ASSOCIATION OF NEWLY SYNTHESIZED MAJOR f1 COAT PROTEIN WITH INFECTED HOST CELL INNER MEMBRANE

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The bacteriophage f1 major coat protein becomes associated with the host cell inner membrane very shortly after it is synthesized. Pulse-chase experiments suggest that the virus is never stably associated with the host cell outer membrane; we propose that it passes directly from the inner membrane to the growth medium.

1. INTRODUCTION

Escherichia coli infected with the filamentous f1-type bacteriophage continue to divide exponentially at a slower rate than uninfected cells while producing and excreting several hundred new phage per cell generation (1). Infectious phage cannot be found inside the infected cells (2-3) and it is likely that newly formed phage are exported quickly from the cell (4). To facilitate the study of this unique mode of bacterial virus replication a method was developed to follow the synthesis and fate of the major f1 coat protein in normal unirradiated f1 infected cells (5). This technique was used to show that after a lag of about 1 min at 37° newly synthesized phage coat rapidly disappears from the cell and appears in the growth medium; the half time for the excretion at 37° is 3 1/2 min (5). The present study was undertaken to determine the location of the f1 coat protein immediately after its synthesis.

2. RESULTS

Non-permissive *E. coli* K38 was grown in M9 medium supplemented with 0.4% glucose and 19 amino acids, each at 20 µg per ml with lysine omitted (5). The culture was split into aliquots of 20 ml. Fraction I remained uninfected. Fraction II was infected with wild type f1 and fraction III was infected with an amber mutant in gene 1(R2). Twenty-five min after the time of infection each culture was labeled for 1 min with 2 µCi of [^{14}C]lysine. Ten ml of each culture was precipitated with trichloroacetic acid and assayed for the major f1 coat protein peptide. The remaining 10 ml was quickly cooled and centrifuged. The pelleted cells were suspended in 10 mM Tris·HCl-20% sucrose of pH 7.4, spheroplasted according to the method of Birdsell and Cota-Robles (6) and sonicated at 0° for 30 second intervals until clearing occurred. The sonicate was layered over a shelf of 10% sucrose and centrifuged at 1000 x g for 30 min. This removed unbroken cells and large debris which were assayed for their content of the major f1 coat protein. The supernatant fraction was treated with RNase (10 µg per ml) for 20 min at room temperature and then centrifuged at 250,000 x g for 2 hr. The pellets and supernatant fractions were precipitated with trichloroacetic acid and assayed for their content of the N-terminal tryptic peptide of the major f1 coat protein. The results in

TABLE 1

Location of the major f1 coat protein in cultures of E. coli *K38 infected with wild type f1 and an amber mutant in gene 1 (R2)*

Incorporation of L-lysine into acid insoluble material (counts per min)

	Total incorporation	High speed supernatant	Pellet
I. uninfected	1,669,000	953,000	403,000
II. f1	1,654,000	820,000	417,000
III. R2	1,166,000	748,000	409,000

Distribution of the N-terminal tryptic peptide of the major f1 coat protein (counts per min)

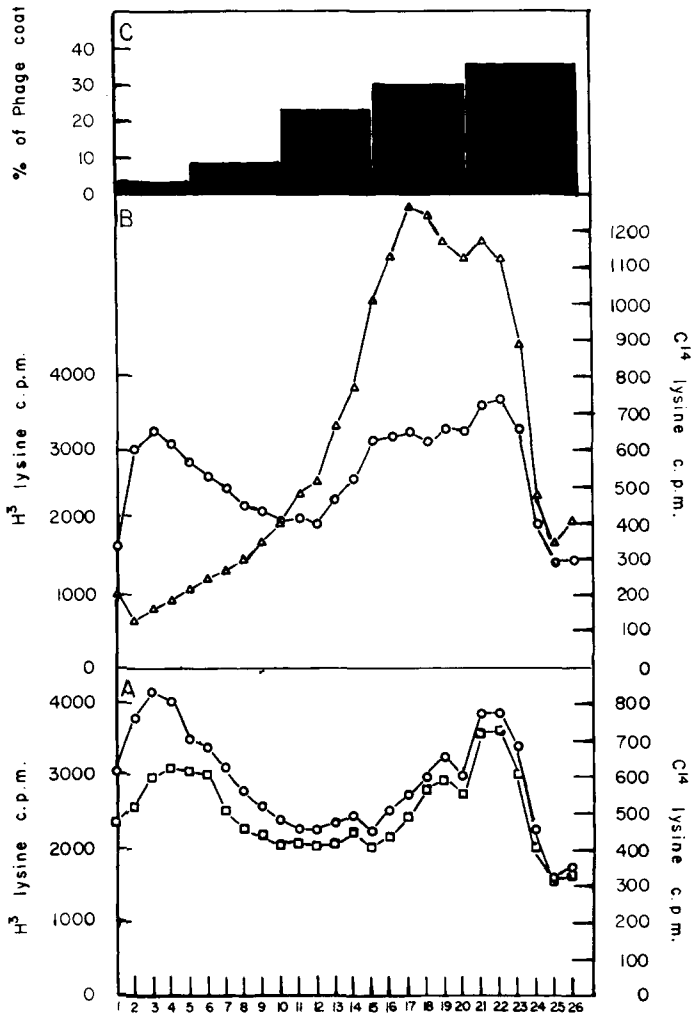
	Total	Supernatant solution	Pellet	Growth medium	Unbroken cells
I. uninfected	12,210	8,975	2,590	236	1,524
II. f1	30,630	9,229	28,400	576	14,720
III. R2	25,850	8,872	21,050	237	8,828

