Modification of Escherichia coli Membranes in the Prereplicative Phase of Phage T4 Infection

SPECIFICITY OF ASSOCIATION AND QUANTITATION OF BOUND PHAGE PROTEINS

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Bela J. Takacs† and Jurg P. Rosenbusch

From the Department of Microbiology, Biozentrum University of Basel, 4056 Basel, Switzerland

SUMMARY

Infection of Escherichia coli with the bacterial virus T4 causes modifications of the properties of the host cell envelope during the prereplicative phase of the lytic cycle. These changes include altered densities of cell envelopes and their subfractions, morphological modifications of membrane vesicles, and association of newly synthesized proteins with the host cell envelope. Polypeptide analysis by high resolution electrophoresis on polyacrylamide slab gels in dodecyl sulfate revealed that most of some 30 prereplicative phage-coded polypeptides are attached to this structure.

Different means of cell disruption and selective extraction procedures, such as variations of ionic strength, removal of divalent cations, and the addition of chaotropic agents or detergents were used to study the characteristics of these attachments. Many proteins appeared to be artifactually adsorbed or weakly bound to the envelope. Separation of cell walls from plasma membranes showed that all of the tightly bound proteins were associated with the cell membrane fraction. The partitioning of phage proteins between the different fractions was monitored using 12 polypeptides which were identified as products of distinct phage genes. Of these, 8 were eliminated as potential membrane markers. Four polypeptides, the products of genes rIIA, rIIB, 39, and 52 were operationally defined as membrane proteins.

The number of molecules of the 12 identified phage gene products, synthesized during a single lytic cycle, was determined. The results allowed the estimation of the concentration in the membrane of those proteins which were found to be quantitatively associated with that structure. Association of phage proteins with the cell envelope was found to be unaffected by mutations in any of the identified phage polypeptides.

Recently, several studies (1–4) have appeared which reported an association of two bacteriophage T4 coded proteins, the products of genes rIIA and rIIB (5), with the envelope of the host cell, Escherichia coli. Since circumstantial evidence accumulated in the past two decades indicated that these gene products contributed to changes affecting the host cell envelope upon infection (6), such a finding was to be anticipated. When we applied high resolution gel electrophoresis in dodecyl sulfate to the investigation of envelope-associated phage polypeptides, however, we saw over 30 phage gene products associated with the bacterial envelope fraction. We therefore had to ask not whether any of the prereplicative phage proteins was associated with the envelope of the host cell, but whether any of these attachments was specific. It was also of interest to know with which subfractions of the cell envelope the proteins were associated. An interaction of the rIIA and rIIB proteins with the cytoplasmic membrane has been postulated on the basis of the observation that these polypeptides were found in the envelope fraction of spheroplasts (2–4). However, this fraction contains portions of the cell wall in addition to the plasma membrane (7, 8). Attempts to localize unidentified T4 phage-coded proteins by fractional solubilization with detergents yielded ambivalent results. On the basis of extractions with nonionic detergents, phage proteins were assigned to the outer membrane of E. coli (9), but solubilization with dodecyl sarcosinate indicated partitioning between outer envelope and inner membrane (10).

In this report, we characterize the association of phage proteins to cell envelopes using preparative procedures and selective extractions (11, 12). In view of the large number of envelope-associated polypeptides, we have limited our investigations to gene products which we could identify at all stages of the preparation procedure by the use of amber (13) and deletion (5) mutations in bacteriophage T4 genes. Of the 12 gene products so identified, we tentatively defined 4 phage gene products as integral membrane proteins (14).

EXPERIMENTAL PROCEDURES

Chemicals—SDS, purchased from Sigma, was recrystallized from 95% ethanol. Dodecyl sarcosinate, 30% solution, was a gift from the Ciba-Geigy Corp. (Sarkosyl NL30). Guanidinium chloride (ultrapure) was obtained from Schwarz-Mann. The proteins

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used were from the following sources: ovalbumin, ribosomal proteins (from both subunits), glyceraldehyde-3-phosphate dehydrogenase, and RNA polymerase were the gifts of Drs. J. T. Edsall (Harvard University), J. Gordon (F. Miescher Institute, Basel), K. Kirechever and A. Taugita (University of Basel). Aspartate transcarbamylase and immunoglobulin are prepared routinely in this laboratory. β-Galactosidase, phosphorylase α, catalase, and cytochrome c were from Boehringer Mannheim. Bovine serum albumin was from Pentex, and chymotrypsinogen A and lysosome (grade A, 3 times recrystallized) were from Sigma and Calbiochem, respectively. Pancreatic deoxyribonuclease I (grade A, Boehringer Mannheim) was pretreated with phenylmethane sulfonylfluoride (Schwarz-Mann) according to Gold (15) and found to be entirely free of proteolytic activity as judged by an assay able to detect tryptic and other proteolytic activities at the picomolar level. Bovine pancreatic ribonuclease (grade A, Calbiochem) was protease-free according to the same criterion. Radiochemicals were from Radiochemical Centre (Amersham, England) and had the following specific activity: 14C-aminocaproic acid (containing all amino acids except histidine, cysteine, methionine, and tryptophan), 57 mCi/mmol; 14C-proline, 285 mCi/ mmol; and Na14CO3, 90 mCi/mmol. All other chemicals used were analytical grade from commercial sources.

Polypeptide Gel Electrophoresis in SDS—Proteins were subjected to electrophoresis in a discontinuous slab gel system according to Maizel. The vertical slab apparatus has been described by Studier (16) and the buffer system by Laemmli (17). Subjecting to electrophoresis in a discontinuous slab gel system. Protein standards were applied according to the method of Lowry et al. (20). Amino acid analyses were performed as described by Osborn et al. (21), using 3-oxoziyl)benzene (2,5-diphenyloxazole) per liter, and G parts of Triton X-100. For the quantitation of radioactivity in gels, slices of 1 mm were dissolved in 0.5 ml of 30% H2O2 at 60° overnight in capped scintillation vials prior to the addition of scintillation fluid.

