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## Comparative Properties of Bacteriophage $\phi 6$ and $\phi 6$ Nucleocapsid<sup>1</sup>

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Nonionic detergent treatments released a nucleocapsid from the enveloped bacteriophage  $\phi 6$ . The nucleocapsid sedimented at nearly the same rate as the whole phage in sucrose density gradients, but the buoyant density in  $\text{Cs}_2\text{SO}_4$  changed from 1.22 g/cm<sup>3</sup> for the whole phage to 1.33 g/cm<sup>3</sup> for the nucleocapsid. The detergent completely removed the lipid and 5 of the 10 proteins from the phage. Surface labeling of the phage and nucleocapsid with <sup>125</sup>I revealed that protein P3 was on the outer surface of the whole phage and P8 was on the surface of the nucleocapsid. Both the phage and the nucleocapsid were stable between pH 6.0 and 9.5. Low concentrations of EDTA (10<sup>-4</sup> M) dissociated the nucleocapsid but had no effect on the whole phage. The nucleocapsid contained all three double-stranded RNA segments, as well as RNA polymerase activity.

The bacteriophage  $\phi 6$  of *Pseudomonas phaseolicola* is surrounded by a lipid-containing envelope that is required for infectivity (11, 12, 16). In this respect,  $\phi 6$  is similar to many enveloped mammalian viruses (9). Detergent treatment of such mammalian viruses often results in the release of nucleocapsids. This report describes the isolation of  $\phi 6$  nucleocapsids and compares biochemical properties of the nucleocapsids with those of the whole phage. Portions of these results have been published in abstract (J. Van Eten, L. Lane, C. Gonzalez, and A. Vidaver, *Annu. Proc. Am. Phytopathol. Soc.* for 1975, p. 43).

### MATERIALS AND METHODS

**$\phi 6$  Production and the isolation of  $\phi 6$  nucleocapsids.** Growth of the host, *P. phaseolicola* HB10Y, and production of lysates were previously described (16). The phage was purified after DNase treatment and polyethylene glycol concentration by equilibrium centrifugation in CsCl gradients or by centrifugation in sucrose density gradients as previously described (16). Phage  $\phi 6$  nucleocapsids were obtained by incubating the polyethylene glycol-concentrated virus or purified  $\phi 6$  with 0.25% to 2.5% (wt/vol) nonionic detergents at 0 C for 15 min in the presence of 12.5 mM KPO<sub>4</sub>, pH 7.2. The nucleocapsids were isolated from 10 to 40% (wt/vol) linear sucrose density gradient columns containing 12.5 mM KPO<sub>4</sub>, pH 7.2, after centrifugation at 23,000 rpm for 150 min in a Spinco SW27 rotor. The zone of material at an absorbance (A) of 254 nm was collected and pelleted

by centrifugation in a Spinco Ti50 rotor at 40,000 rpm for 2 h. The resultant  $\phi 6$  nucleocapsid was suspended in a small volume of 12.5 mM KPO<sub>4</sub>, pH 7.2, and stored at -80 C.

**Labeling of  $\phi 6$  with [<sup>3</sup>H]glycerol.** The host was grown overnight on a minimal medium (20) containing 1% glycerol and 1% Casitone (Difco) and subcultured to about 8 × 10<sup>7</sup> cells/ml. When the culture reached 4 × 10<sup>8</sup> cells/ml it was chilled to 0 C, and phage was added at a ratio of 5 PFU per cell. After an adsorption period of 30 min at 0 C, infection was initiated by warming the culture to 25 C. [2-<sup>3</sup>H]glycerol (200 mCi/mmol) was added at a concentration of 0.8  $\mu$ Ci/ml at the start of infection as well as 35 min later. After lysis of the cells, the <sup>3</sup>H-labeled phage was purified as described above.

**Electron microscopy.** Purified  $\phi 6$  and  $\phi 6$  nucleocapsids were applied to a carbon-backed collodion-coated grid. The grid was drained, floated on a solution of 15% (vol/vol) glycerol for 15 min (10), drained, and negatively stained with 1% (wt/vol) uranyl acetate in distilled water.