Table 1 show that most of the radioactivity in the N-terminal tryptic peptide of the major f1 coat protein can be recovered in the high speed pellet. This is also true if the f1 infected cells are labeled for 10, 20, 30 or 60 seconds. Since there is a 60 second interval between the synthesis of phage coat and its appearance in the growth medium, phage coat protein in the high speed pellet does not represent excreted phage. Since no phage can be found inside the cell, phage coat found in the high speed pellet does not represent viable intracellular phage. Therefore it is likely that the major phage coat protein in some intermediate stage of phage f1 maturation is associated with the cell envelope.

The *E. coli* cell envelope can be divided into 3 distinct layers. The cytoplasmic or inner membrane is rich in phospholipids and proteins; the mucopeptide fraction is thought to give the cell its characteristic shape and can be digested with lysozyme; and the outer membrane contains phospholipid, protein and lipopolysaccharide. The inner membrane is less dense than the outer membrane having a density of 1.18 g per cm^3. The outer membrane has a density of 1.22 g per cm^3. Recently it has become possible to isolate separately the inner and outer membranes of *E. coli* and *Salmonella* (7,14). We have utilized this technique to further study the association of the major f1 coat protein with the cell envelope.

(a) *Separation of inner and outer membranes*

Three cultures of *E. coli* were grown. One was labeled with [^{3}H]lysine for several generations. The second was infected with wild type f1 and labeled for 30 seconds with [^{14}C]lysine. A third culture (uninfected) was also labeled for 30 seconds with [^{14}C]lysine. Membranes were prepared from all 3 cultures. An aliquot of the long term [^{3}H]lysine labeled membranes was mixed with the [^{14}C]lysine pulse labeled membranes prepared from the uninfected cells. A second aliquot of [^{3}H]lysine labeled membranes was mixed with the



[^{14}C]lysine labeled membranes prepared from the f1 infected cells. Each mixture was centrifuged to equilibrium in a preformed, linear sucrose density gradient. Radioactivity was assayed along both gradients (Fig. 1A and 1B respectively). Fractions 1-5, 6-10, 11-15, 16-20 and 21-25 were pooled from gradient B and assayed for the N-terminal tryptic peptide of the major f1 coat protein (Fig. 1C). The details of this experiment are presented in Table 2. A similar experiment was done in which cultures of permissive *E. coli* K37 were pulse labeled with [^{14}C]lysine at 0, 15 and 25 min after being infected with f1. Membranes were made and mixed with aliquots of [^{3}H]lysine labeled membranes prepared from the uninfected cells. Each mixture was again centrifuged to equilibrium in a sucrose density gradient (Fig. 2).