Bacterial Growth, Infection with Bacteriophage T4, and Radioactive Labeling Procedures Escherichia coli B, E. coli CR 63, and a proline auxotroph E. coli B 970 (25) were the kind gifts of E. Kellenberger and W. Arber (University of Basel). The description and sources of wild type and mutant bacteriophages used are summarized in Table I. Cells were cultured in M-5 medium (26) at 37° C. Infection (a multiplicity of 5 was used throughout) was performed in the presence of tryptophan (10 μg/ml) when bacteria had reached a density of 4 x 10^9 cells/ml. Less than 1% of the labelled nitrogen, as monitored by plating. Unless otherwise indicated, radioactive labeling with 14C-aminocaproic acid (1 μCi/ml of culture) was initiated 4 min after infection. A large excess of casamino acids (final concentration 1%) was added 3 min later. Samples were cooled rapidly by immersion in Dry ice-ethanol mixture. Cells were collected by centrifugation at 4°, washed, and harvested. Radioactive labeling of uninfected cells was initiated at a cell density of 2 x 10^6 cells/ml by the addition of 1 μCi of 14C-aminocaproic acid (containing 0.01% casamino acids). [14C]Palmic acid was used for the radioactive labeling of phospholipids. At a density of 10^9 cells/ml, [14C]palmic acid (2 μCi/ml of culture) was added. At 10^6 cells/ml, the bacteria were either harvested or centrifuged at 37° and resuspended in an equal volume of M-9 medium. In the latter case, bacteria were infected 15 min later with T4 phage and the cells were chilled and harvested as described. To ascertain that radioactive palmic acid was incorporated exclusively into phospholipids, whole cells were dissolved in sample buffer and subjected to electrophoresis in the presence of SDS. Autoradiograms of the resulting gels showed

* Stocks of T4 phase mutants were grown on Escherichia coli CR 63. b Infections with phage-carrying mutations with a phenotype showing a delay in DNA synthesis were performed at 30°. At this temperature, the products of genes 39 and 52 were not detected although plating on E. coli B yielded as many plaques as on E. coli CR 63. The positions of the products of genes 60 and 58/61 could not be detected.

The nature of this mutation is not known to us.

\[ \text{wt} \]

\[ \text{am N 52} \]

\[ \text{am B 22} \]

\[ \text{del r88H} \]

\[ \text{am N 130} \]

\[ \text{am H 39 X} \]

\[ \text{am N 81} \]

\[ \text{am N 116} \]

\[ \text{am EA 142} \]

\[ \text{NG 164} \]

\[ \text{am HL 618} \]

\[ \text{del r 106} \]

\[ \text{del r 2226} \]

\[ \text{am E 10} \]

\[ \text{am HA 0 M} \]

\[ \text{am E 24} \]

\[ \text{am HL 625} \]

\[ \text{am HL 627} \]

\[ \text{am E 219} \]

\[ \text{am N 81; N55; R22; J. Hosoda} \]

\[ \text{N52; E10} \]

\[ \text{E. Kellenberger} \]

\[ \text{E. Kellenberger} \]

\[ \text{A. Bolle} \]

\[ \text{A. Bolle} \]

\[ \text{J. Hosoda} \]

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\[ \text{W. Wood} \]

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\[ \text{N52; E10} \]

\[ \text{J. P. Rosenbusch, manuscript in preparation.} \]
Preparation of Cell Envelopes—Cell breakage was performed by agitation in the presence of glass beads in a Mickle apparatus (27). Preparations were layered on top of gradient tubes containing 70% (10 ml), 50% (12 ml), and 40% (12 ml) sucrose (w/v) in 10 mM Tris-HCl, pH 7.5. Centrifugation in a SW 40 rotor was at an average force of 40,000 g in an International Ultracentrifuge for 12 to 14 hours at 4°C. The visible band at the interface between 50 and 70% sucrose was collected with a syringe through the side of the tube, diluted with the same buffer without sucrose, and sedimented at 100,000 g x 1 for 1 hour. Envelopes thus purified were suspended or lyophilized. Prior to electrophoresis, this fraction was dissolved in sample buffer.

Extractions of Envelope Preparations with Chaotropic Agents and Detergents—Extractions of envelopes with 4 M guanidinium chloride were performed in one of two ways. Envelopes were either prepared from cells broken with glass beads and then suspended in 50 mM Tris-HCl, 10 mM 2-mercaptoethanol, and 4 mM guanidinium or, alternatively, cell disintegration was performed in the presence of 4 mM guanidinium. After such extractions, the supernatant fractions obtained after centrifugation were washed three times with distilled water before suspension in sample buffer. Supernatants were lyophilized after extensive dialysis against 25 mM NH4HCO3 containing 10 mM 2-mercaptoethanol and were dissolved in sample buffer.

Extractions of envelope fractions with dodecyl sarcosinate (0.5%) were performed at 30°C for 30 min in 10 mM Tris-HCl, 40 mM KC1, and 10 mM EDTA, pH 7.5. Preparations were then centrifuged at 160,000 g x 1 for 2 hours. The well drained pellets were washed twice with the same buffer without detergent. Proteins in the supernatant fraction were precipitated in an ice bath by the addition of 2 volumes of 90% ethanol, precooled at −20°C. Precipitates were collected by centrifugation at 16,000 x g for 15 min. Supernatants were decontaminated and the residues were dried with a gentle stream of N2. Both dodecyl sarcosinate soluble and insoluble fractions were dissolved in sample buffer and subjected to electrophoresis. Addition of dodecyl sarcosinate to protein standards did not affect the mobility of proteins in SDS gel electrophoresis.

Preparation and Fractionation of Spheroplast Envelopes into Membranes and Cell Walls—The procedures used were those described by Osborn et al. (8). Spheroplast formation was monitored by phase-contrast microscopy and was always greater than 98%. With uninfected cells, spheroplasts were lysed osmotically by pouring the suspension into 5 volumes of ice-cold distilled H2O with constant agitation. With infected cells, the same procedure was used, although Mg2+ of the cells was present during addition of the EDTA solution. An alternative procedure with magnesium present at all times was also used (29).

Preparation of Cell Envelopes—Cell breakage was performed by agitation in the presence of glass beads in a Mickle apparatus (27). Disintegration was monitored by phase contrast microscopy and was always greater than 98%. Subsequent centrifugation at 150,000 g x 2 (5 min) removed residual glass beads and whole cells. Sedimentations of envelopes was performed at 30,000 g x 2. Pellets were resuspended in 1 ml of the same buffer containing 20% sucrose. A 10-fold dilution with buffer without sucrose was performed to remove soluble components trapped in vesicular structures by osmotic shock. The envelope fraction was then washed twice in 10 mM Tris-HCl, 40 mM KCl, containing either 6 mM MgCl2 or 10 mM EDTA. Further purification was obtained by stepwise or linear sucrose density centrifugation. In stepwise gradients, pellets were suspended in the same buffer containing 20% sucrose to give a final protein concentration of 5 to 8 mg/ml. One to two milliliters of this preparation were layered on top of gradient tubes containing 70%, 50%, and 40% sucrose. Centrifugation in a SW 27 rotor was at an average force of 80,000 g in an International Ultracentrifuge for 12 to 14 hours at 4°C. The visible band at the interface between 50 and 70% sucrose was collected with a syringe through the side of the tube, diluted with the same buffer without sucrose, and sedimented at 100,000 g x 1 for 1 hour. Envelopes thus purified were suspended or lyophilized. Prior to electrophoresis, this fraction was dissolved in sample buffer.

