Proc. Nat. Acad. Sci. USA
Vol. 72, No. 12, pp. 4749–4753, December 1975
Biochemistry

Asymmetric orientation of a phage coat protein in cytoplasmic membrane of *Escherichia coli*

(membrane assembly/radioimmune assays)

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Communicated by Arthur Kornberg, August 29, 1975

ABSTRACT **The coat protein of a filamentous phage (M13) enters the cytoplasmic membrane from two directions: from the outside upon infection and from the cell interior late in the viral life cycle prior to phage assembly and extrusion. Binding of ^{125}I-labeled anti-coat protein antibody to spheroplasts or to inverted vesicles was used to assay the orientation of coat protein in the membrane. Both parental and newly synthesized coat protein were found to be exposed on the outer surface of the cytoplasmic membrane. Coat protein in intact infected cells is also accessible to external antibody. Thus two different processes of assembling a protein into membrane, each starting from a different membrane surface, appear to produce similar surface orientations.**

Several striking features of membrane structure and function raise questions as to how this organelle is assembled. Integral membrane proteins are hydrophobic and tend to aggregate in the absence of detergent (1, 2), in contrast to the hydrophilic character of the known machinery of protein synthesis. Different proteins, and perhaps lipids, are oriented asymmetrically towards the inner or outer surface of the bilayer (3–6), despite the apparent localization of protein and lipid synthetic enzymes to inside the cell and despite the very low rates at which lipids and proteins "flip" through the hydrocarbon membrane core (7).

The M13 coliphage offers several apparent advantages for the study of these assembly problems. The filamentous M13 virion consists of a single-stranded circular DNA of 2×10^6 daltons (8) encapsulated in a helical array of coat proteins (9). The coat protein is a 5260 dalton peptide of known sequence with a central hydrophobic region (10). This small peptide is 98% of the virion protein (8). Each virion also has two to four copies of a 70,000 dalton protein, important in several stages of viral growth (11). When M13 infects a male cell, all of the parental coat protein enters the cytoplasmic membrane as the viral DNA is replicated (12–14). This parental coat protein is later incorporated dispersively into progeny virus (15). Late in the infectious process, new coat protein is made and inserted into the cytoplasmic membrane at a prodigious rate, reaching 26% of the membrane protein synthesis (13). Viral DNA, extruding through the membrane, is encapsulated by these coat protein molecules. Approximately 1000 virus particles, each with 1800 coat protein molecules, are made during each cell generation (8). The extraordinary rate of synthesis of this small, defined peptide and the existence of two means of inserting it into membrane, one during infection and one during phage production, recommend it as a system for the study of membrane protein synthesis and assembly.

This communication reports the development of rapid immunological assays for the protein, both in the membrane-bound state and as solubilized by detergents. Using these assays, both the parental coat protein and the progeny coat protein are found to be oriented with their antigenic sites facing the cell exterior.

Abbreviation: NP40, Nonidet P40.

MATERIALS AND METHODS

Organisms. *Escherichia coli* Q13 was obtained from Dr. T. Landers of this department. M13 and *E. coli* strain K37 (*su* I^+) was obtained from Dr. D. Pratt, University of California at Davis.

Phage Preparation. Strain Q13 (or, for amber phage preparation, K37) was grown to $OD_{600\,nm}$ of 0.2 in 90 liters of L Broth (16) at 37° and infected with phage at a multiplicity of 2. After 9 hr of growth, cells were removed by continuous flow centrifugation in a Sharples refrigerated centrifuge, and polyethylene glycol and NaCl were added to the medium to 2.5% and 0.5 M, respectively. After 5 days in the cold, medium was decanted and the settled debris was stirred at 25° for 1 hr with 0.01 volume of Triton X-100 (method of Dr. J. Griffith, personal communication). Virus, collected by sedimentation (0°, 10 min, 12,000 × *g*), were suspended in 1 liter of 1 M NaCl, 0.1 M Tris·HCl (pH 7.5), and 1% Sarkosyl 97, stirred (1 hr, 25°), and sedimented after addition of 25 g of polyethylene glycol. Phage were dissolved in 150 ml of 0.05 M Tris·HCl (pH 7.5), 1 mM EDTA, and 1 M NaCl. Solid CsCl was added to a density of 1.29 g/ml, and the phage were centrifuged (36 hr, 15°, 25,000 rpm in a Beckman SW27 rotor). Pooled phage bands were dialyzed against 0.05 M Tris·HCl (pH 7.5), 0.1 mM EDTA.

