Adsorption Protein of the Bacteriophage fd: Isolation, Molecular Properties, and Location in the Virus†

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ABSTRACT: The adsorption (minor coat) protein of the bacteriophage fd has been implicated to function in several steps of viral morphogenesis. The protein has been purified by sodium dodecyl sulfate gel filtration after dissociation of the virus. The adsorption protein preparation was estimated to have less than 5% contamination by analysis on sodium dodecyl sulfate-polyacrylamide gels and by the results of semiquantitative dansyl-Edman degradation. The amino-terminal sequence of the adsorption protein is: H_2N-Ala-Glx-Thr-Val-Glx-Ser-Pro-Leu-Pro-. Carboxypeptidase A plus B digestion of the protein under a variety of denaturing conditions did not release any amino acids. There are 3-4 adsorption proteins per virion as estimated by the distribution of [^{14}C]leucine between the major and minor coat protein peaks on sodium dodecyl sulfate-polyacrylamide gels. Adsorption protein-specific antibodies were induced in the rabbit and used as electronmicroscopic markers to determine the position of the adsorption proteins in the viral particle. The adsorption proteins were found at only one end of the filamentous viral particle.

The bacteriophage fd and its very close relatives f1 and M13 are viruses which infect *Escherichia coli* carrying an F sex factor (for reviews see: Marvin and Hohn, 1969; Kornberg, 1974). These filamentous bacteriophages are rod-shaped nucleoprotein particles of dimensions 60 × 8800 Å and are composed of a single-stranded circle of DNA surrounded by a protein coat (Marvin and Hoffmann-Berling, 1963; Marvin, 1966; Marvin and Schaller, 1966; Frank and Day, 1970). The viral coat is composed of about 2400 major coat or B protein subunits arranged in a helix around the DNA (Marvin et al., 1974; Berkowitz and Day, 1976). In addition, there are 3-4 copies of a minor coat or adsorption protein (Rossomando and Zinder, 1968; Henry and Pratt, 1969; Beaudoin, 1970; Wiseman et al., 1972). These viruses are among the simplest biological systems known requiring only eight viral gene products to direct their entire life cycle (Pratt et al., 1966, 1969). The simplicity of these viruses facilitates the study of individual proteins which are responsible for morphogenesis. The adsorption protein, a product of gene 3, plays an important role in this process (Rossomando and Zinder, 1968; Pratt et al., 1969; Henry and Pratt, 1969). It functions in attachment of the virus to the host receptor (Pratt et al., 1969) and it may also function in penetration (Marco et al., 1974) and in the initial stages of viral DNA replication (Jazwinski et al., 1973). The protein has been called a "pilot" protein by Arthur Kornberg (1974) because it may lead the viral DNA into the host cell and to its presumed site of DNA replication.

Up to now, the function of the adsorption protein has been inferred from genetic, electron microscopic, and molecular biological studies. A prerequisite to an understanding of the function of the adsorption protein is to isolate it in pure form. In this paper, a method is described for the isolation of milligram quantities of the adsorption protein of bacteriophage fd. This purification scheme was developed following the suggestion of Rossomando and Zinder (1968) that the protein could be purified by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis because of the molecular weight difference between the major and minor coat proteins. The results of the molecular weight, amino acid composition, and amino-terminal sequence determinations are also presented. Antiserum against the purified adsorption protein has been prepared and used to locate the position of the adsorption protein on the virus by electron microscopy. A report on the isolation of the adsorption protein from the bacteriophage fd has appeared (Segawa et al., 1975). The following paper in this issue contains the results of similar studies on the adsorption protein of the bacteriophage f1 (Woolford et al., 1977).

Experimental Procedure

Materials. All chemicals were obtained from conventional sources except as noted below. Protein chemistry reagents were purchased from Pierce except formic acid (97%) (Aldrich) and polyamide layer sheets (Gallard-Schlesinger). Carboxypeptidase A-DFP and B-DFP were obtained from Worthington. Radioactive amino acids [^{3}H]histidine (NET-146) and [^{14}C]leucine (NEC-169) were purchased from New England Nuclear and [^{14}C]leucine (3122-41) from Schwarz/Mann. The goat antiserum to rabbit IgG was obtained from Kallestad and the sodium dodecyl sulfate from Alcolac. The ferritin (six times recrystallized) was from Miles and was a gift from Ms. B. Wallace.

Large-Scale Bacteriophage Preparation and Purification. Large quantities of fd virus (Marvin and Hoffmann-Berling, 1963) were grown in 15 L of 4YT medium (Osborn et al., 1970). The medium was innoculated with a 600-mL culture of *E. coli* strain DM 48 (Hohn et al., 1971) in N7 medium (Tseng et al., 1972) and was incubated at 37 °C until the cell density reached 10^8 cells/mL. The culture was then infected with 10^9 virus/mL and growth was allowed to proceed for 5-6 h. Yields of 3 × 10^{12} virus/mL were common. The bacteria were removed from the medium by centrifugation at 15 000*g* for 30 min and the virus was precipitated from the supernatant by the addition of 3.4% polyethylene glycol and 0.5 M NaCl (Yamamoto et al., 1970). The precipitated virus settled out of

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solution after 48 h. Most of the supernatant was removed by siphoning and the precipitated virus was collected by centrifugation at 15 000*g* for 30 min. The pellet was slowly resuspended in 200–300 mL of N1 medium (Tseng et al., 1972) and the virus was purified by differential centrifugation with a short centrifugation at 52 000*g* for 45 min and then a longer centrifugation at 35 000*g* for 12–15 h to pellet the virus. The pelleted virus was resuspended slowly in N1 medium. The virus was recentrifuged at 52 000*g* for 45 min to remove debris and diluted to a final concentration of 3.4 mg/mL ($E_{260}^{1\%} = 37.4$) (Hoffmann-Berling et al., 1963). The preparation was further purified by a velocity–density gradient centrifugation according to a modified method of Yamamoto et al. (1970) in which KBr was substituted for CsCl. The virus band was collected and dialyzed against distilled water. The preparation was centrifuged at 13 000*g* for 30 min and the supernatant was divided into aliquots and pelleted at 52 000*g* for 12 h. The virus pellets were stored at −70 °C.