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Determination of Kinetics of Synthesis of Phage Gene Products—To determine the kinetics of synthesis of several gene products during a single lytic cycle, a 40 ml culture of E. coli B at a density of 4 X 10^8 cells/ml was infected with a phage strain with a conditional lethal mutation in a tail fiber gene (ann652 in gene 37). One minute after infection, T4 specific antiseraum (kindly provided by E. Kellenberger) was added to neutralize nonadsorbed phages. These conditions ensured complete lysis within 25 min. After adding the antiseraum, the culture was divided into 3 ml aliquots and individual tubes were grown with further aeration. A radioactive pulse (1 μCi of 14C-amino acid/mixture/ml of culture medium) was added to the first tube 2 min after infection. Successive tubes were labeled with radioactive amino acids every 3 min. At the end of the 2-min pulses, a large excess of carrier amino acids (final concentration 1% casamino acids) was added and the cells were washed twice with the same buffer without detergents. Proteins in the supernatant fractions obtained after centrifugation with the EDTA solution were dissolved in sample buffer. Aliquots (20 μl) from each time point were subjected to electrophoresis on a single polyacrylamide slab gel and the individual bands were quantitated by scanning densitometry of autoradiograms.

The integrated areas of identified gene products were divided by the respective molecular weights of the polypeptides. The ratios thus obtained in this analysis were corrected for the number of molecules synthesized per cell during the pulse. They were thus comparable with numbers obtained at other pulse times and with other gene products.

Determination of Absolute Number of T4 DNA Polymerase Molecules Synthesized Per Cell—In order to assess the absolute number of molecules made during a lytic cycle, the experiments described above were extended in this analysis. Cells were harvested using precisely measured volumes. Cell densities were determined by cell counting in a Petroff-Hauser bacteria counter or in a Coulter counter. A proline auxotroph of E. coli D was grown to a density of 4 X 10^8 cells/ml in M-9 medium supplemented with 5 μg of proline/ml. The culture was centrifuged at 37°C and the pellets were suspended in 50 mM Tris-HCl, 10 mM 2-mercaptoethanol, and 4 mM guanidinium without adding proline. An incubation for 20 min with aeration assured the exhaustion of the intracellular proline pool. Subsequently, T4D+ phages were added and allowed to adsorb for 2 min. The lytic cycle was initiated by the addition of radioactive proline (0.18 μg/ml of culture) with a final specific activity of 127 mCi/mmol. Identical amounts of radioactive proline were added every minute. Ten minutes after infection, the cells were rapidly chilled and sodium azide was added to prevent further incorporation of radioactivity. An aliquot was withdrawn for cell counts and exactly 4 ml of the culture were harvested by centrifugation and the pellet was resuspended in exactly 200 μl of sample buffer. Aliquots of 20 μl were subjected to polyacrylamide gel electrophoresis. The autoradiograms obtained showed the typical pattern of repressed phage proteins without any noticeable trace of host protein synthesis. They were scanned and the percentage of radioactivity in the T4 DNA polymerase band (molecular weight of 108,000) was estimated. From the total number of cpm applied to the gel, the amount of radioactivity in this band could be calculated. Alternatively, the band corresponding to T4 DNA polymerase was cut out from a gel and solubilized with H2O2. Its radioactivity was then counted and corrected for quenching. Recovery from the gel slices was complete. Both procedures yielded values within 5% of each other. The figure thus obtained was corrected for radioactivity incorporated into glutamic acid. From this result and the number of cell equivalents applied to the gel, the number of T4 DNA polymerase molecules synthesized per cell between 6 and 10 min could be calculated on the basis of the specific activity of proline and the number of proline residues per polypeptide chain (40 residues/108,000 daltons, cf. Ref. 31). Conversion to other time periods was obtained by integrating the corresponding areas of

1 We have observed that chromatographically pure [14C]proline was incorporated into proteins as proline and glutamic acid. The conversion of proline into glutamic γ-semialdehyde, and then into glutamic acid (and to minor extents into other amino acids) is possible via an alternate pathway (30). To correct for this incorporation, an aliquot of soluble proteins from infected cells was hydrolyzed after removal of free amino acids by gel filtration. Under the experimental conditions used, 15% of the radioactivity co-chromatographed with glutamic acid. Longer periods of labeling (two generations) led to up to 50% conversion.
kinetic plots and expressing them as fractions of the entire cycle.

**RESULTS**

**Characterization of Envelope Preparations from Infected Cells—**

When uninfected bacteria were labeled by the addition of $^{14}$C-aminoacids for two generations, and the purified envelopes were subjected to slab gel electrophoresis in SDS, scanning densitometry revealed that between 60 and 100 bands were resolved. The largest peak in Fig. 1A corresponds to the major envelope protein of *Escherichia coli* with a molecular weight of 36,500 (27). The second largest peak with an apparent molecular weight of 44,000 represents the major periplasmic protein from *E. coli* whose characteristics are described elsewhere. A doublet to the far right of the gel pattern with apparent molecular weights of 155,000 and 165,000 (32) corresponds to the large subunits ($\beta$ and $\beta'$) of RNA polymerase. An identical profile was obtained by a labeling period of 3 min instead of two generations.

When exponentially growing cells were infected with bacteriophage T4 and labeled with $^{14}$C-aminoacids between 4 and 7 min, the autoradiograms obtained from the envelope and supernatant fractions showed the patterns of Fig. 1A, B and C. The profile of the envelope fraction was clearly different from that obtained with host cell envelopes, as was expected since host protein synthesis is drastically reduced in the first few minutes after phage infection (33). To have easy access available for the individual bands in subsequent analyses, and to learn more about the functional significance of the association of phage proteins to the host envelope, we have used the amber and deletion mutants of Table I for the identification of radioactive bands. At least 12 peaks in the envelope fraction and 4 peaks in the supernatant are the products of distinct and unique phage genes. Our assignments are essentially in agreement with those obtained by O’Farrell *et al.* (34) in whole cells. The use of phage mutants, deficient in polypeptides necessary for the synthesis of postreplicative proteins (35), showed that unidentified proteins were also of pre-replicative origin. The radioactive patterns obtained with envelope preparations of cells infected with T4 phages mutated in genes 33 and 55 were indistinguishable from the one shown in Fig. 1B.