**Buoyant density determinations.** About 1 optical density unit at A<sub>260</sub> of purified  $\phi 6$  or  $\phi 6$  nucleocapsid was centrifuged to equilibrium (24 h at 20 C at 39,000 rpm) in a gradient in a Spinco SW50.1 rotor. The gradients consisted of 30% (wt/wt) Cs<sub>2</sub>SO<sub>4</sub> equilibrated with 12.5 mM KPO<sub>4</sub>, pH 7.2. The tubes were fractionated into 0.15-ml fractions, and the densities were determined by direct weighing of the samples in preweighed, self-filling capillary tubes.

**Polyacrylamide gel analysis of  $\phi 6$  and  $\phi 6$  nucleocapsid proteins.** Proteins from  $\phi 6$  and  $\phi 6$  nucleocapsid purified on sucrose density gradients and Cs<sub>2</sub>SO<sub>4</sub> equilibrium density gradients were examined on polyacrylamide slab gels. The gel electrophoretic apparatus was that of Reid and Bielski as described by Studier (15). The buffer system was that of Laemmli (5) except that all buffers contained 0.2% sodium

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dodecyl sulfate (SDS). Virus or protein samples were mixed with an equal volume of 30% (wt/vol) sucrose, 1% (wt/vol) SDS, 0.01% crystal violet, and 5 mM dithiothreitol dissolved in 0.05 M boric acid, which had been adjusted to pH 9.0 with NaOH. The solution was boiled for 1 min, cooled, brought to 20 mM in iodoacetamide and incubated for 15 min at 50 C. The samples were subjected to electrophoresis in 5 to 20% polyacrylamide gradient gels with a 3.5% polyacrylamide stacking gel. The running gel was 14 cm long and 1.3 mm thick. Samples were subjected to electrophoresis at a constant current of 15 mA/cm<sup>2</sup> until the marker dye approached the bottom. Gels were stained with Coomassie brilliant blue and destained as described previously (6). Molecular weight standards are listed in the legend to Fig. 5. Molecular weights of the viral proteins were estimated by plotting the log of the molecular weight versus the log of mobility.

**Iodination of surface proteins.** Purified  $\phi 6$  and  $\phi 6$  nucleocapsids were iodinated by the method of Palacios (8). Thirty optical density units at  $A_{260}$  of  $\phi 6$  or  $\phi 6$  nucleocapsids were dispersed in 1 ml of 50 mM NaPO<sub>4</sub>, pH 7.8, containing 400  $\mu$ g of lactoperoxidase and 5  $\mu$ l of <sup>125</sup>I (New England Nuclear Corp., carrier-free, 20  $\mu$ Ci/ $\mu$ l). The reaction was initiated by the addition of 100  $\mu$ l of freshly diluted H<sub>2</sub>O<sub>2</sub> (to 90  $\mu$ m) and incubated at room temperature for 2 h. The samples were then dialyzed overnight against three changes of 1,000 volumes of 12.5 mM KPO<sub>4</sub> buffer, pH 7.2, and labeled virus was purified on 10 to 40% sucrose density gradients as described above.

Protein from the <sup>125</sup>I-labeled phage particles were examined by SDS-polyacrylamide gel electrophoresis (7.5, 10, and 15% acrylamide) (21), stained with Coomassie blue, destained, and scanned on a Gilford linear transport gel scanner. The stained bands were excised, swollen in 9:1 (vol/vol) Nuclear Chicago solubilizer-water (23), and counted in an Omnifluor-toluene counting solution.

**RNA in  $\phi 6$  and  $\phi 6$  nucleocapsid.** The phage or phage nucleocapsids were incubated in STE buffer (50 mM Tris, 0.1 M NaCl, mM EDTA, pH 8.0) containing 2.5% sodium *N*-lauroyl sarcosine for 15 min at room temperature to dissociate the RNA from the viral particles (3). The mixtures were layered directly on linear log sucrose density gradient columns (2) containing 2 $\times$  SSC (SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and centrifuged for 15 h at 14 C at 30,000 rpm in a Spinco SW41 rotor.