Labeled phage were purified by the above method after growth in minimal medium 63 (17), glucose, and the indicated radioactive supplement.

Coat Protein. Fifty milligrams of M13, 300 mg sodium dodecyl sulfate, and 0.04 ml of 2-mercaptoethanol were mixed in a final volume of 4 ml, heated (100°, 3 min) and dialyzed against 0.02 M Tris·HCl (pH 7.5), 0.1% sodium dodecyl sulfate, and 0.1% 2-mercaptoethanol. This was applied to a 100 ml Sephadex G-150 (Pharmacia) column equilibrated with this buffer at room temperature. An included peak of coat protein, assayed by its absorbance (280 nm) was pooled (26 mg, 11 ml).

Antibodies. Antibody to the M13 coat protein was prepared by injecting 1 mg of the sodium dodecyl sulfate-peptide in complete Freund's adjuvant into the footpads of a New Zealand white rabbit, followed by 1 mg of peptide in incomplete adjuvant 2 weeks later. Blood was removed through ear veins and serum was collected.

Anti-coat γ globulin was purified by combination with antigen. Antiserum (10 ml) and intact M13 phage (200 mg) were mixed in a 16-ml reaction with potassium phosphate buffer (0.04 M, pH 7.0). After incubation (20 min, 37°), the flocculent suspension was centrifuged (12,000 × *g*, 10 min,

0°) and the precipitate was twice suspended in 0.15 M NaCl (20 ml each) and centrifuged (12,000 × g, 10 min, 0°). The washed complex of antibody with phage was suspended in 0.15 M NaCl (17 ml) and mixed with acetic acid (1.9 ml of 1 M), incubated 10 min at room temperature and 10 min on ice, and centrifuged (0°, 40,000 × g, 20 min) to remove isoelectrically precipitated virus. The supernatant was filtered through a column of Bio-Gel A-5m (100–200 mesh, Bio-Rad Laboratories, 2.7 × 26 cm) in 0.1 M acetic acid at 25°. An included peak of antibody, detected by its absorbance (280 nm), was pooled and neutralized with 2 M Tris base. This antibody preparation (10 ml, 15 mg) stored at 0°, retained full phage-precipitating activity for at least 2 months.

Anti-coat protein antibodies, purified as above, were labeled with ^{125}I by the method of Syvanen *et al.* (18). Labeled antibody had a specific activity of 1.3×10^6 cpm/μg; 85% of the antibody was capable of combining specifically with M13 virions.

Gamma globulin was purified from nonimmunized rabbit serum by $(NH_4)_2SO_4$ precipitation (40% saturation at 0°), dialysis (0.02 M potassium phosphate, pH 7.0), and passage through DEAE-cellulose (equilibrated with 0.02 potassium phosphate, pH 7.0) at room temperature.