A comparison of envelope and supernatant fractions of infected cells shows that most of the bands found in the supernatant were also present in the particulate fraction. On the other hand, many of the bands found in the envelope were not in the supernatant. On the basis of these results, we have assigned individual phage polypeptides to either of two classes. Proteins which partitioned between envelope and supernatant fractions were assigned to Class I. T4 DNA polymerase (p43) is a representative example. Table II shows that upon cell breakage in the presence of magnesium, one sixth of the product of gene 43 was found in the supernatant. If cell disintegration was performed in the presence of EDTA, 50% of this gene product was found in the supernatant after centrifugation at 160,000 × g. Proteins were assigned to Class II if they were found associated quantitatively with the envelope fraction. As a representative example, the product of gene 45.

When cell disintegration was obtained with methods other than cell disruption with glass beads (e.g. passage through a French pressure cell or spheroplast lysis), this protein was released quantitatively from the particulate fraction. It may therefore be a useful indicator for the destructiveness of the method used. Extractions of envelope preparations containing this polypeptide with dodecyl sarcosinate also solubilized this protein. Its presence in detergent extracts was the only change in the polypeptide composition observed in plasma membrane preparations obtained by isopycnic centrifugations. A manuscript (B. J. Takacs, G. R. Jacobson, and J. P. Rosenbusch) describing the properties of this protein is in preparation.

6 Apparent molecular weights are given in Table IV. Due to the good agreement with O’Farrell *et al.* (34), we have adopted their assignment of the second largest polypeptide as a product of gene X.

**FIG. 1** Comparison of protein profiles from envelopes of uninfected cells (Panel A) with the envelope fractions of phage T4-infected cells (Panel B). Panel C represents the supernatant fraction from infected cells. The profiles were obtained by scanning densitometry of autoradiograms from polyacrylamide slab gels. Cells were labeled with $^{14}$C-aminoacids for two generations, and the purified envelopes were subjected to slab gel electrophoresis in SDS, scanning densitometry of autoradiograms, individual peaks corresponding to the identified gene products were integrated and expressed as fractions of the entire band pattern. At least 12 peaks in the envelope fraction and 4 peaks in the supernatant are the products of distinct and unique phage genes. Our assignments are essentially in agreement with those obtained by O’Farrell *et al.* (34) in whole cells. The use of phage mutants, deficient in polypeptides necessary for the synthesis of postreplicative proteins (35), showed that unidentified proteins were also of pre-replicative origin. The radioactive patterns obtained with envelope preparations of cells infected with T4 phages mutated in genes 33 and 55 were indistinguishable from the one shown in Fig. 1B.

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Cells were infected, radioactively labeled, and disintegrated with glass beads 7 min after infection. Envelope fractions were collected by centrifugation at 30,000 \( \times g \). The supernatant fraction (I) was then centrifuged at 160,000 \( \times g \), yielding a high speed pellet and a supernatant (II) fraction. The latter was lyophilized after extensive dialyses against 25 mm NIH/HCO\(_3\)-10 mm 2-mercaptoethanol. Envelope, high speed pellet, and supernatant (II) were dissolved in equal amounts and applied to a slab gel. After electrophoresis in SDS, gels were obtained from autoradiograms and the distributions between the fractions were calculated for each peak.

<table>
<thead>
<tr>
<th>Class</th>
<th>Gene product</th>
<th>In presence of Mg(^{2+}) (6 min)</th>
<th>In presence of EDTA (10 min)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Envelope</td>
<td>High speed pellet</td>
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<td>-----------------</td>
</tr>
<tr>
<td>I</td>
<td>p43</td>
<td>58</td>
<td>26</td>
</tr>
<tr>
<td>II</td>
<td>prIIA</td>
<td>100</td>
<td>0</td>
</tr>
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</table>

* An arbitrary limit was set for the assignment of individual gene products to Classes I and II. If >90% of a gene product was contained in the envelope fraction, association was considered complete. This was to account for the small amounts of proteins which may have dissociated from the envelope as a consequence of damage caused by cell breakage. Using this criterion, other identified gene products assigned to Class I were: p imm, p36, and p ip III. Those belonging to Class II were: pX, p46, p30/41, p39, p52, p32, and prIIA.

The rIIA gene is shown in Table II. The distribution of these proteins was affected only a little by the presence of chelating agents. Four of the 12 identified bands belonged to the first class and eight bands belonged to the second class. Complete or partial envelope association of identified and unidentified bands was reflected in the observation that 80% of the total radioactivity incorporated into proteins remained associated with the particulate fraction. The corresponding value in the presence of EDTA was 70%.

The preparation procedure used in these experiments is a relatively mild one. Over 90% of the lipopolysaccharides remained associated with the particulate fraction (27) and the major periplasmic protein was recovered quantitatively with it. Also, these preparations contained very few small vesicular structures, as demonstrated by electron microscopic examination of negatively stained specimen (data not shown).

**Properties of Spheroplast Envelopes and Membrane and Wall Fractions after T4 Infection**—When spheroplasts were prepared from infected cells by the method of Osborn et al. (8) and the particulate and supernatant fractions of osmotically lysed preparations were separated by centrifugation at 160,000 \( \times g \), 30% of the radioactivity incorporated into proteins was retained in spheroplast envelopes as compared to 70% retained in the envelope fraction after cell disintegration with glass beads. To determine whether the distribution of specific proteins between pellet and supernatant fractions was different or whether all radioactively labeled polypeptides were released nonselectively, autoradiograms from electrophoretically fractionated spheroplast envelopes (Fig. 2c) were compared with those obtained from envelope fractions prepared after cell disintegration with glass beads (Fig. 2b). Such a comparison indicated that three proteins (pX, p32, and p46) were released essentially quantitatively, but showed that most of the other proteins were present also in spheroplast envelopes.