Fig. 1 shows that during a 30 second labeling period, less total label is incorporated into the outer membrane and more label is incorporated into the inner membrane of f1 infected cells, as compared with incorporation into the comparable fractions of uninfected cells (see also Table 2). Fig. 2 shows that most of this change occurs between 15 and 25 min after infection, during which time phage coat synthesis becomes maximal (5). In addition, Fig. 1 shows that all of the phage coat protein that is in the cell envelope can be

Fig. 1. *Separation of inner and outer membranes from uninfected and f1 infected cells.* Three cultures of *E. coli* were grown in M9 medium supplemented with 0.4% glucose and 19 amino acids, each at 20 µg per ml, with lysine omitted. One culture was supplemented with 10 µg per ml of lysine and 0.3 ml of [^{3}H]lysine (3 Ci per mmole). At an A_{600} of 0.2 the second culture was infected with wild type f1. A third culture was left uninfected. At an A_{600} of 0.5 (5 x 10^8 cells per ml) both the uninfected culture and the f1 infected culture were labeled for 30 seconds with 10 µCi of [^{14}C]lysine (>220 mCi per mmole) and quickly cooled. Membranes were prepared using a modification of the method described by Miura and Mizushima (7). All of the cultures were centrifuged for 10 min at 10,000 x g. The pelleted cells were suspended to an A_{600} of 5.0 in cold 0.75 M sucrose-10 mM Tris·HCl of pH 7.5. Lysozyme was added at 100 µg per ml of Tris-sucrose solution, and the suspension stirred in the cold. Two volumes of cold 1.5 mM EDTA were added dropwise over 10 min with constant stirring. The spheroplasts were broken by brief (45 second) sonication. Unbroken cells were removed by centrifuging the sonicate for 30 min at 1000 x g. The membranes were then pelleted by centrifugation for 2 hr at 250,000 x g. The membranes were suspended in cold 0.3 M KCl-5 mM EDTA, sonicated for 2 min and pelleted again. The washed membranes were suspended in 5 mM EDTA. An aliquot of the long term [^{3}H]labeled membranes (—○—○—) was mixed with the [^{14}C]lysine pulse labeled membranes prepared from the uninfected cells (—□—□—) (gradient A). A second aliquot of [^{3}H]lysine labeled membranes (—○—○—) was mixed with the [^{14}C]lysine labeled membranes prepared from the f1 infected cells (—△—△—) (gradient B). The sucrose density gradients (35 to 52.5% sucrose-5 mM EDTA) were spun for 40 hr at 200,000 x g and radioactivity was assayed along both gradients. Fractions 1-5, 6-10, 11-15, 16-20 and 21-25 were pooled from gradient B and assayed for the N-terminal tryptic peptide of the major f1 coat protein. The amount of the peptide found is expressed as the percentage of the total amount of the peptide present in the gradient (Fig. 1C).

found in the inner membrane; and most of the phage coat protein can be found in the least dense fractions of the inner membrane.

[^{3}H]lysine labeled f1 and [^{14}C]lysine labeled inner membrane prepared from uninfected cells were mixed. Both sonicated and unsonicated mixtures were centrifuged on sucrose density gradients. Fig. 3 illustrates the extent of separation obtained in both experiments. While the [^{14}C]labeled membranes banded according to their density, the [^{3}H]lysine labeled phage merely sedimented slowly. This reconstruction experiment suggests that the phage coat protein associated with the cell envelope is not present in newly formed phage particles which adhere nonspecifically to the cell membrane. This is further substantiated by the fact that the phage coat produced in non-permissive *E. coli* infected by a gene 5 amber mutant becomes associated with the host cell inner membrane. Gene 5 amber mutants of phage f1 are defective in phage DNA synthesis and do not form phage particles.

Table 2 also shows that while f1 infected cells incorporate 40 to 50% less radioactivity during a 30 second labeling with [^{14}C]lysine than do uninfected cells, they incorporate more radioactivity into the cell envelope than do uninfected cells. Also, f1 infected cells and uninfected cells incorporate nearly identical amounts of [2-^{3}H]glycerol into phospholipid for 60 min after infection (8). This suggests that the rate of total membrane

TABLE 2

The effect of phage f1 infection on host membrane synthesis

	uninfected	f1 infected
Centrifuged cells (cpm)	5,540,000	4,000,000
Acid insoluble material (cpm)	5,569,000	2,922,000
Membrane (cpm)	667,000	787,000
Doubling time (min)	40	60

	inner membrane (fractions 12-24)		outer membrane (fractions 2-8)	
	cpm	% of total cell incorp.	cpm	% of total cell incorp.
Uninfected	373,000	6.8	284,000	5.2
f1 Infected	669,000	22.8	80,500	2.8