Labeled Bacteriophage Preparation and Purification. Several radioactive labels were incorporated into the virus. [^{3}H]Histidine-labeled viruses were grown in 50 mL of N13 medium (Tseng et al., 1972) plus 5 μg/mL histidine and 20 μg/mL all other amino acids, [^{14}C]leucine-labeled viruses were grown in 20 mL of N13 medium plus 0.05% casamino acids, and [^{14}C]leucine/[^{3}H]histidine labeled viruses were grown in 10 mL of N13 plus 5 μg/mL histidine and 20 μg/mL all other amino acids. In all cases, a culture of DM48 in N7 medium was diluted 1:100 into the various media and the bacteria were grown at 37 °C to a cell density of 10^8 cells/mL. The culture was infected with 10^9 virus/mL, the label (1 mCi of [^{3}H]histidine, 10 μCi of [^{14}C]leucine, or 0.5 mCi of [^{3}H]histidine and 0.03 mCi of [^{14}C]leucine) was added, and the infected cells were allowed to grow for 5–6 h. The yield was approximately 3×10^{11} virus/mL. Bacteria were removed from the infected cultures by centrifugation at 6500*g* for 30 min. The virus was precipitated from the supernatant with polyethylene glycol, and the viral precipitate was collected by centrifugation at 6500*g* for 30 min. The resuspended virus was purified by differential centrifugation, first with a low-speed centrifugation at 40 000*g* for 30 min, followed by a high-speed centrifugation at 190 000*g* for 3 h. The viral pellet was resuspended and purified by velocity sedimentation through a 5 to 20% (w/w) sucrose gradient which was centrifuged at 150 000*g* for 70 min in a Beckman SW50.1 rotor. The material in the peak which contained the virus was collected and the virus was pelleted by centrifugation at 190 000*g* for 3 h and the pellet was resuspended in 0.5 mL of distilled water. The [^{14}C]leucine/[^{3}H]histidine labeled virus for the estimation of the number of adsorption proteins per virion was further purified by a second sucrose gradient centrifugation. Following both sucrose gradients, only the center fractions of the viral peak were pooled to avoid the inclusion of multiple-length viral particles and mini or incomplete particles.

Adsorption Protein Purification. The viral pellets were resuspended in distilled water to give a final concentration of 16 mg/mL. Approximately 10^4 cpm of [^{3}H]histidine-labeled virus was routinely added to each preparation. Radioactive labeling of the adsorption protein was necessary because its concentration was too dilute for optical monitoring. The major coat protein was followed by the addition of [^{14}C]leucine-labeled virus to some of the preparations. An equal volume of a solution containing 10% sodium dodecyl sulfate, 2% 2-mercaptoethanol, and 0.02 M Tris[1]-acetate (pH 5.4) was added to the virus solution and the resulting mixture was stirred gently until the virus was disrupted. After dissociation of the virus, the solution was brought to neutrality with saturated sodium hydroxide. Magnesium chloride was added to give a final concentration of 0.1 M and the mixture was centrifuged at 150 000*g* for 12 h which pelleted 90% of the DNA. The protein solution was passed over a 5.5 × 85 cm Bio-Gel A1.5m (200–400 mesh) column equilibrated with a solution of 1% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol, and 0.01 M Tris-HCl (pH 7.5). The proteins were eluted from the column and the [^{3}H]histidine-labeled peak (80–90% of the total ^{3}H counts) were pooled and concentrated to 10 mL by pressure dialysis in an Amicon ultrafiltration cell (Model 52) with an XM50 membrane. During Amicon filtration, the solution was diluted with water and reconcentrated several times in order to reduce the concentration of sodium dodecyl sulfate in the buffer. The protein was precipitated by adding the solution to nine volumes of acetone (Weiner et al., 1972). The precipitated protein was then collected by centrifugation at 6500*g* for 10 min and stored as a frozen dry powder for future experiments. Before use, the protein was resuspended in 0.5 mL of 0.1% sodium dodecyl sulfate. The yield of purified adsorption protein from this procedure was approximately 1.25 mg/240 mg of virus.

Amino Acid Composition. Amino acid analysis was performed on a Beckman 121-M amino acid analyzer. The protein (0.5 nM in 0.15 mL of 6 N HCl containing 0.2% phenol) was hydrolyzed in vacuo at 110 ± 2 °C for 24, 48, and 72 h. The relative concentration of most of the amino acids was determined by averaging the results of six analyses (two at each hydrolysis time). The values for threonine and serine were corrected for destruction during acid hydrolysis and the value for valine was determined from the 72-h hydrolysis samples. The concentration of cysteine was determined following performic acid oxidation and acid hydrolysis of the adsorption protein (Hirs, 1967). Tryptophan was determined following hydrolysis in 3 N mercaptoethanesulfonic acid (Penke et al., 1974). A blank containing lysozyme and the sodium dodecyl sulfate column elution buffer was analyzed in parallel with the sample to determine a correction factor for the percentage of tryptophan destruction under these conditions.

Dansyl–Edman Degradation. The dansyl-Edman procedure for amino-terminal sequencing of the adsorption protein was essentially the method of Weiner et al. (1972) with the following modifications. The adsorption protein was resuspended to a final concentration of 40 nM/mL in 0.5 M $NaHCO_3$, 1% sodium dodecyl sulfate. The protein solution was divided into nine 4 nM aliquots which were put into small Pyrex test tubes. All steps of the procedure including hydrolysis were performed in these tubes to eliminate transfer losses. The coupling reagent PITC (0.01 mL) was added to each aliquot and the tubes were incubated at 50 °C for 1 h. The coupling procedure was repeated to keep the degradation completely in phase. The coupled amino-terminal residue was cyclized and cleaved by the addition of 0.1 mL of F_3AcOH. After dansylation, the protein plus the sodium bicarbonate was precipitated with 2 mL of acetone. It was washed with 10% Cl_3AcOH, which dissolved the sodium bicarbonate precipitate, and then with 1 N HCl. The dansylated protein was hydrolyzed, the residue after drying was resuspended in 0.01 mL of ethyl acetate or ethyl acetate–pyridine–water (60:1:1), and 2 μL was spotted for chromatography. The dansylated amino acids were separated by chromatography and were identified by comparison with a set of standards which had been chromato-

[1] Abbreviations used are: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; TEMED, *N,N,N′,N′*-tetramethylethylenediamine.