In contrast to cell envelopes obtained by cell breakage with glass beads, most of the bands present in spheroplast envelopes were also found in the concentrated lysis supernatant (Fig. 2b). However, a detailed comparison of the two patterns indicated that several phage proteins were preferentially recovered in the lysis supernatant, whereas others were enriched in spheroplast envelopes. The latter proteins include the products of genes rIIA, 46, 39, 52, rIIIB, and the immunity protein (p imm). The release of a fraction of most proteins into the supernatant was consistent with the electron microscopic observation that spheroplast lysis caused the formation of very small vesicular structures which could not be sedimented even by the high centrifugal forces used.

**Table II**

<table>
<thead>
<tr>
<th>Class</th>
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<tr>
<td>-------</td>
<td>--------------</td>
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</tr>
<tr>
<td>I</td>
<td>p43</td>
<td>58</td>
<td>26</td>
</tr>
<tr>
<td>II</td>
<td>prIIA</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

The product of the "immunity gene" (identified by the use of the double mutant with which this gene (36) was first discovered) appeared to be partitioned between the envelope and supernatant fraction. If the band with an apparent molecular weight of 30,000 indeed corresponded to the immunity gene product, this protein would be expected to bind to the cell envelope (36). We attempted to examine the possibility of a periplasmic location by subjecting bacteria to an osmotic shock treatment (37) 7 min after infection. However, infected cells proved too fragile, so that release of periplasmic polypeptides could not be obtained without concomitant cell lysis. The proof that this gene product is indeed the immunity protein, and its localization, must therefore await further study.

**Fig. 2.** Autoradiographic patterns of proteins in spheroplast preparations. Samples for Gels b to f were obtained from spheroplasts prepared according to Osborn et al. (8); that in Gel h was from spheroplasts prepared according to Haywood (26). Cells were labeled from 4 to 7 min after infection with T4 phage. Gels a and g represent envelope preparations obtained from cells disrupted with glass beads (included as reference); b, concentrated supernatant from lysed spheroplasts; c, spheroplast envelopes; d, Fraction I; e, Fraction II; and f, Fraction II. *Samples d to f* were prepared by isopyknic centrifugation. The identity of the major band in Gel d is not known; it appears not to be the product of gene 1. Gels a to f were gradient gels (7.5 to 15% acrylamide, stabilized with 10% glycerol in the 15% solution). Gels g and h were uniformly 10%. *P. lipid* indicates the position of phospholipids.
and the notations of the different bands (H, M, and L) are those of Osborn et al. (8). From T4 phage-infected cells (Panel B). The preparation method and the notations of the different bands (H, M, and L) are those of Osborn et al. (8).

Peaks L1 and were isolated by isopyknic centrifugations and were derived from membranes and cell walls from uninfected and T4-infected cells. Radioactive labeling was as described in Fig. 1. Membranes and cell walls from uninfected cells are very similar to scans derived from Fig. 2, d.

The profiles from infected cells were compared in Fig. 3A. The enzymatic activities assayed were in good agreement with earlier findings (38). Electron microscopic observations (see below) confirmed the assignments of cell wall to the H peak and minor peak L2 varied from one experiment to another. Chemical composition and enzymatic activities, as assayed by the distributions of lipopolysaccharides and two enzymes, NADH oxidase and succinate dehydrogenase, were in good agreement with previous results (8,22). Electron microscopic observations (below) confirmed the assignments of cell wall to the H peak and of plasma membrane to the L fractions. The polypeptide chain compositions of membrane and cell wall fractions of uninfected cells are shown in Fig. 4, A and B, respectively. The distribution of polypeptides between these two fractions is in agreement with earlier findings (38).

When spheroplast envelopes from infected cells were fractionated by isopyknic centrifugation, the separation shown in Fig. 3B was obtained. The distribution of host material, monitored by the absorbance of light at 280 nm, was roughly similar to that of uninfected cells. However, the separation of cell walls and membranes was not as good as in uninfected cells, as evidenced by the considerable amount of material banding at the intermediate position, M. The density of the H band was consistently higher than that observed of the control (p = 1.24 g/ml versus 1.23 g/ml). The major peak of absorbance migrated reproducibly in the position of L2 (p = 1.16 g/ml) in infected cells. The enzymatic activities assayed were also found mostly in this peak, whereas in uninfected cells, the highest enzymatic activities were found in the position of L2. The profile of radioactivity, representing proteins synthesized between 4 and 7 min after phage infection, exhibited a major peak which invariably migrated in the position of L2, exhibiting a shoulder in the position of L4. Virtually no radioactivity was observed at a density of 1.24 g/ml. Electron microscopic examination of thin sections of cell wall and membrane fractions of both uninfected and infected cells resembled those described in detail previously (7,8,22). Negatively stained specimens of cell wall fractions of uninfected and infected cells did not reveal differences either (data not shown). However, negatively stained membrane vesicles from infected cells reproducibly showed wrinkled surfaces not observed in the uninfected counterpart (Fig. 5).

The distribution of individual phage proteins in the different fractions is shown in Fig. 2, d to f. Some viral polypeptides, such as p11A and p11B, were enriched in L2, whereas others were pre-eminent in L3 (e.g. p52). To obtain clearly detectable bands on autoradiograms of the cell wall fraction (Fig. 2, Gel f), an 80-fold higher amount had to be applied to the gels. Most of the radioactivity in this fraction was incorporated into a peak which by its temperature-dependent electrophoretic mobility was identified as the major envelope protein of the host cell (27). The small amount of radioactivity incorporated demonstrated that host protein synthesis was continuing between 4 and 7 min after infection, but that its rate was drastically reduced. An alternative preparation procedure (29) for spheroplasts was also used. This method consisted of repeated cycles of freezing and thawing to allow the hydrolysis of the peptidoglycan by lysozyme in the presence of magnesium and the subsequent lysis of the spheroplasts. The autoradiogram of a spheroplast envelope preparation thus obtained is shown in Fig. 2h. In contrast to the lysozyme-EDTA method, the products of genes 43, 46, and 30/41 were entirely absent from the autoradiogram, whereas p45 was not quantitatively removed by this method.