To determine the sensitivity of endogenous nucleocapsid double-stranded RNA (dsRNA) to exogenous RNase, purified  $\phi 6$  and  $\phi 6$  nucleocapsids were incubated for 15 min at 25 C in 12.5 mM KPO<sub>4</sub>, pH 7.2, containing 10  $\mu$ g of RNase A per ml and subsequently centrifuged on sucrose density gradients. The virus zones were collected, and the RNA was extracted and analyzed by sucrose density gradient centrifugation.

**Stability of  $\phi 6$  and  $\phi 6$  nucleocapsid.** The effect of pH on the integrity of  $\phi 6$  and  $\phi 6$  nucleocapsids was determined by incubating the particles at 0 C for 1 h in the presence of 0.02 M buffers containing 0.4 M NaCl. The buffers were succinate (pH range, 4 to 6), KPO<sub>4</sub> (pH range, 6 to 8), Tris (pH range, 7 to 9), and glycine (pH range, 8 to 11). After incubation the

mixtures were layered on 10 to 40% (wt/vol) linear sucrose density gradient columns equilibrated with 12.5 mM KPO<sub>4</sub> buffer, pH 7.2, and centrifuged at 23,000 rpm for 150 min at 4 C in a Spinco SW27 rotor fitted with buckets (<sup>5</sup>/<sub>8</sub> by 4 inches [ca. 1.6 by 10 cm]). The gradients were scanned photometrically at  $A_{254}$  with an ISCO density gradient fractionator (Instrumentation Specialties Co.).

The effects of NaCl, magnesium acetate, manganese acetate, and EDTA on  $\phi 6$  and  $\phi 6$  nucleocapsids were determined after incubating the mixtures for 1 h at 0 C and then centrifuging the samples on sucrose density gradients equilibrated with 12.5 mM KPO<sub>4</sub>, pH 7.2.

**Other determinations.** RNA polymerase activity of phage nucleocapsids was assayed by procedures previously described (19). Protein was determined by the method of Lowry et al. (7) with bovine serum albumin as a standard. RNA content was determined by the orcinol procedure (13) with yeast RNA as a standard.

Antisera to  $\phi 6$  and  $\phi 6$  nucleocapsid were prepared as previously described (17). The ability of  $\phi 6$  or  $\phi 6$  nucleocapsids to react with various dilutions of the two antisera was determined by the procedure of Ball and Brakke (1).

## RESULTS

**Isolation of  $\phi 6$  nucleocapsids.** Sucrose density gradient-purified  $\phi 6$  was incubated for 15 min at 0 C with various detergents (Triton X-100 [nonionic, Rohm and Haas, Inc.], Nonidet P-40 [nonionic, Shell Chemicals Co.], Emulphogene [nonionic, General Aniline and Film Corp.], sodium deoxycholate [anionic, Sigma Chemical Co.], SDS [anionic, Sigma Chemical Co.], Igepon T-73 [anionic, General Aniline and Film Corp.], sodium *N*-lauroyl sarcosine [anionic, Sigma Chemical Co.], ethylhexadecyldimethyl ammonium bromide [cationic, Eastman Kodak Co.], or dodecyltrimethyl ammonium bromide [cationic, Eastman Kodak Co.]) and then centrifuged on sucrose density gradients. Figure 1 shows centrifugation profiles of untreated  $\phi 6$  or  $\phi 6$  treated with 2.5% (wt/vol) Triton X-100 or 0.25% (wt/vol) sodium *N*-lauroyl sarcosine. Sodium *N*-lauroyl sarcosine treatment dissociated the phage (Fig. 1 C), whereas Triton X-100 (Fig. 1 B) yielded a component that sedimented just slightly slower than untreated  $\phi 6$  (Fig. 1 A). Treatment of the phage with 0.25 to 2.5% (wt/vol) Nonidet P-40 or Emulphogene gave the same effect as Triton X-100. The other detergents dissociated the virus. As will become apparent, Triton X-100, Nonidet P-40, or Emulphogene treatment of  $\phi 6$  removes the  $\phi 6$  envelope.

