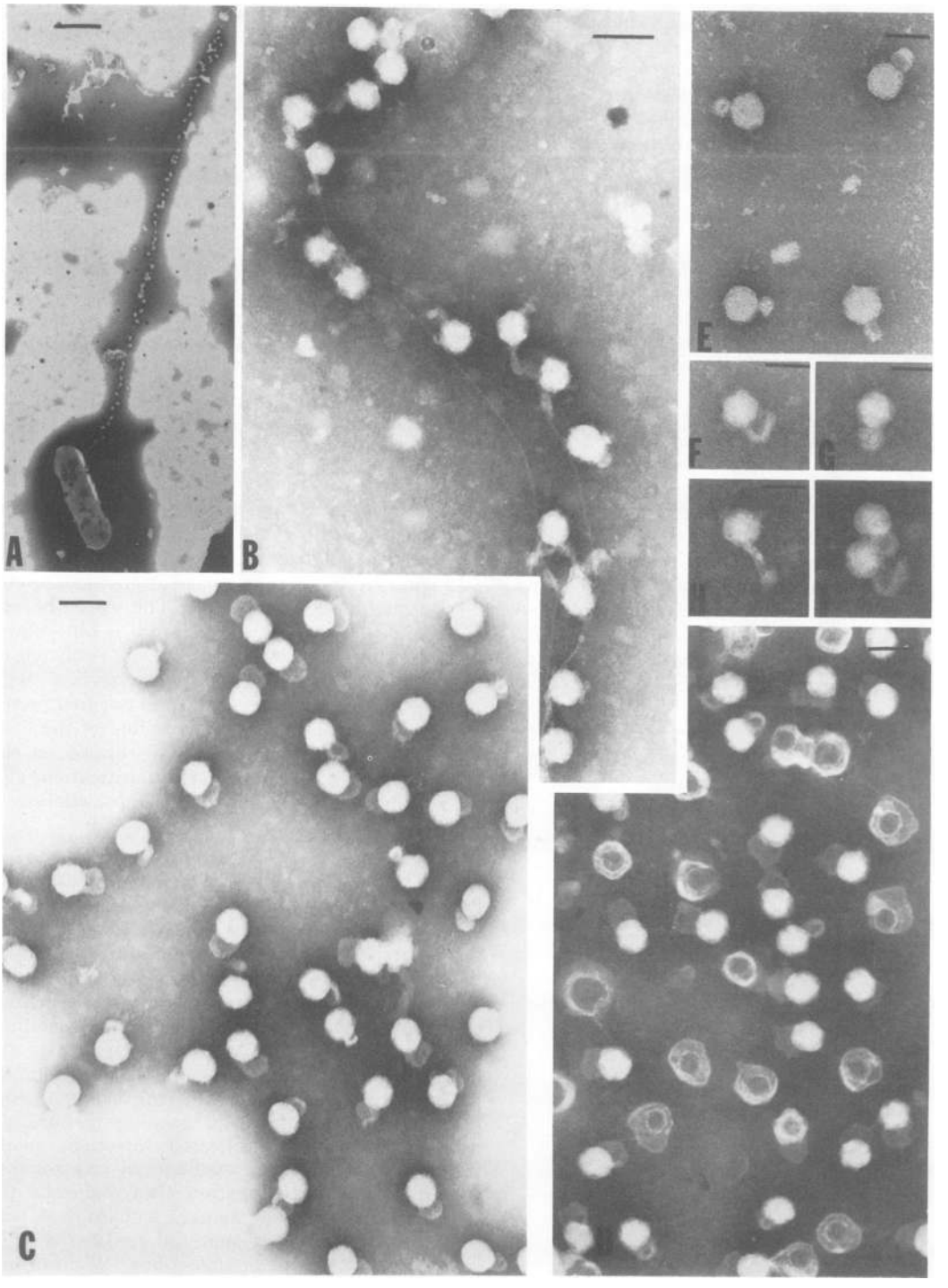

FIG. 4. *Electron micrographs of phage φ6. (A) Phage and bacterium from crude lysate (NBY grown). (B) Higher magnification of phage and bacterium from the same crude lysate; note "tails" attached to pili. (C) Purified lysate; note envelope structure. (D) Purified lysate showing intact and empty particles; note preservation of head structure with amorphous envelope material attached. (E) Particles showing tails in different positions, some with electron-dense material evident. Particles were obtained from bacteria grown in SSM and treated with DNAse. (F-I) Particles as in E, except without the DNAse treatment. Line markers indicate 1.0 μm in A, and 100 nm in B to I.*

sucrose density gradients or on CsCl gradients. (iv) The only nucleic acid derived from the peak infectious material was double-stranded RNA (manuscript in preparation).

The data presented indicate that bacteriophage ϕ6 contains lipid; it may be arranged as an envelope around the phage particle. The data supporting the presence of lipid in ϕ6 are the sensitivity to lipid solvents, deoxycholate, and phospholipase A, fatty acid analysis of the purified phage, and buoyant density of the whole particle.

The envelope of ϕ6 was clearly seen in electron microscopy studies, which showed a membranous, compressible, saclike structure surrounding the polyhedral head. The apparent elongation of the envelope upon attachment to pili may be a drying artifact, or the envelope may have one or more specific attachment sites. The morphology of phage ϕ6 thus differs from PM2 (4, 13) which has a compact-appearing membrane surrounding the head. Because of the appearance of the envelope, phage ϕ6 differs from all other morphological types of phage thus far described (2).

A 25% lipid content of ϕ6 is high, when compared with 10.5% for PM2 (4), but in the range of lipid content (18–27%) for large RNA viruses (10). The 62% protein content for ϕ6 is in the same range (60–75%) as myxoviruses (10) and within the range (39–88%) for other phages (9). The 13% RNA for ϕ6 compares with 25 to 31% RNA for known RNA phages and picornaviruses (7), with 1 to 4% RNA for myxoviruses (10) and about 11 to 20% for double-stranded RNA-containing viruses (12). Phage ϕ6 also contains double-stranded RNA which sediments as three components (Semancik, Van Etten, and Vidaver, manuscript in preparation; Abstr. Annu. Meet. Amer. Soc. Microbiol., 72nd, Philadelphia, p. 221, 1972.

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