Effects of Selective Extraction Procedure on Envelope-associated T4 Proteins—To study the interaction of phage-coded polypeptides with the plasma membranes, membrane fractions (either in their isolated form or in association with the cell wall) were subjected to extractions with guanidinium chloride or dodecyl sar-
FIG. 5. Negatively stained preparations of membrane fractions from uninfected (upper panel) and infected (lower panel) cells. Specimens were from the L, fractions in Fig. 3. The bars indicate 0.5 μm. The preparations shown are representative samples of five independently prepared specimens of each kind. To exclude preparation artifacts, equal amounts of vesicles from infected and uninfected cells were mixed and prepared on a grid. The resulting micrograph showed a 1:1 ratio of smooth and wrinkled structures.

cosinate. The former solvent has been shown not to disrupt protein-lipid interactions (39), while the latter is thought to extract selectively membrane proteins, leaving those of the cell wall unperturbed (40).

When isolated envelopes from uninfected cells were incubated in 4 or 6 M guanidinium chloride, the insoluble residue was highly enriched in the major envelope protein of E. coli. A number of minor bands also remained in the insoluble fraction, such as the large subunits (β and β') of RNA polymerase. Extraction with 4 M guanidinium of infected cells, labeled with 14C-amino acid from 4 to 7 min after infection, left 40% of the radioactivity in the insoluble fraction. Fig. 6 shows the autoradiograms of extracted and washed pellets of two mutant strains (Gels b and d) in comparison with unextracted envelopes (Gels a and c). It is evident that several bands were quantitatively extracted by this treatment, while others appear to be affected very little. In both

* Since during the preparation of the cell envelopes used for these extractions damage to these structures was unavoidable,
uninfected and infected cells, this extraction solubilized 45 to 50% of the phospholipids.

When uninfected cells were extracted with dodecyl sarcosinate and the supernatant and pellet of a high speed centrifugation analyzed by SDS polyacrylamide gel electrophoresis, patterns indistinguishable from those obtained from plasma membranes and cell walls prepared by isopyknic centrifugation (Fig. 4, A and B) were obtained. The host envelope protein is completely insoluble under the conditions used; the presence of a single additional peak in the supernatant fraction of uninfected cells is explained in Footnote 4. Extractions of envelopes from infected cells labeled with 14C-amino acids with this detergent yielded the result shown in Fig. 4C (soluble fraction) and Fig. 4D (insoluble fraction). Again, the agreement of these patterns with those obtained by isopyknic isolation of cell membranes and walls from spheroplast envelopes (Fig. 2, d and f) was clear and provided supporting evidence for the membrane-association of phage-coded proteins. Similar extractions of envelopes labeled in their phospholipid moiety solubilized 60% of the radioactivity in uninfected cells and 75% in infected cells. Thus, partial extraction of phospholipids and proteins with dodecyl sarcosinate shows the selectivity of this detergent. The results of the different preparation and extraction procedures are summarized in Table III.

**Table III**

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Quantitative association with envelopes (Fig. 1)</th>
<th>Proteins in spheroplast envelopes</th>
<th>Quantitative extraction by 4 M guanidinium</th>
<th>Operational assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>p43</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Membrane</td>
</tr>
<tr>
<td>pX</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Membrane</td>
</tr>
<tr>
<td>prII A</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Membrane</td>
</tr>
<tr>
<td>p46</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Yes</td>
</tr>
<tr>
<td>p30/41</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>p39</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Yes</td>
</tr>
<tr>
<td>p32</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Membrane</td>
</tr>
<tr>
<td>prII B</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Yes</td>
</tr>
<tr>
<td>p imm</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Membrane</td>
</tr>
<tr>
<td>p ip III</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Membrane</td>
</tr>
</tbody>
</table>

* An association of >90% was considered quantitative, since the damage set by cell disintegration may release small quantities of even tightly bound proteins.

(43, 44). Also, several phage-coded polypeptides bind to DNA-dependent RNA polymerase of the host (35). Association of these structures with the membrane has been described (45-50). We therefore examined whether any such binding of envelope-related phage proteins to the membrane through intermediary structures could be excluded.

Treatment of envelopes from cells or spheroplasts with pancreatic deoxyribonuclease I or pancreatic ribonuclease (50 µg/ml at room temperature for 10 min in the presence of a free magnesium concentration of 5 mM) effected no change of the resulting autoradiographic patterns. Although such preparations contain only about 1% of the original DNA and RNA even without nuclease treatment (51), and although the nucleases used hydrolyze available substrates to small fragments (51, 52), these results do not exclude binding of T4-coded proteins to small fragments which remained attached to, and were protected by, the membrane. Unfortunately, it is difficult to design experiments to prove or disprove such a possibility conclusively. We are planning to approach this question by reconstitution experiments.

**Indirect Binding of Proteins to Cell Membranes**—Several pre-replicative proteins tightly bind to T4 DNA (42) and association of T4-coded proteins with host ribosomes have been reported. Partial extractability of all proteins was to be expected. Furthermore, increasing chaotropic effects will eventually lead to the solubilization of all proteins (41) including the host envelope protein. For this reason, a guanidinium concentration of 4 M was used here. Experiments with 6 M guanidinium gave similar results, but the intensities of all bands were decreased. The presence of 4 M guanidinium during, rather than after, cell disintegration yielded similar results.
membranes. Inspection of stained gels led to the observation that β and β' were present in spheroplast envelopes prepared by the lysozyme-EDTA method (8), and were found associated with L1, but not with L0, upon isopyknic isolation of these fractions. Spheroplasts prepared by an alternate procedure (29) did not contain them. Extraction of envelope preparations with guanidinium ions also failed to extract quantitatively these subunits. A functional association of this enzyme with the membrane has been suggested (48-50), but it is difficult to interpret the significance of the binding of both large subunits (since the smaller subunits escape detection, we do not know whether they also bind). In the present context, the observation that the intensity of radioactivity labeled phage proteins was independent of the amount of RNA polymerase present was critical. Thus, a role of this enzyme as an intermediary in envelope binding is unlikely.

Concentration of T4-Coded Proteins in Host Cell Membrane

If the number of molecules per cell and the approximate membrane surface are known, an estimate of the density of distribution can be obtained for membrane-bound proteins. To this end, we first determined the kinetics of synthesis of the identified prereplicative proteins of T4 phage during a single lytic cycle. These results provided the basis for an estimation of the absolute number of molecules synthesized during a lytic cycle. Fig. 7 shows the relative number of molecules for the individual gene products. The patterns of protein synthesis described here are very similar to the transcriptional patterns observed by O'Farrell and Gold (53).