**Evidence for a  $\phi 6$  nucleocapsid.** Electron micrographs of  $\phi 6$  and  $\phi 6$  treated with Triton X-100 are shown in Fig. 2. Treatment of the intact virion removed the envelope and re-

vealed an icosahedral particle with a diameter of 60 nm (Fig. 2B). Equilibrium density gradient centrifugation of untreated  $\phi 6$  and Triton X-100-treated  $\phi 6$  in  $\text{Cs}_2\text{SO}_4$  is shown in Fig. 3. Untreated  $\phi 6$  had a buoyant density of 1.22 g/cm<sup>3</sup> (Fig. 3A), and Triton X-100-treated  $\phi 6$  had

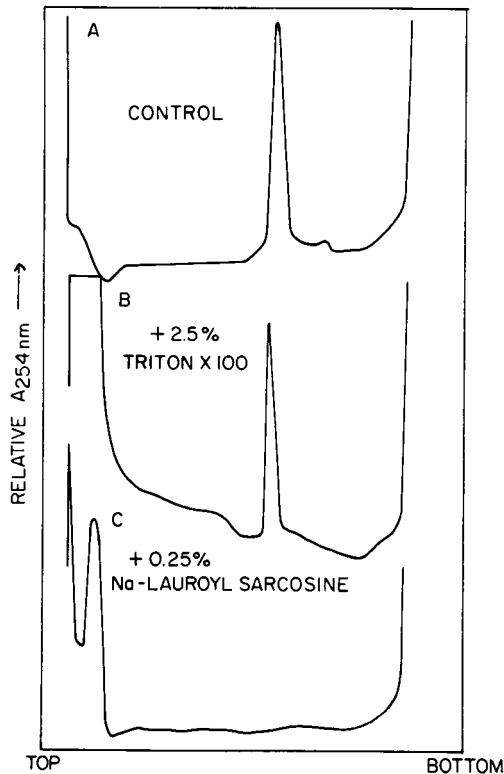


FIG. 1. Centrifugation of 0.5 optical density units at  $A_{260}$  of  $\phi 6$  on 10 to 40% (wt/vol) linear sucrose density gradients for 140 min at 23,000 rpm at 4 C in a Spinco SW27 rotor fitted with buckets ( $\frac{3}{8}$  by 4 inches [ca. 1.6 by 10 cm]). The phage was (A) untreated, (B) treated with 2.5% Triton X-100, or (C) treated with 0.25% sodium N-lauroyl sarcosine prior to centrifugation.

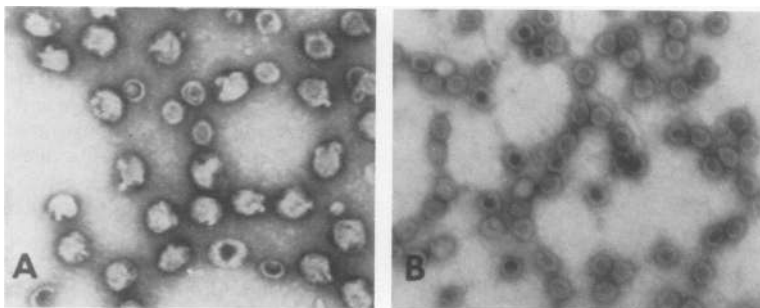


FIG. 2. Uranyl acetate negatively stained (A)  $\phi 6$  and (B)  $\phi 6$  nucleocapsid.  $\times 65,000$ .

a buoyant density of 1.33 g/cm<sup>3</sup> (Fig. 3B). Removal of a lipid-containing envelope from a virus would be expected to increase its buoyant density. Phage  $\phi 6$  treated with either Triton X-100, Nonidet P-40, or Emulphogene formed a precipitated band during equilibrium density gradient centrifugation in  $\text{CsCl}$ . Estimates of the buoyant density of this band were 1.35 to 1.37 g/cm<sup>3</sup>. These densities are similar to those of other dsRNA viruses that lack a lipid envelope (22).