	[^{14}C]lysine (cpm)	% of whole cell
Whole cell (f1 infected)	2,932,000	100
Membrane	787,000	26
N-Terminal tryptic peptide of major coat protein	42,400	1.4
Phage coat protein	212,000	7.0

	[^{14}C]lysine (cpm)	% of total phage coat
Total phage coat	212,000	100
Phage coat in supernatant	60,000	28
Phage coat in KCl wash	8,500	4
Phage coat in membrane	141,000	66.5

The experimental details are described in Fig. 1. This table provides a detailed quantitation for this experiment.

synthesis is not affected by f1 infection while other cell functions, such as protein synthesis, and cell division are slowed by 40 to 50%. Since the size of an f1 infected cell is identical to that of an uninfected cell (9) it is likely that f1 infected cells contain more cell envelope per cell than do uninfected cells. This is also suggested by the electron microscopic examinations performed by Bradley (10).

(b) *Protein composition of the f1 infected cell envelope*

To determine the protein composition of the f1 infected cell envelope, two cultures of *E. coli* K37 were grown. Culture I remained uninfected while culture II was infected with wild type f1. Sixty min after the time of infection, culture I was labeled with [^{3}H]proline. At the same time culture II

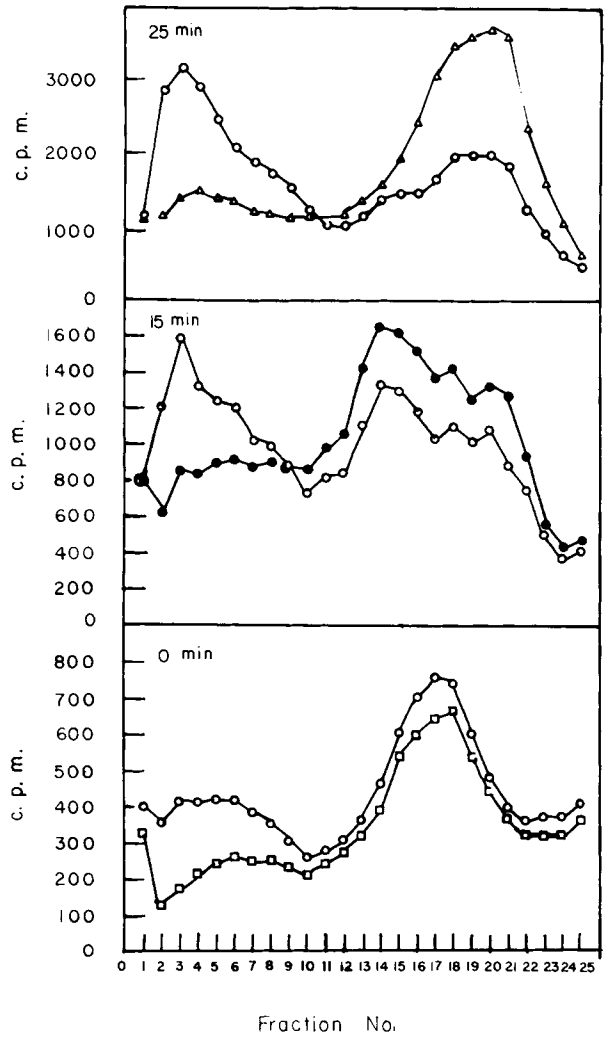


Fig. 2. *Separation of inner and outer membrane at various times after infection.* A culture of *E. coli* was grown and infected with wild type f1 under the same conditions as detailed in the legend of Fig. 1. At 0, 15 and 25 min after infection, an aliquot of the culture was pulse labeled with 10 μCi of [^{14}C]lysine for 1 min and quickly centrifuged in the cold at 10,000 x g for 10 min. Membranes were prepared as detailed in the legend of Fig. 1. An aliquot of long term [^{3}H]labeled membranes (—○—○—) was mixed with the [^{14}C]lysine pulse labeled membranes (—□—□—, —●—●—, —△—△—; 0 min, 15 min, 25 min, respectively). The mixture was layered on a linear 35 to 25.5% sucrose density gradient containing 5 mM EDTA and centrifuged for 40 hr at 200,000 x g. Radioactivity was assayed along the gradients.