Cell Infection and Disruption. Strain Q13 was grown in L-Broth (16) at 37° with rotary shaking to $OD_{600\,nm}$ of 0.2, infected with M13 at a multiplicity of 100, and allowed to grow until the $OD_{600\,nm}$ reached 0.8. KCN was added to 10 mM and the cells were sheared for 1 min in a Waring Blendor. After centrifugation (20°, 5 min, 13,000 × g), cells were suspended in 0.04 M Tris·HCl (pH 8.0, 30 ml/g of cell pellet). To prepare spheroplasts (19), 80% sucrose was added to the cell suspension to a final concentration of 20% and the cells were stirred for 10 min at 25°. EDTA and lysozyme were added (0.04 volume of 0.5 M EDTA with 5 mg of lysozyme per ml) and stirring was continued for 90 min at room temperature, at which time at least 80% of the cells were spherical. After centrifugation (0°, 35,000 × g, 5 min) spheroplasts were suspended in buffer A [20% sucrose, 0.03 M Tris·HCl (pH 8.0), 10 mM KCN, 0.1 mM EDTA, 10 mM SO_4] at 50 mg of protein per ml. Spheroplasts were sonicated in 1 ml portions at 0° in an MSE sonicator by six 30 sec, maximum-power bursts separated by 1 min cooling periods.

Coat Protein Assays (0.1 ml) were performed in buffer A plus 0.25 mg of bovine serum albumin per ml with ^{125}I-anti coat γG (0.2 μg), unlabeled affinity-purified anti-coat γG where indicated, and cell fractions. After 20 min at 30°, assay tubes were centrifuged (0°, 45,000 × g, 30 min) and 50 μl of the supernatant was assayed for ^{125}I in a Nuclear Chicago γ-counter. Binding was calculated from depletion of the supernatant.

RESULTS

Membrane Orientation. In agreement with studies of uninfected *E. coli* (5, 20), membrane marker enzymes such as NADH oxidase are inaccessible to substrate in intact, M13-infected cells or in spheroplasts prepared from these cells (Table 1). This activity can be assayed when the permeability barrier is destroyed by toluene (5), which indicates that its active site is localized to the inner surface of the cell membrane. Sonicated spheroplasts are inverted (20) as judged by the accessibility of NADH oxidase to its polar substrate and the failure to measure increased activity after permeabilization.

Parental Coat Protein Orientation. The processes of uncoating and initial DNA replication, while requiring no new protein synthesis, are known to be coupled (14, 22). Rifampicin, by specifically preventing the priming of viral replication, blocks the transfer of parent coat protein into the membrane; chloramphenicol has no effect on either event. Male *E. coli* (strain Q13) were treated with chloramphenicol and infected with wild-type M13. Cells were vigorously blended in the presence of KCN to remove any adsorbed phage (14), and then converted to spheroplasts. Affinity-purified antibody was labeled with ^{125}I to provide a specific reagent for assaying the accessibility of membrane-bound M13 coat protein in cells, spheroplasts, and sonicated (inverted) spheroplasts. ^{125}I-Labeled anti-coat protein antibody was found to bind to spheroplasts from these cells (Fig. 1). This binding was not seen to spheroplasts from uninfected cells (data not shown) and was diluted by excess unlabeled anti-coat protein antibody but not by control antibody. Binding was, therefore, specific for the presence of the coat protein antigen and for its antibody and is a measure of exposed coat protein antigenic sites. Sonicated, inverted vesicles did not bind antibody and mixing spheroplasts with sonicated vesicles failed to reveal any diffusible inhibitor of binding. In control experiments, 88% of the coat protein in cells infected with labeled M13 remained membrane-bound

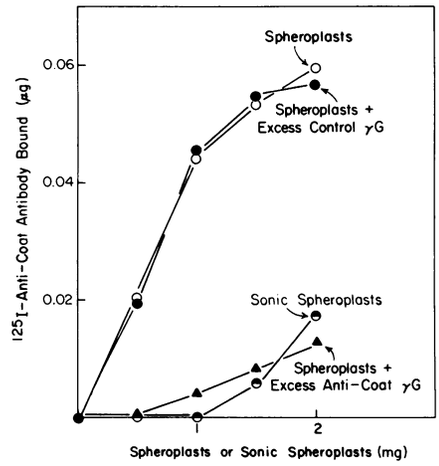

FIG. 1. Parental coat protein antigenic sites are exposed only to the outer surface of the cytoplasmic membrane. Strain Q13 was grown to $OD_{600\,nm}$ of 0.5, mixed with chloramphenicol (200 μg/ml, Parke-Davis), and infected with M13 at a multiplicity of 100 per cell. After 15 min at 37°, KCN was added to 10 mM and cells were converted to spheroplasts and sonicated spheroplasts. Each binding assay contained 0.1 μg of ^{125}I-antibody to coat protein and, where indicated, 5 μg of nonimmunized rabbit γ-globulin or 5 μg of nonradioactive antibody to coat protein.