TABLE I: Denaturing Conditions for the Solubilization, Dissociation, and Digestion of the Protein.[a]

Dissociation conditions	Digestion conditions
0.02% $NaDodSO_4$, 0.02 M $NaHCO_3$; heated to 100 °C for 3 min	0.02% $NaDodSO_4$; 0.02 M $NaHCO_3$
0.2% $NaDodSO_4$, 0.2 M $NaHCO_3$; heated to 100 °C for 3 min	0.2% $NaDodSO_4$; 0.2 M $NaHCO_3$
8 M urea, 0.2 M $NaHCO_3$; heated to 100 °C for 3 min	5 M urea, 0.12 M $NaHCO_3$
8 M urea; 0.1% $NaDodSO_4$, 0.2 M $NaHCO_3$; heated to 100 °C for 3 min	5 M urea, 0.06% $NaDodSO_4$, 0.12 M $NaHCO_3$
10 M urea, 0.2 M $NaHCO_3$; incubated 25 °C for 15 min	4 M urea, 0.08 M $NaHCO_3$
10 M urea, 0.2 M $NaHCO_3$; incubated 25 °C for 15 min	3 M urea, 0.06 M $NaHCO_3$
10 M urea, 0.2 M $NaHCO_3$; incubated 25 °C for 15 min	2 M urea, 0.04 M $NaHCO_3$
Succinylated protein (Klotz, 1967) in 0.1% $NaDodSO_4$, 0.2 M $NaHCO_3$; heated to 100 °C for 3 min	0.1% $NaDodSO_4$, 0.2 M $NaHCO_3$
0.1% $NaDodSO_4$, 0.2 M sodium acetate (pH 6.0); heated to 100 °C for 3 min	0.1% $NaDodSO_4$, 0.2 M sodium acetate (pH 6.0); carboxypeptidase A only

[a] Abbreviation used: $NaDodSO_4$, sodium dodecyl sulfate.

graphed on the reverse side of the polyamide plate.

Carboxypeptidase Digestion. Carboxypeptidases A and B were used in an attempt to release amino acids from the carboxy-terminal end of the adsorption protein (Ambler, 1972). The carboxypeptidase A solution was prepared by diluting 5 μL of (70 mg/mL) enzyme stock solution into 0.1 mL of 1 M $NaHCO_3$, 37 °C for 2 min. This solution was diluted with 1.9 mL of 0.1 M $NaHCO_3$ and incubated at and divided into 0.1-mL aliquots. For the carboxypeptidase B solution, 0.05 mL of (8 mg/mL) enzyme stock solution was diluted into 1.95 mL of 0.1 M $NaHCO_3$ and divided into 0.1-mL aliquots. The aliquots were stored at −20 °C until needed. For each digestion, aliquots were defrosted and utilized, and the unused portion was discarded. The protein was solubilized, dissociated, and digested under a variety of denaturing conditions which are listed in Table I. The digestion conditions, at the bottom of the list, were selected to facilitate the release of acidic residues (Titani et al., 1962). However, the effectiveness of this procedure as a general method for the removal of these residues has yet to be demonstrated (Ambler, 1972). For all experiments except where noted, 0.01 mL of each enzyme was added to 5 nM protein and the digestion was allowed to proceed at 37 °C for 1 h. The digestion was terminated by the addition of 0.01 mL of 6 N HCl and the samples were lyophilized and then run on the amino acid analyzer. A variety of controls with the α and β chain of hemoglobin were run to ensure that the carboxypeptidases B and A were active. The controls were run alone and together with the adsorption protein to confirm that there was no material in the adsorption protein preparations which would inactivate the enzymes.

Sodium Dodecyl Sulfate-Polyacrylamide Gels for Molecular Weight Determinations. The two sodium dodecyl sulfate-polyacrylamide gel systems which were used to determine the molecular weight of the adsorption protein were the sodium dodecyl sulfate-urea system of Henry and Pratt (1969) and the method of Weber and Osborn (1969). Slab gels were used so that the mobilities of the proteins could be accurately compared. The protein dissociation mixture for both gel systems was 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, and 0.01 M sodium phosphate, pH 7.0. The protein samples (1 mg/mL maximum concentration) were dissociated by heating at 100 °C for 3–5 min. The standard proteins used for the molecular weight determinations were transferrin (mol wt 76 600), catalase (mol wt 60 000), γ-globulin, heavy chain (mol wt 50 000), ovalbumin (mol wt 43 000), glyceraldehyde phosphate dehydrogenase (mol wt 36 000), and γ-globulin, light chain (mol wt 23 500) (Fish et al., 1970; Weber and Osborn, 1969). No carbohydrate was detected when the gel was treated with periodic acid–Schiff reagent.

Polyacrylamide Disc Gel Electrophoresis. The polyacrylamide gels contained 7.5% acrylamide (acrylamide-methylenebisacrylamide = 29:1), 0.1 M sodium phosphate (pH 7.2), 0.1% sodium dodecyl sulfate, and 0.1% TEMED. The gels were polymerized just prior to use by adding 0.05 volume of 1% ammonium persulfate to a stock solution of all of the above reagents and immersing the filled gel tubes in warm water. After polymerization, the gels were prerun in 0.1 M Na_2HPO_4, 0.1% sodium dodecyl sulfate, and 0.2% thioglycolic acid (final pH 7.2) for 15–30 min (Wiseman et al., 1972). For the sample dissociation, 0.05 mL of [^{14}C]leucine/[^{3}H]histidine-labeled virus was dissociated as described for the adsorption protein purification. The samples plus 0.1 volume of glycerol saturated with bromophenol blue were layered on the gels and the gels were run until the tracking dye had migrated half way down the tubes. The gels were crushed and the gel particles were eluted in 0.5 mL of 0.1% sodium dodecyl sulfate. Approximately 50 fractions were taken from each gel. The fractions were incubated at 45 °C for 2–3 days to elute the radioactive material. After the elution, 3.5 mL of Aquasol was added and the fractions were counted. No differential quenching of counts along the length of the gel was observed.

Calculation of the Number of Adsorption Protein Molecules per Virion. Using whole virus labeled with a single radioactive amino acid, the number of adsorption protein molecules per virion can be calculated from the distribution of the label between the minor and major coat protein peaks in sodium dodecyl sulfate-polyacrylamide gels and the ratio of the amino acid (in this case leucine) in the two proteins as follows

$$\left(\frac{\text{counts in A peak}}{\text{counts in B peak}}\right)\left(\frac{\text{mol \% leucine in B}}{\text{mol \% leucine in A}}\right) \times \left(\frac{\text{mol wt B}}{\text{mol wt A}}\right)\left(\frac{\text{no. of B}}{\text{virion}}\right) = \text{no. of A/virion}$$

where A = adsorption protein; B = major coat protein; mol % leucine in B = 4.0 (Asbeck et al., 1969; Nakashima and Konigsberg, 1974); mol % leucine in A = 4.7 (Table I); molecular weight of B = 5240 (Asbeck et al., 1969; Nakashima and Konigsberg, 1974); molecular weight of A = 60 000; number of B/virion = 2400 ± 120 (Berkowitz and Day, 1976).