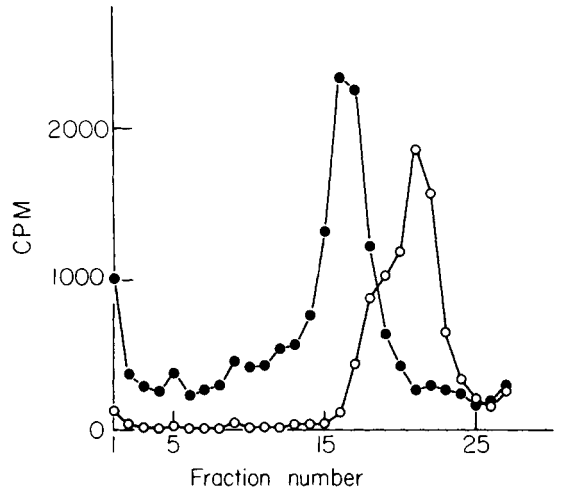


Fig. 3. *Sucrose density gradient separation of phage f1 and cell inner membrane.* [^{3}H]lysine labeled f1 (—●—●—) and [^{14}C]lysine labeled inner membrane (—○—○—) prepared from a culture of uninfected K37 cells as described in the legend of Fig. 1 were mixed. The unsonicated mixtures were layered on a linear 35 to 52.5% sucrose density gradient containing 5 mM EDTA and centrifuged for 40 hr at 200,000 x g. Radioactivity was assayed along the gradient.

was labeled with [^{14}C]proline. Membranes were then prepared. The two membrane preparations were mixed and layered on a sucrose density gradient and centrifuged for 12 hr. The outer membrane fractions of the gradient were pooled and precipitated with trichloroacetic acid. Half of the trichloroacetic acid precipitate was assayed for f1 coat protein while the other half was subjected to SDS-acrylamide gel electrophoresis. The inner membrane fractions were pooled and treated in a similar fashion. The results from gel electrophoresis of the outer membrane and inner membrane proteins are shown in Fig. 4A and 4B respectively. The [^{14}C] and [^{3}H] counts have been normalized to represent equivalent moles of proline. Table 3 shows the comparative results of the peptide assay and gel.

Membranes from f1 infected cells contain all of the normal protein species found in uninfected cells as well as phage coat protein which is present almost exclusively in the inner membrane. All membrane protein radioactivity is present in decreased amounts in the f1 infected cell; the amount of phage coat protein present on gels is equivalent to the amount of phage coat peptide that is formed. All of the excess label in inner membranes found in f1 infected cells labeled with proline can be accounted for by the f1 phage coat protein. The decreased amount of outer membrane protein radioactivity in f1 infected cells may therefore reflect a decrease in the synthesis of all membrane proteins in f1 infected cells.

The following pulse-chase experiment also shows that all of the excess label in the inner membrane of f1 infected cells can be accounted for by the f1 phage coat protein. Uninfected cells were labeled with [^{3}H]proline for 1 min while f1 infected cells were labeled with [^{14}C]proline for 1 min. A 10,000-fold excess of unlabeled proline was added to each culture and samples were withdrawn and mixed immediately, after 1 min, 3 min and 9 min of incubation. Inner and outer membranes were prepared from each mixture and their

TABLE 3

Assay of the distribution of the major f1 coat protein by paper electrophoretic peptide analysis and gel electrophoresis of the membrane proteins

	Peptide assay (cpm)	Gel electrophoresis (cpm)
Unseparated membrane	100,000	99,200
Inner membrane	74,300	101,000
Outer membrane	6,250	5,885

See the legend of Fig. 4 for experimental details.

proteins analyzed by SDS-acrylamide gel electrophoresis. Fig. 5 shows that at the end of a 1 min labeling the major f1 coat protein constitutes 50% of the proline label in infected cell inner membrane; by 4 min it constitutes only 20%. The small amount of phage coat protein present in the outer membrane is a reflection of contamination of outer membrane with inner membrane. By 10 min a small amount of small molecular weight material is still associated with host cell inner membrane. This material is most likely phage coat protein. The fact that the phage coat protein chases out of the inner membrane also suggests that the phage coat protein associated with the inner membrane is not non-specifically attached.

