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ALTERATIONS IN THE CELL ENVELOPE OF *ESCHERICHIA COLI* LATE IN BACTERIOPHAGE T4 INFECTION *

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Summary

The cell envelope of *Escherichia coli* was examined for changes during late stages of bacteriophage T4 infection. Late events in T4 infection are shown to result in (i) a reduction in the effectiveness of membrane separation procedures employing either isopycnic sucrose gradient centrifugation or selective solubilization of inner membrane by detergent (Sarkosyl or Triton X-100), (ii) the appearance of a 54 000 dalton host protein in membrane preparations, (iii) the adventitious presence of detergent-resistant phage morphogenetic structures in membrane preparations, and (iv) a decrease in the activity of NADH oxidase and an apparent alteration in its association with inner membrane. These modifications occur regardless of the state of the *e* and *t* genes of T4.

Introduction

The cell envelope of *Escherichia coli* is involved in many phases of the T4 infective cycle. Initially the phage interacts with a specific lipopolysaccharide receptor in the outer membrane [1]. The effective T4 receptors appear to be those positioned at sites of adhesion between the cell wall and the inner membrane [2,3]. Once the T4 genome has entered the cell a number of changes in the host components of the cell wall occur. These include accelerated loss of outer membrane material [4,5], detachment of the host chromosome from membrane [6,7], the disruption of host nucleoids [8] and reduction in the rate of synthesis of the major outer membrane proteins [9,10], phospholipids [11, 12], and lipopolysaccharide [13]. Other envelope-related events include the attachment to and replication of the T4 genome on the membrane [6,7], the association of bacterial DNA polymerase I with the cell membrane [14], alterations in the permeability barrier of the cell envelope, the strengthening of

* Preliminary reports of this work were presented at the Annual Meetings of the American Society for Microbiology in 1975 (New York) and 1976 (Atlantic City).

the cell envelope which is manifested by super infection exclusion and resistance to killing by ghosts, and the development of lysis inhibition [15]. Later in infection, precursors of the T4 head are seen associated with membrane [6, 16] and the infective cycle is ended with the cessation of oxygen uptake and the subsequent lysis of the infected cell [17].

The large number of cell envelope-related modifications following T4 infection and the concentration of previous studies on changes that occur soon after infection prompted this study of phenomena occurring late in T4 infection. Alterations which were observed late in permissive infection include an increased sensitivity of outer membrane to Sarkosyl and Triton X-100, an apparent tighter association or mixing of outer and inner membrane which prevents their separation in sucrose gradients, and a decrease in NADH oxidase activity with increasing time after infection. It was also observed that outer membrane preparations isolated late in infection contained apparent T4 head precursors and a preexisting host protein which was not present in membrane prior to infection.

Materials and Methods

Bacteria and phage. E. coli strains B, BB, CR63 and PL-2 (defective in UDP-galactose epimerase [18], provided by H. Wu) were employed in these experiments. Bacteriophage used included $T4D_0$, T4*rII*187, T4*am*N82 (gene 44, defective in DNA synthesis), T4*am*H26 (*e* gene, defective in lysozyme production), T4*amt*A3 (*t* gene, defective in lysis), and T4*am*B17 (gene 23, defective in the major T4 head protein). $T4D_0$ was grown on *E. coli* B or BB and the amber mutants were grown on *E. coli* CR63; phage were concentrated and purified as described previously [19].

Media and reagents. Tryptone-NaCl broth, top agar, plates, dilution fluid [19], F medium [20], T medium [21] and HB medium [22] have been described.

Sodium lauryl sarcosinate (Sarkosyl) was a gift from Geigy Corp., Ardsley, NY, and Triton X-100 was obtained from Sigma Chemical Co., St. Louis, MO. The following radiochemicals were purchased from New England Nuclear, Boston, MA: L-[4,5-^{3}H]leucine, D-[1-^{14}C]galactose, and carrier-free $H_2{}^{35}SO_4$: L-[U-^{14}C]leucine was obtained from Amersham Searle, Arlington Heights, IL, or Schwarz-Mann, Orangeburg, NY, and [2-^{3}H]glycerol was purchased from Amersham-Searle. NADH, disodium, was obtained from P-L Biochemicals, Milwaukee, WI.