The determination of the absolute number of molecules synthesized for one specific phage protein constituted the next step. We chose T4 DNA polymerase, since its amino acid composition is known (31) and it gives a unique, isolated band on autoradiograms (amber mutations in gene 43 completely delete this band). A proline auxotroph derivative of E. coli B was used since the pool size of this amino acid is known to be small (54). After an appropriate starvation period, the lytic cycle was initiated by the addition of radioactive proline of known specific activity, and the number of polymerase molecules synthesized per cell was estimated (for details, see "Experimental Procedures"). The value obtained was 660 copies per cell, compared to a figure of 750, calculated from the purification scheme given by Goulian et al. (31). The agreement between the two figures may be coincidental to some extent, as the conditions used by these authors probably led to a higher yield but also to losses due to the removal of the particulate fraction in their purification procedure. However, the agreement indicated that the figure could serve as a basis for the estimation of the mass of other viral proteins after uniform labeling of a culture with a 14C-amino acid mixture. In this step, the amount of radioactivity incorporated into each identified gene product was compared with that incorporated into T4 DNA polymerase. The number of molecules of the individual phage protein was calculated by correcting for the size of the polypeptide (Table IV). A reasonable reliability of the results obtained is again indicated, in this instance by the good agreement between the number of molecules of DNA unwinding protein determined here and that published by Alberts (58).

The molar ratio of rIIIB to rIIIA protein is also in agreement with a previous report (53). For the proteins thought to be quantitatively associated with the membrane (prIIA, prIIIB, p39, and p52), the concentration in the membrane was calculated assuming a surface of the bacterial membrane of 3 μm² (60). Thus, an area 55 nm square was calculated to contain, on the average, 1 molecule each of prIIA, p39, and p52, and 5 molecules of the product of gene rIIIB. The total mass of these 4 most tightly membrane-associated proteins constitutes approximately 10% of the proteins contained in the plasma membrane. This calculation is based on our estimate that the proteins of the plasma membrane constitute 10% of the total cell protein.

Interrelation between Identified Phage Proteins—In all experiments performed for the identification of phage proteins, the ratios of the peak areas corresponding to the products of non

![Fig. 7. Comparison of the kinetics of synthesis of 11 identified phage gene products. The units on the ordinates are arbitrary, but the bar heights reflect the relative amounts for each protein. The results shown were obtained in an experiment in which lysis was complete 25 min after infection. Solid bars reflect synthesis of proteins which have been operationally defined as membrane components.](http://www.jbc.org/)

**TABLE IV**

<table>
<thead>
<tr>
<th>Gene Product</th>
<th>Known of synthesized</th>
<th>Molecular weight</th>
<th>Number of Copies Determined</th>
<th>Protein labelled after infection 10³ copies/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>p31</td>
<td>DNA-polymerase (31)</td>
<td>170000</td>
<td>1000</td>
<td>1.4</td>
</tr>
<tr>
<td>p30</td>
<td>nucleoside (31)</td>
<td>100000</td>
<td>7000</td>
<td>1.3</td>
</tr>
<tr>
<td>p39</td>
<td>Mg²⁺ pump (31)</td>
<td>70000</td>
<td>30000</td>
<td>1.0</td>
</tr>
<tr>
<td>p58</td>
<td>DNA unwinding protein (31)</td>
<td>120000</td>
<td>100000</td>
<td>5.0</td>
</tr>
<tr>
<td>p32</td>
<td>Mg²⁺ pump (31)</td>
<td>70000</td>
<td>40000</td>
<td>3.0</td>
</tr>
<tr>
<td>p15</td>
<td>Mg²⁺ pump (31)</td>
<td>70000</td>
<td>30000</td>
<td>2.1</td>
</tr>
<tr>
<td>p32</td>
<td>Mg²⁺ pump (31)</td>
<td>70000</td>
<td>30000</td>
<td>1.5</td>
</tr>
<tr>
<td>p58</td>
<td>DNA unwinding protein (31)</td>
<td>120000</td>
<td>100000</td>
<td>5.0</td>
</tr>
</tbody>
</table>

a Presumed functions are indicated with an asterisk.
b The remaining 43% are accounted for by unidentified peaks.
c Value calculated from the data given in ref. 31.
d Experimentally determined value (58).
mutated genes were carefully monitored to determine whether the absence of one gene product would affect the presence of any other polypeptide in whole cells and particularly in envelope preparations. With the mutants used, no such interdependence was observed. This result indicated that the absence of bands was likely to be due to the nonsense and deletion mutations used rather than to pleiotropic effects. The kinetics of synthesis of the prerplicative proteins and the size of the deletion fragments detected also support our identifications. That the association of polypeptides with the envelope fractions appeared to be independent of the presence of the other polypeptides studied in that fraction may be significant for future reconstitution experiments.

To examine whether any of the identified phage proteins was released from the envelope prior to cell lysis, a culture was labeled at 4 min after infection and the pulse was terminated 3 min later by the addition of a large excess of carrier amino acids. Samples were harvested in 3-min intervals and processed for gel electrophoresis. The ratio between the different bands remained approximately constant between 7 min after infection and the onset of cell lysis. These data indicated that lysis is not triggered by an early dissociation of one of the observed phage-coded proteins.

**DISCUSSION**

Our experimental results clearly show that plasma membranes of host cells undergo alterations upon infection with coliphage T4. Infected cells are more sensitive toward osmotic lysis than uninfected cells and the densities of their plasma membranes and cell walls are subject to small but reproducible changes. Also, membrane vesicles from infected cells exhibit distinct morphological differences when compared to their uninfected counterparts. We do not know whether these membrane modifications are due to alterations of phospholipids at early times after infection (61, 62), to changes in the peptidoglycan (61), to the association of newly synthesized phage-coded proteins with the membrane, or to a combination of these factors. The observation that nearly all prerplicative phage proteins were found attached to the host cell envelope, however, suggested that many of them may be bound nonspecifically. In an attempt to select proteins which may be inserted into the cytoplasmic membrane, we have used different procedures to characterize their envelope association and have eliminated those polypeptides which did not satisfy all of the individual criteria discussed below.

As a first approach, we observed which of the phage-coded proteins would partition between the soluble and particulate fraction after cell disintegration with glass beads. This relatively mild procedure does not create small vesicles which cannot be removed by high centrifugal forces. The rationale of this approach was the assumption that an integral membrane protein would not partition between the membrane and an aqueous phase even upon such harsh treatment as cell breakage. Four phage proteins were observed both in cell envelopes and in the supernatant fraction. Thus, the products of genes 43 and 45, the immunity protein and internal protein III, were eliminated as integral membrane proteins.