Double-labeled viruses ([^{14}C]leucine/[^{3}H]histidine) were grown and the proteins were separated on sodium dodecyl sulfate-polyacrylamide disc gels as described above. The [^{3}H]histidine label was used to define the extent of the adsorption protein peak and the [^{14}C]leucine label was used to calculate the ratio of the two coat proteins. The estimation of the number of ^{14}C counts in the adsorption protein peak is complicated, since the passage of highly labeled B protein through the gel causes an elevated background of ^{14}C counts.

The elevated baseline (as determined by averaging the cpm of the fractions on either side of the adsorption protein peak) was subtracted from the cpm in each fraction to avoid inclusion of any counts contributed by B protein. Had a blank gel sample been used to determine the background, the number of adsorption proteins per virion would have been 12% higher. The estimation of the number of adsorption protein molecules per virion is further complicated because all sodium dodecyl sulfate extraction procedures investigated fail to dissociate completely all of the adsorption protein from the DNA. If [^{3}H]histidine-labeled virus was phenol extracted at 60 °C (Marvin and Schaller, 1966), only 8% of the ^{3}H counts remained with the DNA in the aqueous phase. The ^{3}H counts associated with the DNA after phenol extraction are most likely due to low levels of radioactive contaminants in the [^{3}H]histidine, since the percentage of non-phenol-extractable ^{3}H counts varied depending upon the batch of [^{3}H]histidine label used. In these double-label experiments, however, 19% of the [^{3}H]histidine counts remained near the top of the gel with the DNA. Since phenol extraction of sodium dodecyl sulfate extracted virus was found to release additional adsorption protein as judged by sodium dodecyl sulfate-polyacrylamide gel analysis of the extracted material, all phenol-extractable [^{3}H]histidine counts (92% of the total) were presumed to be from the adsorption protein, even though some of the ^{3}H counts did not migrate in the adsorption protein peak. A corrected value for the number of ^{14}C counts in the adsorption protein peak was used in determining the number of adsorption proteins per virion. Had this correction not been made, the final result would have been 13% lower. The results of seven polyacrylamide gels were averaged (standard deviation = 0.15).

Preparation of Antiadsorption Protein Antiserum. A white male New Zealand rabbit was used for the production of antiserum against the adsorption protein of the bacteriophage fd. An emulsion of 2 mg of adsorption protein in 0.5 mL of 0.1% sodium dodecyl sulfate and approximately an equal volume of Freund's complete adjuvant was injected into the foot pads of the rabbit. Approximately 5 weeks later, the animal was boosted by the injection of 2.5 mg of adsorption protein in 0.5 mL of 0.1% sodium dodecyl sulfate into an ear vein. A week later, 30 mL of blood was removed from the animal via cardiac puncture. The blood was incubated at 37 °C for 1 h and at 4 °C overnight to allow the clot to retract. The serum was collected and the remaining red blood cells were removed by centrifugation in a clinical centrifuge for 15 min. The serum was stored at −20 °C until use. The activity of the serum against the virus was checked by an infectivity assay. In this assay, 0.1 mL of serum from the rabbit before and after immunization was suspended in 5 mL of N1 medium and heated to 60 °C for 30 min. After cooling, 0.01 mL of a 4×10^{13} virus/mL solution was added to the antiserum and the mixtures were incubated at 20 °C for 30 min. These two samples plus an untreated virus sample were titered in parallel. Under these conditions, 0.1 mL of antiadsorption protein antiserum inactivated 3.6×10^{11} virus particles relative to the control serum or untreated virus.

Purification of Antiadsorption Protein Antibodies. The serum (1 mL) was centrifuged at 3000*g* for 15 min to remove precipitated material. An equal volume of saturated ammonium sulfate was then added and the mixture was incubated at 4 °C for 30 min. The precipitate was collected by centrifugation at 3000*g* for 15 min and then resuspended in 0.5 mL of water. The ammonium sulfate fractionation, which selects for the γ-globulin fraction of the serum, was repeated twice and the final precipitate was dialyzed against 0.15 M NaCl, 0.01 M sodium phosphate (pH 7.3). To select for viral-specific antibodies, 1 mL of the γ-globulin fraction was mixed with 1 mL of 10 mg/mL purified virus and incubated at 4 °C overnight. The viral-antibody complex was collected by centrifugation at 190 000*g* for 3 h. The pellet was resuspended in 0.5 mL of 0.15 M NaCl overnight and then diluted into 5 mL of 0.15 M NaCl, 0.01 M glycine (pH 2.9). The mixture was then incubated for 6 h at 4 °C. The low pH dissociates the antibody-antigen complex. The virus was removed by centrifugation at 190 000*g* for 3 h and the supernatant was dialyzed against 0.15 M NaCl, 0.01 M sodium phosphate (pH 7.3). The antiadsorption protein antibodies were stored at −20 °C.

Ferritin Conjugation. The γ-globulin fraction of the goat anti-rabbit serum was prepared by ammonium sulfate fractionation, as just described, except that the antibodies were dialyzed against 0.1 M sodium phosphate (pH 7.0). Ferritin was recrystallized and the coupling reaction with 2 mg of antiserum was carried out according to the method of Heitzmann and Richards (1974). The ferritin-antibody conjugate was dialyzed against 0.15 M NaCl, 0.01 M sodium phosphate (pH 7.5) and stored at 4 °C in 0.2% sodium azide.

Electronmicroscope Sample Preparation and Electron Microscopy. The viral-antibody complex was prepared by mixing 50 μL of a solution of 10^{11} virus/mL with 5 μL of purified antiadsorption protein antibodies and incubating at 20 °C for 30 min. The ferritin-antibody conjugate was diluted 1:10, 5 μL of the conjugate was added to the viral-antibody complex, and the mixture was incubated for 30 min. A drop of this sample was put on a formvar-coated 200-mesh copper grid with a thin carbon coating and the material was allowed to settle onto the grid for 30 min. The grid was washed and negatively stained with 2% uranyl acetate for 30 s. Finally, the samples were viewed in a Phillips Model 300 electron microscope.