Figure 4 provides evidence that most, if not all, of the lipid in the  $\phi 6$  virion is associated

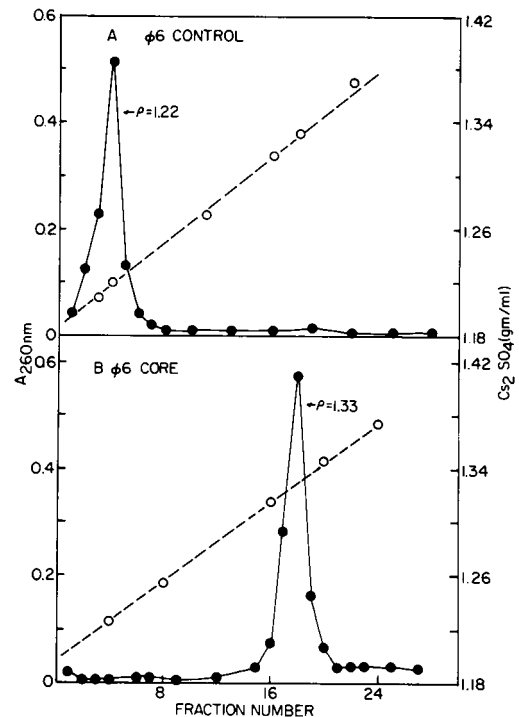


FIG. 3. Density equilibrium centrifugation in  $\text{Cs}_2\text{SO}_4$  of (A)  $\phi 6$  and (B)  $\phi 6$  treated with 2.5% Triton X-100.

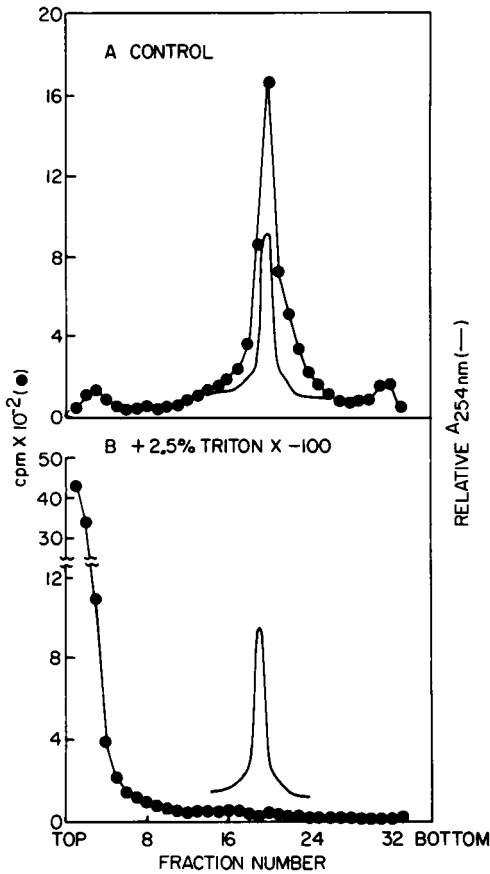


FIG. 4. Centrifugation of phage  $\phi 6$  labeled with [ $2\text{-}^3\text{H}$ ]glycerol on 10 to 40% (wt/vol) linear sucrose gradients for 140 min at 23,000 rpm at 4 C in an SW27 rotor. The phage was (A) untreated, or (B) treated with 2.5% Triton X-100 prior to centrifugation.

with the envelope. The phage was purified from lysates of infected cells growing on a medium containing [ $2\text{-}^3\text{H}$ ]glycerol. Radioactivity from [ $^3\text{H}$ ]glycerol labeled in the 2 position can only be incorporated into glycerol containing lipids, since the tritium label is lost to the medium in the pathways leading to amino acid biosynthesis (4). Treatment of this  $^3\text{H}$ -labeled phage with 2.5% Triton X-100 completely dissociated the radioactivity from the phage. Identical results were also obtained with Nonidet P-40 or Emulphogene.

The nucleocapsid consisted of about 28% RNA and 72% protein. Sucrose density gradient centrifugation of RNA isolated from the nucleocapsids showed the normal distribution of the three dsRNA components. Treatment of the nucleocapsids with 10  $\mu\text{g}$  of RNase A per ml in a low-salt buffer had no effect on the sedimenta-

tion properties of dsRNA subsequently isolated from these treated particles.