3. DISCUSSION

On the basis of these data we propose that the f1 coat protein becomes associated with the host inner membrane very shortly after it is synthesized. Attempts to find the coat protein in the cytoplasm after very short pulse-labels have been unsuccessful. The association of coat protein with the membrane is not covalent since it is released by SDS. In some unknown way the coat protein and DNA combine and the resulting phage is released from the membrane. We propose that the virus is never stably associated with the host cell outer membrane but passes directly from the inner membrane to the growth medium. The evidence presented also suggests that f1 infected cells contain less outer membrane and more inner membrane than do uninfected cells. During a short labeling period less total label is incorporated into the outer membrane and more label is incorporated into the inner membrane of f1 infected cells as compared to identically treated uninfected cells. A pulse-chase experiment in which inner and outer membranes were prepared at various times after the beginning of the chase shows the continued absence of label and phage coat in the outer membrane fractions of the gradient. When corrected for growth rate, f1 infected cells contain a normal complement of host cell inner membrane proteins and a decreased amount of host cell outer membrane

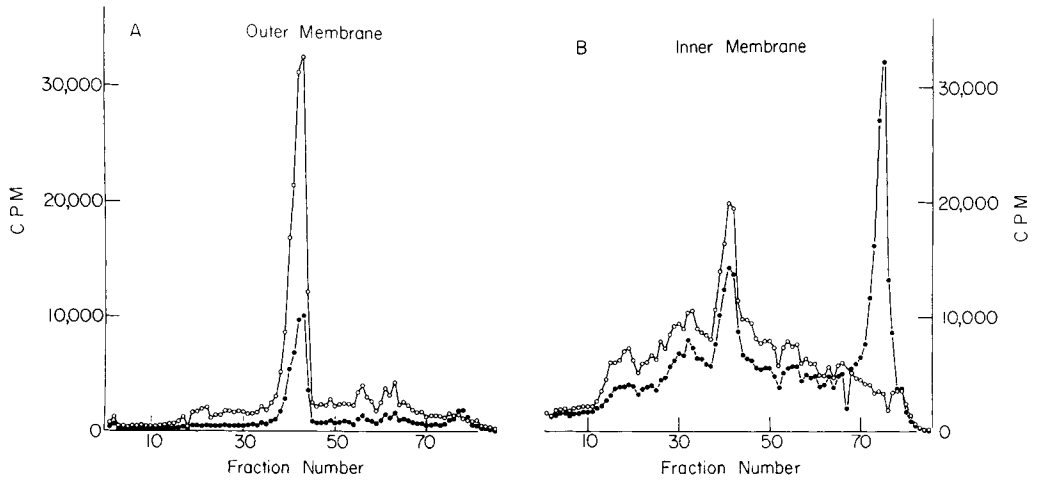


Fig. 4. *SDS-acrylamide gel electrophoresis pattern of outer and inner membrane proteins from infected and uninfected cells.* A 200 ml culture of *E. coli* K37 was grown in M9 medium supplemented with 0.4% glucose and 19 amino acids, each at 20 µg per ml with proline omitted. The culture was split into two 100 ml portions. Culture I remained uninfected while culture II was infected with wild type f1. Sixty min after the time of infection, culture I was labeled for 1 min with 200 µCi of [^{3}H]proline (2 Ci per mmole) (—○—○—). At the same time culture II was labeled for 1 min with 20 µCi of [^{14}C]proline (200 mCi per mmole) (—●—●—). Membranes were then prepared as described in the legend to Fig. 1. Half of each membrane preparation was mixed and layered on a linear 35 to 52.5% sucrose density gradient containing 5 mM EDTA and centrifuged for 40 hr at 200,000 x g. The outer membrane fractions of the gradient were pooled and precipitated with trichloroacetic acid. Half of the trichloroacetic acid precipitate was assayed for f1 coat protein while the other half, was subjected to electrophoresis on a 2% SDS, 7.5% acrylamide gel according to the method of Fairbanks (13). The inner membrane fractions were pooled and treated in a similar fashion. The distribution of radioactivity in the outer membrane and inner membrane gels is shown in Fig. 4A and 4B respectively. The [^{14}C] and [^{3}H] cpm have been normalized to represent equivalent moles of proline. Table 3 shows the comparative results of the peptide and gel assays performed on unseparated membranes, and the outer and inner membrane fractions from f1 infected cells.