Results

Attempts to prepare milligram amounts of the adsorption protein in highly purified form have been hampered by a number of problems. One of the difficulties is that the coat proteins are soluble only in detergents or under denaturing conditions (Knippers and Hoffmann-Berling, 1966; Chun et al., 1974; Marco, 1975). This property restricts the type of purification procedures that can be used for the isolation of the adsorption protein. The available approaches are also limited by the tendency of the B protein (mol wt 5240) (Asbeck et al., 1969; Nakashima and Konigsberg, 1974) to aggregate, forming oligomers of molecular weight close to that of the adsorption protein and which copurify with it when the separation is based on molecular size. The degree of B protein aggregation is dependent upon the denaturant or detergent employed, the method of virus disruption, and the subsequent treatment of the viral coat proteins. A disruption procedure using sodium dodecyl sulfate and Tris-acetate (pH 5.4) at 25 °C has given the best results. Heating the virus to 37 °C or above, a procedure commonly used for viral protein disaggregation, was avoided because, in contrast to most systems, this treatment caused aggregation of the B protein. After centrifugation to remove most of the DNA, the major and minor coat proteins were separated by gel filtration using agarose A1.5m equilibrated with 1% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol, and 0.01 M Tris-HCl (pH 7.5), as shown in Figure 1. Two well-resolved peaks of radioactivity were observed. [^{3}H]Histidine, which had been incorporated into whole virus, served as a convenient marker for the adsorption protein, since the B protein lacks histidine, arginine,

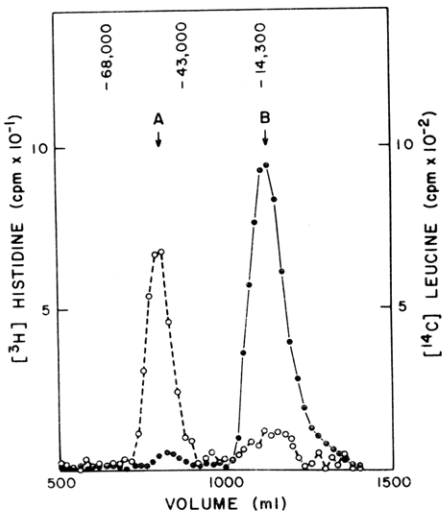

FIGURE 1: Column elution profile of fd viral coat proteins. The elution profile is from a standard protein fractionation as described under Experimental Procedure. The [^{3}H]histidine-labeled material is indicated by open circles. The [^{14}C]leucine material is indicated by closed circles. A marks the position of the adsorption protein. B marks the position of the major coat protein. The position of the molecular weight markers were determined in a separate experiment. For the column standardization, 1.5 mL of 20 mg/mL bovine serum albumin, ovalbumin, and lysozyme was diluted with an equal volume of virus dissociation medium and the mixture was heated to 100 °C for 3 min. Labeled virus was added to the preparation and the proteins were fractionated. The position of the marker proteins was determined by optical density at 280 nm and the viral proteins by radioactivity.

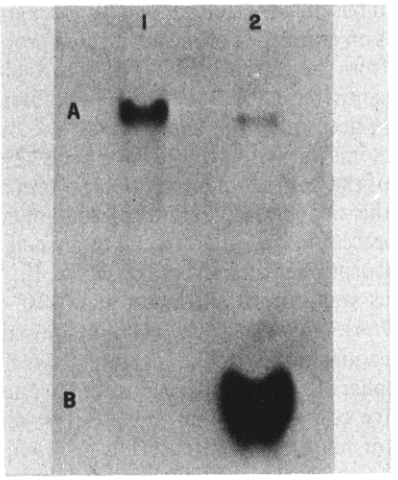

FIGURE 2: Sodium dodecyl sulfate-polyacrylamide gel of purified adsorption protein and whole virus. Approximately 25 μg of purified adsorption protein (slot no. 1) was solubilized as described under Experimental Procedure for the adsorption protein purification except that the mixture was heated to 100 °C for 5 min. Approximately 0.5 mg of whole virus (slot no. 2) was solubilized as described above but without heating. The samples were then analyzed on an sodium dodecyl sulfate-polyacrylamide gel. A marks the position of the adsorption protein. B marks the position of the major cost protein.

and cysteine (Braunitzer et al., 1967). [^{14}C]Leucine was used to identify the location of the B protein. When [^{3}H]arginine was used instead of [^{3}H]histidine, a second peak of tritium appeared at the trailing edge of the B protein peak. This peak, which has also been observed by other workers, may represent a third, very small viral coat protein (Henry and Pratt, 1969; Beaudoin, 1970; Kornberg, 1974). The yield of the adsorption protein was about 30% based on the total amount of tritium in the [^{3}H]histidine-labeled virus used in the purification.

Analytical sodium dodecyl sulfate–polyacrylamide gels of the isolated adsorption protein (Figure 2) indicated that the preparation was greater than 95% pure. Although no band was visible at the position of the B protein, it may have been present in multiple aggregation states which were spread throughout the gel and which do not form a clearly stainable band. The low molecular weight of the B protein means that even a small percentage contamination by weight corresponds to many copies. For this reason, a more stringent criterion of purity was sought. Since amino-terminal sequence analysis, by the dansyl–Edman procedure, is usually capable of revealing low levels of protein impurities, it was applied to the adsorption protein preparation as discussed below.

Sequence Determination. A nine-residue amino-terminal sequence of the adsorption protein was determined in two independent experiments using 4 nM of adsorption protein for each step of the Edman degradation. The sequence is H_2N-Ala-Glx-Thr-Val-Glx-Ser-Pro-Leu-Pro-. Since this sequence does not correspond to any sequence in the B protein and since no contaminating B protein sequences were seen, the adsorption protein preparation appears to be essentially free of B protein (less than 2% contamination by weight).

The adsorption protein preparation was digested with carboxypeptidase A and B but no amino acids were released. The conditions of digestion were such that serine, the carboxy-terminal amino acid of the B protein, would have been liberated if any of this protein were present. The failure to observe this amino acid after carboxypeptidase digestion provides further support for the purity of adsorption protein preparation. It also suggests that either the amino acid at the carboxy terminus of the adsorption protein is one which is not susceptible to the action of these enzymes or that the adsorption protein is not completely denatured under the conditions used with the result that the carboxy-terminal residue is inaccessible.