**Proteins in  $\phi 6$  and  $\phi 6$  nucleocapsids.** The mature phage contained nine clearly defined proteins (Fig. 5) with estimated molecular weights of: P1, 77,000; P2, 67,000; P3, 65,000; P4, 33,500; P5, 23,500; P6, 16,500; P7, 15,500; P8, 14,000; and P9, 12,500. In addition, the phage contained a rapidly migrating, weakly staining, broad band (P10) that is not shown in Fig. 5. Purified nucleocapsids were devoid of P3, P5, P6, P9, and P10. Most viral preparations also contained a band that migrated slightly faster than P4. Under certain conditions, the intensity of this band increased at the expense of the intensity of P4; thus, it is probably a degradation product of P4. In addition, a few other minor bands were observed after electrophoresis of  $\phi 6$ ; at present we do not know if these proteins are integral components of the phage or contaminant proteins.

Enzymatic iodination of  $\phi 6$  or  $\phi 6$  nucleocapsid, which should preferentially label the surface of the particles, revealed that one protein in each type of particle was labeled to a greater extent than the others (Table 1). P3 was the major protein labeled in  $\phi 6$ , and P8 was the major protein labeled in  $\phi 6$  nucleocapsids. In addition, small amounts of radioactivity were associated with all of the phage proteins, indicating that all of the proteins were susceptible to labeling and that a minor amount of nonenzymatic labeling occurred. Thus, these experiments suggest that P3 is located on the outer surface of the  $\phi 6$  lipid envelope, whereas P8 is probably located on the outer surface of the nucleocapsid.

**Serology.** The cross-reactivity of  $\phi 6$  and  $\phi 6$  nucleocapsid antigens and antisera are reported in Table 2. Antiserum to purified  $\phi 6$  nucleocapsid reacted strongly with  $\phi 6$  nucleocapsid and only slightly with purified virus. The reaction of nucleocapsid antiserum with purified  $\phi 6$  varied with the  $\phi 6$  preparation and was probably due to damage to some of the virions during purification.

**Stability of  $\phi 6$  and  $\phi 6$  nucleocapsid.** Both  $\phi 6$  and  $\phi 6$  nucleocapsids retained their structural integrity at pH values of 6 to 9.5 for at least 1 h at 0 C. At somewhat higher and lower pH values, the intact virion was slightly more stable than the nucleocapsid. Both  $\phi 6$  and  $\phi 6$  nucleocapsid were stable in 0.1 to 0.5 M NaCl and 0.001 to 0.1 M magnesium acetate for 1 h at 0 C; however, both particles were dissociated by manganese acetate concentrations greater than 0.01 M. The two particles differed upon treatment with EDTA. Concentrations of 0.05 M EDTA had no effect on  $\phi 6$ , whereas concentra-