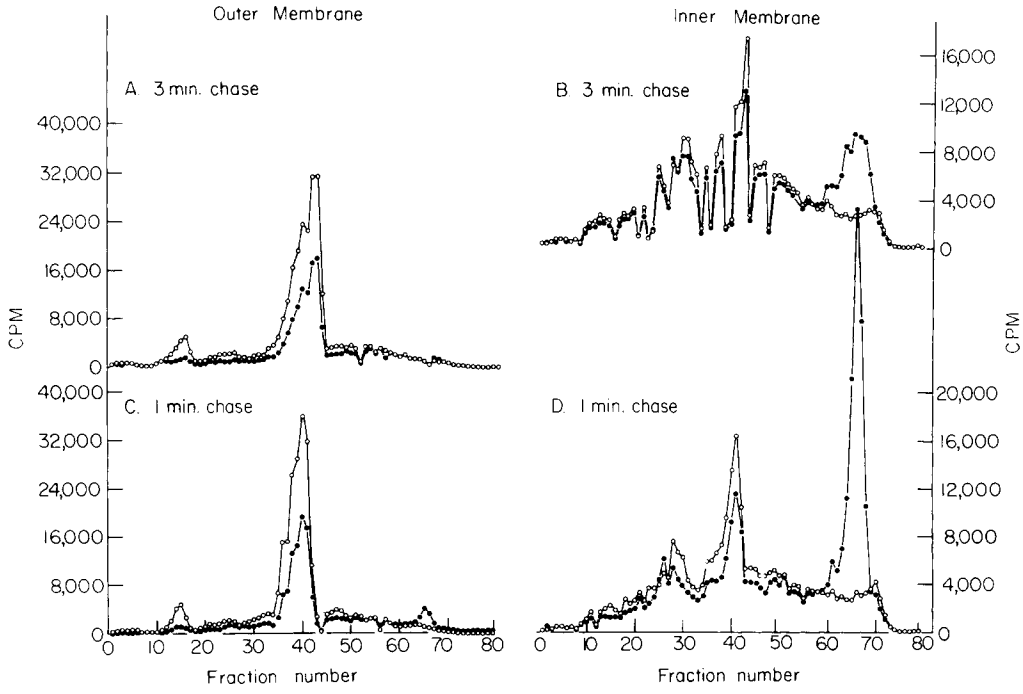


Fig. 5. *SDS-acrylamide gel electrophoretic patterns of inner and outer membrane proteins from a pulse-chase experiment.* Two 300 ml cultures of *E. coli* K37 were grown in M9 medium supplemented with 0.4% glucose and 19 amino acids, each at 20 µg per ml with proline omitted. One culture was infected with wild type f1 at an A_{600} of 0.2. The uninfected culture was labeled at an A_{600} of 0.5 for 1 min with 250 µCi of [^{3}H]proline (1.0 Ci per mmole) (—○—○—). After 1 min a 10,000-fold excess of unlabeled proline was added, and 70 ml of the culture was poured over ice. The chase was continued, and at 1 min, 3 min and 9 min of incubation, 70 ml aliquots were poured over ice and the cells collected by centrifugation. At an A_{600} of 0.5 the infected culture was labeled for 1 min with 50 µCi of [^{14}C]proline (200 mCi per mmole) (—●—●—) and treated in the same way as were the uninfected cells. The pellets were suspended in Tris-sucrose solution and the infected and uninfected cells were mixed. Membranes were prepared and subjected to sucrose density gradient centrifugation as described in the legend of Fig. 1. Inner and outer membranes were pooled from each gradient and centrifuged at 250,000 x g for 2 hr. The pellets were dissolved in 2% SDS and electrophoresis performed on 7.5% acrylamide gels. The inner and outer membrane protein gel patterns are shown for the 1 min chase and the 3 min chase.

proteins. These findings are consistent with reports that f1 infected cells are permeable to actinomycin, sensitive to deoxycholate and in general more fragile than uninfected cells (11-12). Of special interest is the fact that f1 infected cells contain as much or more total membrane as do uninfected cells although the rate of synthesis of all host membrane proteins is reduced. The rate of lipid synthesis is slightly stimulated by f1 infection although the amount of lipopolysaccharide made (as measured by galactose incorporation into an epimerase negative mutant) is reduced by 50%. Perhaps the rate of lipid synthesis is regulated by the amount of protein destined for membrane while the amount of lipopolysaccharide synthesis is regulated by the growth rate of the cell.

Further experiments suggest that the f1 infected cell envelope has altered properties. In particular, infected cells are insensitive to a variety of colicins. This will be the subject of a further communication.

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