TABLE II: Amino Acid Composition of the Adsorption Protein from the Bacteriophage fd.[a]

Amino acid	No. of Res.	SD	mol %	mol % of major coat protein
Asp	75.2	0.24	13.2	6.0
Thr	38.2	0.56	6.7	6.0
Ser	43.4	0.60	7.6	8.0
Glu	59.4	0.15	10.4	6.0
Pro	36.7	1.35	6.4	2.0
Gly	91.6	0.25	16.0	8.0
Ala	39.1	0.37	6.8	20.0
Val	28.7	0.09	5.0	8.0
Met	10.3	0.19	1.8	2.0
Ile	12.5	0.19	2.2	8.0
Leu	27.1	0.35	4.7	4.0
Tyr	29.1	0.34	5.1	4.0
Phen	27.0	0.65	4.7	6.0
His	2.9	0.23	0.5	
Lys	19.9	0.93	3.5	10.0
Arg	13.1	0.11	2.3	
Trp	4.6	0.25	0.8	2.0
Cys	12.2	0.93	2.1	
Total	571.0		99.8	100.0

[a] The amino acid composition was determined as described under Experimental Procedure. The mol % of amino acids in the major coat protein was calculated from the amino acid composition (Asbeck et al., 1969; Nakashima and Konigsberg, 1974). Abbreviations used: Res, residues; SD, standard deviation.

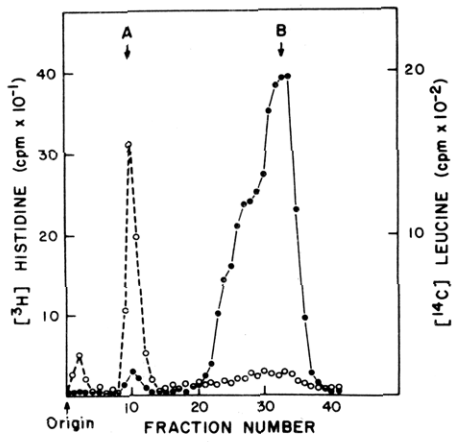

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of [^{14}C]leucine/[^{3}H]histidine virus. Double-labeled virus was dissociated and run on sodium dodecyl sulfate–polyacrylamide gels as described under Experimental Procedure. [^{14}C]Leucine label (●) indicates the distribution of the total viral protein. [^{3}H]Histidine label (○) is associated mainly with the adsorption protein. Direction of migration is left to right. A marks the position of the adsorption protein. B marks the position of the major coat protein.

Amino Acid Composition. The amino acid composition of the adsorption protein is shown in Table II. The maximum variation of these values was ±1.6 residues. In contrast to the B protein, the adsorption protein contains a relatively high percentage of aspartic acid, glutamic acid, and glycine and a low percentage of basic amino acids. The number of residues was calculated using a molecular weight of 60 000 to facilitate a comparison between this preparation and those of Segawa et al. (1975) and Woolford et al. (1977). A search was also made for *O*-phosphorylserine and *O*-phosphorylthreonine as well as amino sugars but none were found.

Molecular Weight of the Adsorption Protein. The molecular weight of the adsorption protein was estimated using polyacrylamide gel electrophoresis in different sodium dodecyl sulfate containing systems. Using the standard 10% sodium dodecyl sulfate–polyacrylamide gels of Weber and Osborn (1969), a molecular weight of 52 000 ± 2000 was obtained. This value is lower than the 60 000 ± 2000 which was found if a sodium dodecyl sulfate system containing 8 M urea was used. It should be noted that urea containing sodium dodecyl sulfate gels are often used for estimating the molecular weight of viral coat proteins. It has become standard practice to use this system with filamentous bacteriophage coat proteins because the major coat protein dimerizes in sodium dodecyl sulfate alone (Knippers and Hoffmann-Berling, 1966; Makino et al., 1975). Since the effect of urea on the molecular weight determination is not known, further investigation will be needed to understand the reasons for the observed differences.

Number of Adsorption Protein Molecules per Virion. Assuming a molecular weight of 60 000 for the adsorption protein, a value of 3.3 adsorption proteins per virion has been found using the distribution of [^{14}C]leucine in labeled virus between the major and minor coat protein peaks on sodium dodecyl sulfate–polyacrylamide gels (Figure 3) and the mol % of leucine in each protein. The accuracy of this value is somewhat uncertain because a portion of the adsorption protein remains associated with the DNA, because the baseline of ^{14}C counts is elevated above background and because the exact molecular weight of the adsorption protein is not known. These factors necessitated several assumptions in the final calculation is discussed under Experimental Procedure.

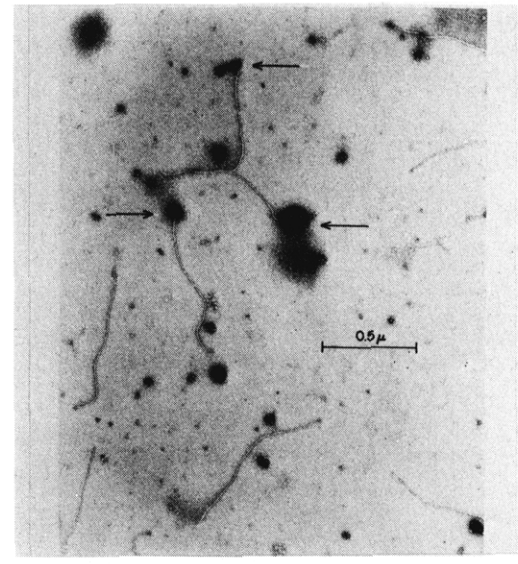

FIGURE 4: Electron micrograph of bacteriophage fd tagged with antiadsorption protein antibodies. The virus was incubated with antiadsorption protein antibodies and with ferritin-conjugated goat anti-rabbit antibodies as described under Experimental Procedure. The field shows some filamentous virus tagged with a cluster of ferritin-conjugated antibodies at one end (arrows), some untagged virus, and some nonspecifically bound ferritin. Magnification is 17 500×.