Table 1. Membrane orientation in M13-infected cell fractions

	NADH oxidase	
	No toluene	With toluene
	(nmol NADH oxidized/min per mg)	
Cells	9	51
Spheroplasts	3	21
Sonicated spheroplasts	26	23

The NADH oxidase activity of M13-infected cells and spheroplasts (prepared without KCN) was assayed at 23° by the method of Hare *et al.* (21), with, or without, prior exposure to toluene (5).

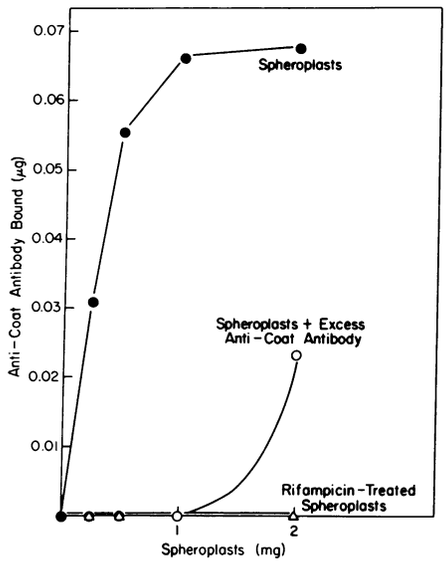

FIG. 2. Decapsidation is linked to viral DNA replication. This experiment was performed as described in the legend to Fig. 1, except that one half of the chloramphenicol-treated culture was mixed with rifampicin (200 μg/ml, Sigma) and both the control and rifampicin-treated cultures were aerated for 15 min at 37° prior to M13 infection.

after sonication. Membrane vesicles from M13-infected cells, prepared by the method of Kaback and coworkers (23), have external coat protein antigenic sites which remain fully exposed during sonication (W. Wickner, manuscript in preparation); therefore, sonication *per se* does not destroy coat protein antigenic sites. Thus coat protein antigen appears to be exposed on the outer surface of the membrane of spheroplasts and is not accessible on inverted sonic vesicles.

In order to establish that binding was to membrane-bound coat protein and not to adsorbed virions, a similar experiment was performed in which chloramphenicol-treated cells were M13-infected in the presence of rifampicin. This drug, which blocks decapsidation by preventing replication, blocked the appearance of coat protein surface antigen (Fig. 2), confirming that it is the membrane-bound form of the protein being measured. Assuming that each antibody molecule binds two coat protein molecules, approximately 900 parental coat protein molecules per cell (one half of a phage equivalent) are bound (Fig. 2); this value was obtained below antibody saturation and is a minimal estimate.