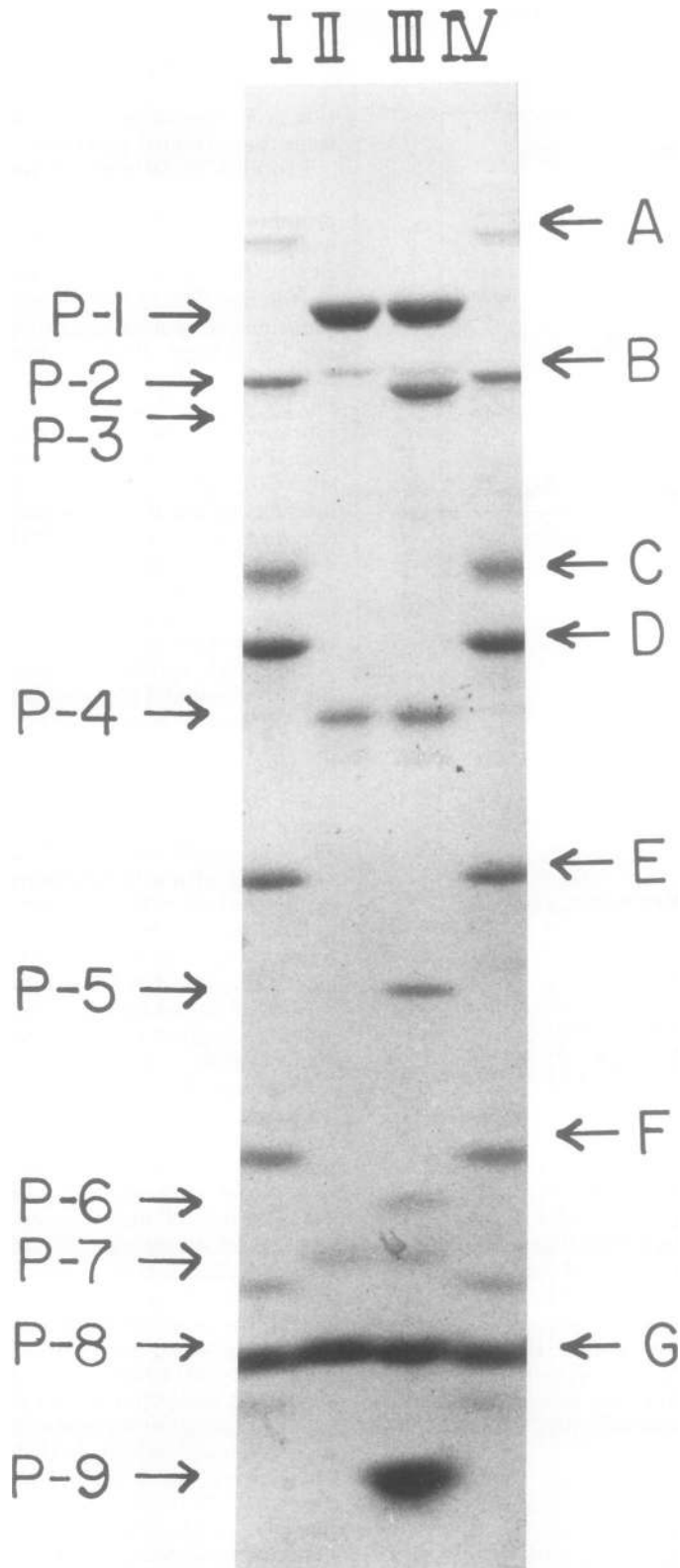


FIG. 5. Polyacrylamide gel electrophoresis of  $\phi 6$  (column II) and  $\phi 6$  nucleocapsid proteins (column III). Molecular weight standards were subjected to electrophoresis in columns I and IV. A, Phosphorylase A (94,000); B, bovine serum albumin (67,000); C, ovalbumin (43,000); D, rabbit muscle aldolase (40,000); E, concanavalin A (25,600); F, tobacco mosaic virus coat protein (17,400); and G, lysozyme (14,400). All samples were electrophoresed in adjacent positions in a single polyacrylamide gel slab.

TABLE 1. Enzymatic labeling of  $\phi 6$  and  $\phi 6$  nucleocapsid proteins with  $^{125}\text{I}$ 

Protein	$\phi 6$		$\phi 6$ Nucleocapsid	
	Counts/min	%	Counts/min	%
P1	2,057	4.2	8,720	19.0
P2	1,646	3.4	2,057	4.5
P3	35,832	73.9	ND <sup>a</sup>	
P4	1,489	3.1	3,632	7.9
P5	1,413	2.9	ND	
P6	13	0.01	ND	
P7	996	2.1	1,186	2.6
P8	4,247	8.8	30,091	65.9
P9, P10	836	1.7	ND	

<sup>a</sup> ND, Not detectable.