Localization of the Adsorption Proteins. The location of the adsorption protein in the virion has been determined using adsorption protein-specific antibodies as electron-microscopic markers. The virus antiadsorption protein–antibody complex was reacted with a ferritin–antibody conjugate to increase the number of electron-dense particles which could be bound to the adsorption protein. This labeling enabled a clear indentification of the attachment site. The ferritin-labeled virus showed clusters of ferritin–antibodies covering one end of the virion (Figure 4). The labeling appeared to be antibody dependent, since a reduction in the quantity of adsorption protein specific antibodies reduced the level of ferritin labeling at the end of the virus. A study of antibody-induced agglutination of the virus showed clusters of virus radiating from a single locus (Figure 5) which suggested that the adsorption proteins are located at only one end of the virion.

Ferritin labeling also occurred to some extent along the entire length of the virion. However, it was impossible to determine whether this labeling represented specific antibody binding or was merely an artifact of sample preparation. Therefore, a chi-square analysis of the data was done (Table III) to determine if the observed labeling was random. The analysis indicated that the probability of obtaining these results by random distribution of antiadsorption protein–antibody along the virus was less than one in a million. The antiadsorption protein antibodies were tested for the possibility of cross-reactivity with B protein by incubating antiadsorption protein antiserum with B protein and looking for a decrease in virus inactivation. Antiadsorption protein antiserum which was not treated with B protein was used as a control. No difference was found between the two antisera, indicating no detectable cross-reactivity and providing further evidence for the purity of the antiadsorption protein antiserum.

Discussion

The ideal method for isolating the adsorption protein from the bacteriophage fd would be to dissociate selectively either the major or minor coat protein from the viral DNA. Attempts

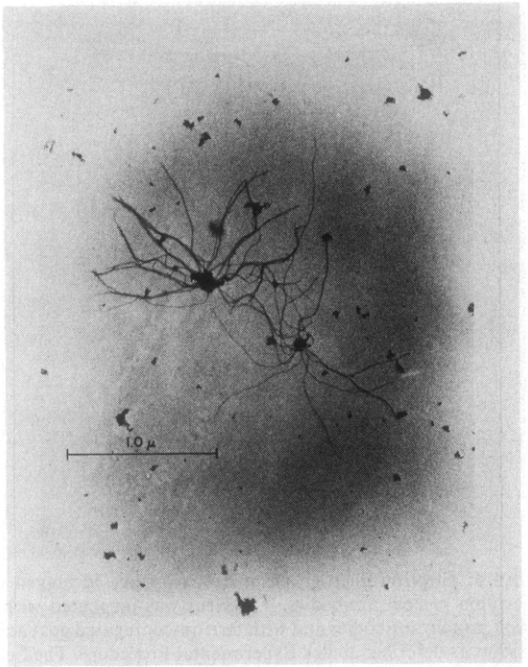

FIGURE 5: Electron micrograph of virus agglutinated by antiadsorption protein antibodies. The aggregates were formed by incubating 0.5 mL of a solution of 10^{12} virus/mL with 0.5 mL of whole serum at 20 °C for 30 min. The complex was washed with 30 mL of 0.15 M NaCl, 0.01 M sodium phosphate buffer (pH 7.5) in a Millipore filter holder with a 0.45-μm membrane. The ferritin-conjugated goat anti-rabbit antibodies (0.05 mL) were added and the material was incubated for 30 min and then washed as before. The aggregates were eluted from the filter into buffer and the samples were prepared for electron microscopy as described under Experimental Procedure. Magnification is 13 000×.

TABLE III: Distribution of Ferritin-Labeling Sites on the Virus.[a]

Label location	No. of Observations	
	Obsd	Expd
End	42	14.6
Middle	31	58.4

[a]Samples for electron microscopy were prepared as described under Experimental procedure and electron micrographs were taken at a magnification of 18 000×. All the clearly distinguishable virus particles (45) in the field were counted. Three of the particles were unlabeled and none were labeled at both ends. If a model for the system is made assuming that the ends of the virus constitute 10% of the length and the middle 80%, a chi-square evaluation of the data may be done. For this system, $\chi^2 = 64.28$. For a system with one degree of freedom, this value means that the probability of obtaining the observed results given the hypothesis of a random distribution is less than 1 in 10^6.

to do this, using methods which worked for the RNA bacteriophages as well as various other procedures such as heat, alkali, urea–detergent, and guanidine hydrochloride treatments, failed to give the desired result. This approach was abandoned in favor of complete dissociation of the virus and separation of the components by molecular sieving. The choice of sodium dodecyl sulfate for gel filtration was dictated by the previously observed effect of urea or guandine hydrochloride on the B protein (Knippers and Hoffman-Berling, 1966). These denaturants cause this protein to form an aggregate which migrates at the position of the adsorption protein. The method of virus dissociation was found to be critical because of the tendency of the B protein to aggregate under most disruption conditions resulting in the comigration of adsorption protein and some of the aggregated B protein. The disruption procedure described here was optimized so that the resulting adsorption protein preparation had very little if any B protein contamination as shown by amino-terminal sequence analysis. In earlier experiments, amino-terminal sequences corresponding to those of the B protein had been observed even though the adsorption protein preparation used gave a single, strongly staining band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. This result indicated the inadequacy, in this case, of the polyacrylamide gel method for estimating purity.

One of the major unsolved problems is whether or not the adsorption protein isolated by this procedure can be renatured for use in functional studies. Essentially all of the sodium dodecyl sulfate can be removed from the isolated adsorption protein but a satisfactory test for the regain of functional properties has not yet been devised. One possible test might be the enhancement of transfection of *E. coli* spheroplasts by fd DNA after addition of adsorption protein. This type of experiment has been successful with the adsorption protein from the RNA bacteriophage R17 (Iglewski, 1976).

It is of interest to compare our results on the amino acid analysis and amino-terminal sequence with those of Woolford et al. (1977) and Segawa et al. (1975). The amino acid analysis of the bacteriophage fl adsorption protein reported by Woolford et al. (1977) which was prepared by gel-filtration in sodium deoxycholate differs markedly from that of the fd adsorption protein prepared by gel filtration in sodium dodecyl sulfate. These differences are not due to differences in the bacteriophages, since analyses of the fl adsorption protein we prepared by gel filtration in sodium dodecyl sulfate was essentially the same as the analysis of the fd adsorption protein prepared in the same way (Woolford et al., 1977). The amino acid analysis of the fd adsorption protein reported by Segawa et al. (1975) can be rationalized in part with our data if one assumes that their preparation contains approximately 2 mol of B protein per mol of adsorption protein as well as another protein component.