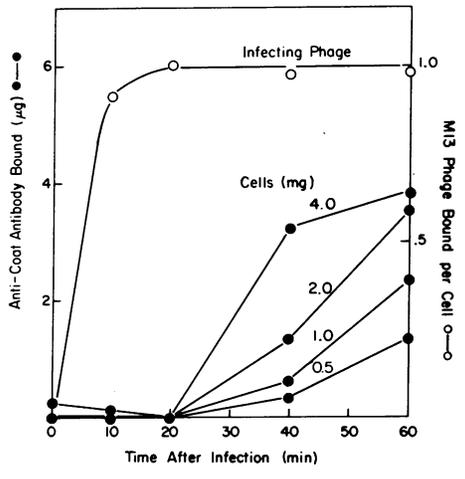

FIG. 3. Newly synthesized coat protein is exposed on the surface of intact cells. Strain Q13 was grown at 37° to an optical density at 600 nm of 0.5, then infected at a multiplicity of 100 per cell with ^{32}P-labeled M13 *amber 5*. Aliquots of infected cells were taken at the indicated times after infection and were treated with KCN, sheared to remove adsorbed virus, and washed as described in *Materials and Methods*. Suspended cells were then collected by centrifugation (0°, 10 min, 10,000 × *g*) and suspended in buffer A at 50 mg of protein per ml. Aliquots were assayed for ^{32}P as a measure of firmly bound phage and for binding of anti-coat protein (7 μg antibody per assay) at the indicated levels of cell protein.

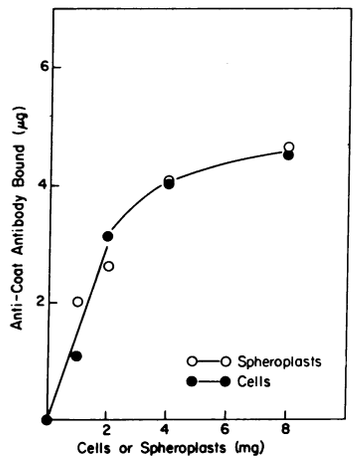

FIG. 4. Similar exposure of newly synthesized coat protein on the surface of cells and spheroplasts. Wild-type M13-infected cells and spheroplasts from these cells, prepared as described in *Materials and Methods*, were assayed for binding of antibody (7 μg) against coat protein. Similar results (not shown) were found when comparing M13 *amber 5*-infected cells and their spheroplasts.

Orientation of Newly Synthesized Coat Protein. Growing cells were infected with ^{32}P-labeled M13 *amber 5* phage in order to block the production of progeny virus while not interfering with coat protein synthesis (24). Portions of the culture were harvested at intervals after infection (Fig. 3). Washed cells, blended in the presence of KCN to remove any loosely adsorbed virus, were assayed for ^{32}P, indicative of firmly bound, infecting phage, and for their ability to bind ^{125}I-labeled, affinity-purified antibody to coat protein. The high levels of antibody (7 μg) used in this experiment preclude detection of parental coat protein molecules. Antigenic sites detected in this experiment therefore represent exposed, newly synthesized, membrane-bound coat protein.

Although the binding of infecting phage to the cells was complete by 10 min, progeny coat protein could be assayed only later in infection (Fig. 3), in agreement with other data on the time course of M13 infection (25) and with the fact that progeny coat protein is being measured. Levels of exposed, newly made coat protein antigens are approximately 100 times higher than those of parental coat protein (compare Figs. 1 and 3).

Assay of a protein localized to the inner, cytoplasmic membrane in intact cells may reflect either the abnormalities in the outer membrane and cell wall during M13 infection (26, 27) or some degree of localization of the coat protein to regions of adhesion or fusion of inner and outer membranes (28). Infected cells and spheroplasts prepared from those cells have similar levels of exposed coat protein antigenic sites (Fig. 4). Similar results were found for the parental coat protein. These results and the fact that M13-infected cells do not release periplasmic enzymes such as endonuclease I or 5′-nucleotidase (26) or alkaline phosphatase

(unpublished observations) support a special localization for the M13 coat protein.

M13-infected cells harvested 2 hr after infection were converted to spheroplasts and sonicated spheroplasts (as described in *Materials and Methods*) and were assayed for exposed coat protein antigens (Fig. 5). As with the parental coat protein, progeny coat protein antigen is exposed on spheroplasts, but is seen only at low levels on sonicated spheroplasts. Even this low level of binding is abolished by pre-absorbing the labeled anti-coat antibody with spheroplasts (Fig. 5, inset), indicating that sonicated spheroplasts are not exposing a different set of "internal" coat protein antigens. The low level of binding to sonic vesicles probably represents some incomplete spheroplast disruption, incomplete inversion upon sonication, or small loss of the natural asymmetry of coat protein in the membrane.