TABLE 2. Effect of  $\phi 6$  or  $\phi 6$  nucleocapsid antisera on  $\phi 6$  and  $\phi 6$  nucleocapsids<sup>a</sup>

Antiserum	Antiserum dilution	% of particles removed	
		$\phi 6$	$\phi 6$ nucleocapsid
None		0	0
$\phi 6$	1:100	100	90
	1:500	89	40
	1:1000	62	22
	1:10,000	0	0
$\phi 6$ nucleocapsid	1:10	19	100
	1:20	7	100
	1:50	4	100
	1:200	0	30

<sup>a</sup> The  $\phi 6$  or  $\phi 6$  nucleocapsids were mixed with the antisera by incubating at room temperature for 1 h, followed by 24 h at 4 C. Samples were then centrifuged on sucrose density gradients, scanned, and the areas under the appropriate peaks were measured with a planimeter.

tions of EDTA as low as  $10^{-4}$  M dissociated the  $\phi 6$  nucleocapsids.

**RNA polymerase activity by the nucleocapsids.** We reported previously that  $\phi 6$  contained RNA polymerase activity that was only detected after the phage was subjected to short heat treatments (19). The data in Table 3 indicate that the  $\phi 6$  nucleocapsid also had RNA polymerase activity. The nucleocapsids, in contrast to the phage particles, did not require heat to activate the enzyme. In fact, heat treatment reduced activity.

## DISCUSSION

The enveloped bacteriophage  $\phi 6$  has a diameter of 75 nm (C. Gonzalez, W. Langenberg, J. Van Etten, and A. Vidaver, in preparation). Treatment of the phage with mild nonionic de-

TABLE 3. Ability of  $\phi 6$  and  $\phi 6$  nucleocapsid to incorporate [ $^3\text{H}$ ]UMP or [ $^3\text{H}$ ]CMP into RNA

Particle	Heat treatment	[ $^3\text{H}$ ]ribonucleotide monophosphate incorporated <sup>a</sup>	
		UMP	CMP
$\phi 6$	None	850	
$\phi 6$	10 s at 60 C	18,267	
$\phi 6$ nucleocapsid	None	10,470	98,466
$\phi 6$ nucleocapsid	10 s at 60 C	3,000	6,960

<sup>a</sup> Counts/min incorporated per 0.55 optical density units at  $A_{260}$  of particle per 20 min.

tergents released a nucleoprotein capsid measuring 60 nm in diameter. In this respect,  $\phi 6$  is the first bacteriophage that resembles the enveloped viruses of many eukaryotic organisms. The removal of the  $\phi 6$  envelope releases 5 of the 10 major proteins as well as all of the viral lipid. Iodination experiments suggest that protein P3 is located on the outer surface of the mature phage, whereas P8 is located on the outer surface of the nucleocapsid.

The nucleocapsids contain all three dsRNA segments and they are protected from degradation by exogenous RNase under conditions (low salt) favorable for degradation of exposed dsRNA. The whole phage contains 13% RNA, 25% lipid, and 62% protein (16), whereas the nucleocapsid consists of about 28% RNA and 72% protein. Thus treatment of  $\phi 6$  with Triton X-100 removed about 50% of the viral weight if one assumes that no RNA was released by the detergent treatment.

The combined molecular weight of the major  $\phi 6$  proteins P1 through P9 is about  $3.25 \times 10^5$ . The combined molecular weights of the three dsRNA's in  $\phi 6$  is estimated to be about  $10^7$  (18), which is sufficient to code for  $5.0 \times 10^5$  daltons of proteins. Thus, the phage contains more than enough genetic information to code for its major proteins.

While this paper was being written, Sinclair et al. (14) reported results of studies on the proteins in  $\phi 6$ . They also concluded that the phage contained 10 major proteins. However, in general, their molecular weight estimates for the  $\phi 6$  proteins were slightly larger (ca. 20%) than ours. This difference probably reflects differences in sample treatment. We found the mobilities of some reduced protein standards, particularly bovine serum albumin, which has 17 disulfide bonds, to be erratic and, therefore, chose to alkylate them prior to electrophoresis. Sinclair et al. (14) reported that after 1% Triton X-100 treatment of the phage, proteins P3, P9, and P10, no longer pelleted with the virus and

that the pellet was depleted in P6. In our experiments, nucleocapsids purified on sucrose density gradients after Triton X-100 treatment completely lost proteins P3, P5, P6, P9, and P10.

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