In contrast to Segawa et al. (1975), we find alanine rather than glycine at the amino terminus. In earlier attempts to isolate the adsorption protein, minor amounts of dansyl-glycine along with dansyl-alanine were found; however, with recent improvements in the isolation and dansyl-Edman technique, only dansyl-alanine was seen. In addition to identifying the amino-terminal residue, the Edman degradation was carried out for an additional eight steps. The nine-residue amino-terminal sequence was then compared with sequences in the B protein (Asbeck et al., 1969; Nakashima and Konigsberg, 1974) and the gene-5 protein (Nakashima et al., 1974a,b) of bacteriophage fd for possible homologies but none were found.

In addition to establishing the purity of the adsorption protein preparation, the amino-terminal sequence will be helpful in establishing the complete chemical structure of the virus. Nuclease cleavage maps (Takanami et al., 1975; Seeburg and Schaller, 1975) and some DNA sequences from the fd DNA (Schaller et al., 1975; Sugimoto et al., 1975; Takanami et al., 1976) have been determined. When further DNA sequencing is done, the nine-residue amino-terminal sequence will provide more than enough information to set the reading frame and to locate the approximate position of the 5′ end of gene 3. Since no residues were released by carboxypeptidase A and B digestion, it was not possible to establish the codons at the 3′ end of gene 3. Proline and glycine are not released by

carboxypeptidase A or B but should be liberated by carboxypeptidase Y (Hayashi et al., 1973), an exopeptidase which has just become commercially available. This experiment will be attempted in the near future.

An accurate molecular weight determination of the adsorption protein posed some special problems. Molecular weight values ranging from 56 000 to 74 000 have been determined by several laboratories using whole virus or viral proteins synthesized in in vitro translating systems (Henry and Pratt, 1969; Wiseman et al., 1972: Ikehara et al., 1973; Jazwinski et al., 1973; Model and Zinder, 1974; Konings et al., 1975). The molecular weight seems to vary depending upon the polyacrylamide gel system used. Even within a single system the values vary widely. For instance, a molecular weight of 60 000 was found for the isolated adsorption protein when an 8 M urea-sodium dodecyl sulfate system was used and a value of 52 000 was observed when urea was omitted from the polyacrylamide gel system. The 60 000 molecular weight for the purified protein agrees with the results of Woolford et al. (1977), who also used urea in their sodium dodecyl sulfate-polyacrylamide gel system. However, the 52 000 value does not agree with the 60 000 figure obtained by Segawa et al. (1975), despite the fact that the standard Weber and Osborn (1969) procedure was used for both determinations. The reason for this discrepancy is not known. Two explanations for the behavior of the adsorption protein in different sodium dodecyl sulfate-polyacrylamide gel systems are possible: The faster mobility of the adsorption protein in sodium dodecyl sulfate may result from the incomplete unfolding of the protein. Alternatively, the lower mobility of the protein in sodium dodecyl sulfate-urea could have been due to the interference of urea with the binding of sodium dodecyl sulfate to the protein.

A knowledge of the molecular weight is important in determining the number of adsorption proteins per virion. The number (3.3) as estimated here is in close agreement with the values found by others (Beaudoin, 1970; Wiseman et al., 1972; Woolford et al., 1977). There are a number of uncertainties, however, in the calculation (see Experimental Procedure) so that it is not possible to assign an exact number using this approach.

The results of the electron microscopy clearly establish the location of the adsorption proteins at only one end of the virion. The same conclusion was reached by Woolford et al. (1977) with bacteriophage f1 using antisera we made against the fd adsorption protein. The location was previously inferred from electron micrographs showing the end of the virus attached to the bacterial F pilus, the host receptor (Caro and Schnös, 1966). The adsorption proteins were believed to be at only one end of the virion, since sheared and unsheared viruses were found equally effective in preventing the attachment of additional virus to the host (Fareed et al., 1966). The clustering of the adsorption proteins at one site on the virion is consistent with the emerging data on the mechanism of viral infection. The adsorption proteins must position the virus for penetration into the host cell. Studies have shown that the complete penetration of the virus is closely coupled to viral DNA replication (Kornberg, 1974) and that viral DNA replication has a unique origin (Tabak et al., 1974; Schaller et al., 1976). Thus, the adsorption protein is likely to have a specific binding site on the DNA close to the site of the origin of DNA replication.

This paper describes a method for isolating purified adsorption protein and several basic studies on the protein. The purification of this protein is one of the essential steps toward gaining an understanding of the molecular mechanisms by which the adsorption protein carries out its multiple functions.

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Adsorption Protein of Bacteriophage fl: Solubilization in Deoxycholate and Localization in the fl Virion†

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ABSTRACT: A complex containing the minor coat protein or adsorption protein (A protein) of bacteriophage fl has been solubilized from the fl virion, using the detergent deoxycholate. This complex was resolved from the fl DNA and from the fl major coat protein, or B protein, by gel filtration in the presence of deoxycholate. The A protein complex migrated as a single band on sodium dodecyl sulfate-urea-polyacrylamide gels corresponding to a molecular weight of 60 000. Analysis of the amino acid composition and amino terminal residues of this preparation indicates that the preparation contains a 20% contamination of additional protein species. Antibody against purified fd A protein is cross-reactive with deoxycholate-purified fl A protein and with fl phage. Electron microscopic observation of negatively stained complexes of fl phage with this anti-fd A protein antibody and ferritin conjugated goat anti-rabbit IgG antibody revealed phages with ferritin particles at their termini or complexes of two or more phages joined together at one end by ferritin, indicating that the complex of A protein molecules is located at one end of the filamentous fl virion.

The closely related filamentous bacteriophages fl, fd, and M13 are flexible rod-shaped particles approximately 8500-9000 Å long and 60-70 Å wide which infect male (F^+) *Escherichia coli* (Marvin and Hohn, 1969). They consist of a single-stranded-circular DNA molecule ensheathed by approximately 2400 molecules of the major coat protein or B protein, encoded by gene 8, and by several molecules of the minor coat protein or adsorption (A) protein,[1] specified by gene 3. The filamentous phages have proven to be most useful model

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[1] Abbreviations used are: A protein, the minor coat protein or absorption protein; B protein, the major coat protein; RF, double-stranded replicative form DNA; IgG, immunoglobulin; TE buffer, 0.01 M Tris, 0.001 M EDTA, pH 7.8; DOC, deoxycholate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; BSA, bovine serum albumin; DEAE, diethylaminoethyl.