Assay of Solubilized Coat Protein. The ability to selectively precipitate M13 coat protein from detergent extracts provided an additional measure of the antibody's specificity. Cells were labeled during growth with [^{3}H]leucine, then infected with ^{35}S-labeled M13. Nonidet P40 (NP40) (0.2%) released 90% of the ^{35}S (coat protein) label and 50% of the ^{3}H (general protein label) from the membrane fraction. Antibody to coat protein specifically precipitated ^{35}S-labeled coat protein without other, ^{3}H-labeled protein (Table 2). Sodium dodecyl sulfate extracts of membranes from these cells had 70% of the ^{35}S-label as a peptide which coelectrophoresed with M13 coat protein (unpublished observations). Label could be efficiently extracted from these membranes with a variety of detergents, including Sarkosyl 97, Triton X 100, Brij 58, deoxycholate, and NP40. Despite the ease of solubilization, anti-coat protein, in conjunction with goat anti-rabbit IgG, was able to recognize and precipitate the coat protein only in deoxycholate and NP40 extracts. This assay will measure both parental and progeny coat protein. It may prove more rapid and convenient for measuring the synthesis of membrane-bound coat protein than the double-label sodium dodecyl sulfate gel assay commonly employed (24, 29).

DISCUSSION

Filamentous coliphages such as M13 are intimately involved with the cytoplasmic membrane at several stages of infection. As the infecting viral DNA is replicated, coat protein is deposited in the cytoplasmic membrane. The pilot protein of the virion is necessary for this event and the parental replicated DNA is recovered in association with cytoplasmic membrane (11). By 30 min after infection, new coat protein is synthesized and assembled into the cytoplasmic membrane. Progeny viral DNA, synthesized in a complex with virus-coded DNA-binding protein (30), sheds this binding protein as it passes through the membrane and is encapsulated in coat protein. Phage assembly, occurring at the membrane, requires several phage-specified proteins of as yet unknown function. The integrity of the cytoplasmic membrane is retained in these M13-infected cells despite the extrusion of up to 1000 virions per generation. Studies of M13 may help to answer questions about how DNA traverses membranes, about the role of membranes in replication, and about how membrane proteins such as the M13 coat protein are synthesized and assembled into a lipid bilayer.

This communication reports a rapid and sensitive assay that detects the coat protein in intact and disrupted cells as well as in detergent extracts. With these assays, the membrane-bound parental and newly synthesized coat proteins

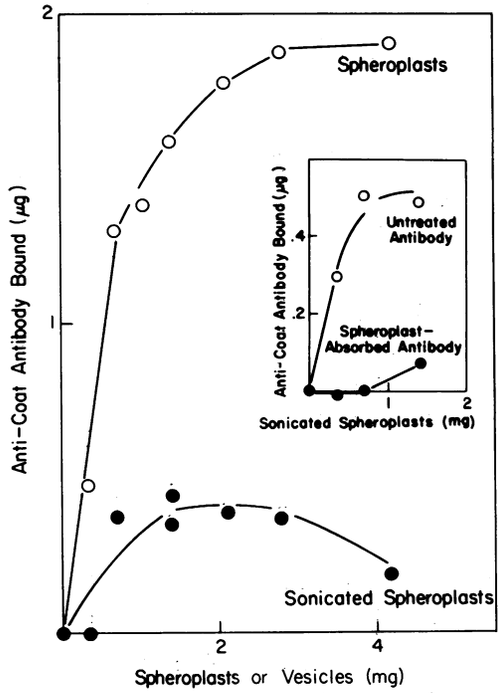

FIG. 5. Newly-synthesized coat protein antigenic sites are concentrated on the outer surface of the cytoplasmic membrane. Strain Q13 was grown, infected with M13, converted to spheroplasts and sonicated spheroplasts, and assayed for binding of anti-coat antibody (2.5 μg per assay) as described in *Materials and Methods*.