The second criterion consisted of the study of the partitioning of proteins between sedimentable and supernatant fractions after

The polypeptide fragments of deletion mutants in the rI1 region (del r88H and del r196, cf. Table I) and the fusion product from r1559 were well resolved with the gel system used and exhibited the molecular weights predicted from the physical map of the rI1 region (§).

The third approach consisted in the extraction of envelopes with guanidinium ions. The combination of high ionic strength and chaotropic effects was expected to remove all of those proteins from envelope preparations which were bound superficially by electrostatic interactions or were artifactually adsorbed to it during the preparation procedure. On the other hand, guanidinium ions are thought not to interfere with protein-lipid interactions (30), and integral membrane proteins should therefore not be extracted. Since the envelopes used had been damaged previously by cell breakage, however, only those proteins which were quantitatively removed by the presence of 4 M guanidinium were excluded as potential membrane proteins. This procedure allowed the elimination of the product of gene 46 as an integral membrane protein.

Taking together the results of these procedures (Table III), four polypeptides remained as potential integral membrane proteins, the products of genes rI1A, rI1B, 39, and 52. These assignments were also supported by yet another procedure (cf. the alternative preparation of spheroplasts in Table III). However, we do not believe that additional but similar screening experiments would conclusively affirm these assignments. As an independent approach, we therefore studied the interaction of these proteins with the subfractions of the host cell envelope.

Separation of plasma membranes from cell walls by isopyknic centrifugation showed that all phage proteins were quantitatively associated with the plasma membrane fraction. Since the density at which this fraction migrated was 1.14 g/ml, a ratio of 60% phospholipids to 40% proteins could be calculated assuming a partial specific volume for proteins of 0.73 ml/g and a density of approximately 1.0 g/ml for phospholipids (64). Thus, if these phage-coded proteins were not artifactually adsorbed to the plasma membrane, their sedimentation behavior would strongly indicate a specific interaction with membrane lipids. On the basis of the results obtained with the extraction of membranes from red blood cell ghosts with guanidinium ions (39), it may be reasonable to expect that such an extraction procedure would remove purely artifactually bound phage polypeptides. However, a critical reading of Table III reveals that such an interpretation may not apply here. Four proteins, the products of genes 43, X, 30/41, and 45, were eliminated by one of the three procedures previously discussed. However, these particular polypeptides were not quantitatively extracted by 4 M guanidinium. A similar observation was made with the large subunits (β and β') of the host cell RNA polymerase. The significance of the results obtained by extraction with 4 M guanidinium, and their applicability to infected bacterial membranes, therefore remains to be established.
The four phage coded proteins that we believe may be associated with the plasma membrane could be bound through direct protein-lipid interactions or through indirect associations with proteins or nucleic acids. Binding to RNA polymerase, ribosomes and nucleic acid fragments which themselves are membrane-bound could occur a priori. On the basis of the data presented, the first two possibilities seem unlikely, but we cannot exclude the third. It is conceivable that these proteins bind to small fragments of DNA which are contained in the membrane and protected by it towards the nucleolytic treatments used. Indeed, it was recently reported that the products of genes rIIA, 39, and 52 bind to DNA prepared from T4 phage or from calf thymus (65). However, before more details on the specificity of these interactions and on the quantitation of the distribution between insoluble and solubilized fractions are available, it is difficult to evaluate these results. In our opinion, this question can only be answered conclusively by reconstitution experiments of isolated polypeptides into membranes under controlled conditions.

Although the elimination of a particular polypeptide as a potential membrane protein should not be interpreted as excluding any interaction with the membrane, it seems appropriate to examine whether the assignment of the products of genes rIIA, rIIB, 39, and 52 as potential membrane proteins agrees with the limited information available on their functional roles. Indirect evidence has indeed suggested that the proteins of the rII region of the genome may be related to the sealing reaction which occurs shortly after infection (6). The phenotype of phages with mutations in genes 39 and 52 is that of a delayed T4 DNA synthesis (66). A membrane association of these gene products would be plausible if the delay in DNA synthesis were due to the effect of these proteins on ion flux across the membrane, as has been postulated earlier (57).

If a protein binds quantitatively to the membrane, the estimation of the number of molecules synthesized during the lytic cycle of bacteriophage T4 should yield the concentration of these proteins in the membrane. The results of such determinations indicated that there are approximately 10^9 polypeptide products of genes rIIA, 39, and 52, and approximately 5 times more for the rIIB protein. An area 55 nm square would therefore contain 1 molecule each of the products of the three former genes and 5 molecules of the rIIB protein if the distribution of these proteins in the membrane were random. The figure for the former three proteins is of the same order of magnitude as that calculated for ATPase in Streptococcus faecalis, but it is considerably lower than that obtained for the major envelope protein from E. coli (1 molecule/5.5 nm square). This protein forms a shell around the peptidoglycan layer of the cell in which the individual protein subunits are contiguous (27).

The screening procedure presented thus yields a small number of proteins whose properties of association with the plasma membrane and whose presumptive function indicate that they may reside in the membrane. Such a procedure allows, of course, merely an operational definition of a membrane protein. The chemical characterization of isolated phage polypeptides and the investigation of their interactions with phospholipids should yield more direct information about their interaction with the membrane. Furthermore, the reconstitution of individual T4 proteins into membranes under controlled conditions may provide an attractive model system for the study of the mechanism of insertion of polypeptides into membranes.

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37. In our opinion, this question can only be answered conclusively by reconstitution experiments of isolated polypeptides into membranes under controlled conditions.
38. We refer the reader to the original references for details on the techniques used.
39. The specificity of these interactions and on the quantitation of the distribution between insoluble and solubilized fractions is difficult to evaluate. In our opinion, this question can only be answered conclusively by reconstitution experiments of isolated polypeptides into membranes under controlled conditions.
40. The results of such determinations indicated that there are approximately 10^9 polypeptide products of genes rIIA, 39, and 52, and approximately 5 times more for the rIIB protein. An area 55 nm square would therefore contain 1 molecule each of the products of the three former genes and 5 molecules of the rIIB protein if the distribution of these proteins in the membrane were random. The figure for the former three proteins is of the same order of magnitude as that calculated for ATPase in Streptococcus faecalis, but it is considerably lower than that obtained for the major envelope protein from E. coli (1 molecule/5.5 nm square). This protein forms a shell around the peptidoglycan layer of the cell in which the individual protein subunits are contiguous (27).
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