were examined. A common orientation, exposing antigenic sites to the exterior of the cytoplasmic membrane, was characteristic of the protein. Surprisingly, these coat protein antigenic sites were as exposed in intact infected cells as in

Table 2. Immunoassay of coat protein in detergent extracts

Fraction	Total protein (^{3}H cpm)	Coat protein (^{35}S cpm)
Extract	558	1210
Immunoprecipitate	11	545

Immunoassay of coat protein in detergent extracts. *E. coli* Q13 was grown at 37° in 100 ml of minimal medium 63 (17), 1% glucose, and [^{3}H]leucine (0.1 mCi, New England Nuclear) to an $OD_{600\ nm}$ of 0.5. Cells, collected by centrifugation (20°, 5 min, 10,000 × *g*) were suspended in L Broth (100 ml) and infected with ^{35}S-labeled M13 (multiplicity of infection 0.05, 10^6 cpm, prepared as described in *Materials and Methods*) at 37°. After 20 min, cells were sedimented (0°, 5 min, 10,000 × *g*), suspended in buffer [10 ml of 0.01 M Tris·HCl (pH 8.0), 1.0 mM EDTA], sheared with an S/P mixer (1 min), and again collected by centrifugation. After resuspension [2 ml of 0.01 M Tris·HCl (pH 8.0), 1.0 mM EDTA], the cells were sonicated (0°, 2 min, maximum power of a Branson sonifier). Unbroken cells were sedimented (0°, 5 min, 10,000 × *g*) and membranes were collected from the supernatant by centrifugation (0°, 30 min, 44,000 × *g*). A suspension of these membranes was mixed with 0.2% NP40 (Shell Limited, England), incubated at 37° for 30 min, and cleared of unextracted material (0°, 30 min, 44,000 × *g*).

Extract (5 μl) was incubated (15 min, 37°) with 4 μg of antibody to coat protein [in 0.1 ml containing 0.05 M Tris·HCl (pH 7.5), 0.05% NP40]. 100 μg of the γ-globulin fraction of goat anti-rabbit-γ-globulin serum was added, incubation was continued for 15 min, and the radioactive immunoprecipitate was collected by sedimentation and assayed for ^{3}H and ^{35}S. Higher NP40 concentrations blocked coat protein precipitation, while the precipitate was contaminated with non-coat proteins at lower detergent levels.

spheroplasts from these cells. The failure of infected cells to release periplasmic enzymes suggests that the outer membrane is not sufficiently permeable to pass antibodies of 150,000 daltons. Possibly the coat protein, which could have a maximum length of 70 Å if entirely α-helical (31), is anchored to the cytoplasmic membrane at regions where the two membranes adhere (28). There is already evidence suggesting that T-phage and ϕX174 adsorption and F-pili protrusion occur at these adhesions (32, 33).

Other techniques will be necessary to decide whether nonantigenic portions of the coat protein are exposed on the inner surface of the membrane. It is remarkable that two means of assembling protein into membrane as different as phage decapsidation and *de novo* synthesis from within the cell lead to a common orientation of the protein in the membrane. This is consistent with the orientation's being determined completely by the structure of the coat protein, independent of the insertion mechanism. Further studies are clearly necessary to decide this question; an understanding of these processes at a molecular level will require their reconstruction *in vitro*.

Note Added in Proof. Recent experiments have shown that the antigenic site of the M13 coat protein is in the first 8 amino-acid residues from its amino terminus (manuscript in preparation).

I thank Ms. Hollis Marriott for skillful and enthusiastic assistance with these experiments. This investigation was supported by grants from the American Cancer Society and National Science Foundation; W.W. is a Mellon Fellow at Stanford University. After April 1, 1976, W.W. may be contacted at the Department of Biological Chemistry and the Molecular Biology Institute, University of California at Los Angeles, Los Angeles, Calif. 90024.

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