Membrane isolation. The procedure of Osborn et al. [23] was used with the modifications described previously [24]. Total membrane preparations were isolated by terminating the procedure after the second 360 000 × *g* centrifugation. Outer, middle and inner membrane fractions were obtained by isopycnic sucrose density gradient centrifugation. Gradient fractions to be pooled were identified by one of two methods: 5-μl portions of each 0.4 ml gradient fraction were precipitated with trichloroacetic acid, collected on filters and washed and the radioactivity was determined as described previously [24] or absorbance (280 nm) was monitored during gradient fractionation. Fractions corresponding to the membrane species were pooled, diluted to 0.25 M sucrose,

1.0 mM EDTA · Na_2, 3.3 mM Tris-HCl, pH 7.8, and collected by centrifugation at 360 000 × *g* for 2 h. Preparations for gel electrophoresis were resuspended in solubilization buffer [25] and those for NADH oxidase assay in 0.05 M Tris-HCl buffer (pH 7.8) containing 0.2 mM dithiothreitol. On one occasion total membrane was isolated by the procedure of Inouye and Guthrie [26].

Polyacrylamide gel electrophoresis. Slab gel electrophoresis was performed by a modification [21] of the discontinuous gel electrophoresis method of Laemmli [25] except that 7.5% gels were run at 160 V for 1.25 h.

Preparation of [^{14}C]leucine-labeled T4D$_0$. A 20 ml culture of *E. coli* BB in F medium supplemented with 5 μg L-leucine/ml was grown to an absorbance at 540 nm of 0.3, supplemented with 50 μg L-tryptophan/ml, and infected with T4D$_0$ at a multiplicity of infection of 7.5. At 9 min after infection 0.2 μCi L-[^{14}C]leucine/ml was added. 3 h later the lysate was concentrated and purified.

Determination of NADH oxidase activity. The membrane fraction (3—100 μg of protein as determined by the method of Lowry et al. [27]) was assayed as described previously [23].

Densitometer scanning of autoradiograms. The portion of the autoradiogram of slab gels corresponding to proteins of molecular weight 90 000 to 30 000 was scanned with a Beckman Analytrol Film Analyzer.

Results

Localization of host membrane constituents after infection

We have shown that *E. coli* outer and inner membranes can still be separated by equilibrium centrifugation after non-permissive infection with the DNA-negative mutant T4*am*N82 [20]. Membranes isolated from cells in which T4 late protein synthesis has occurred cannot be separated (Fig. 1A—C). The distribution of host membrane protein isolated from cultures infected with T4D$_0$ for up to 9 min is indistinguishable from that of uninfected cells or of cultures infected with T4*am*N82 [20]; that is, the outer (fractions 6—12) and inner (fractions 16—26) membrane species are still clearly separable at this stage of infection. (The material at the bottom of the gradient (fractions 1—4 in panels A, B and C) contains outer membrane proteins [20] and is therefore probably related to that characterized by Loeb and Kilner [5].) However, as the infection progresses (Fig. 1C), there is an increase in the material in the middle region of the gradient, which can be correlated with decreases in both inner and outer membrane bands. This effect has also been seen after infection of *E. coli* B with T4*rII*187, T4*am*H26, and T4*amt*A3 and of *E. coli* CR63 with T4D$_0$ (data not shown).

Pre-existing lipopolysaccharide is also delocalized late in infection. Virtually all of the lipopolysaccharide is associated with the outer membrane of uninfected *E. coli* [23], of cells infected for 15 min with T4*am*N82 (data not shown), and of cells infected for 5 min with T4D$_0$ (Fig. 2A). Late in infection (Fig. 2B), pre-existing lipopolysaccharide is no longer localized exclusively in the region of the gradient characterized as outer membrane; some lipopolysaccharide appears in the middle band and some at a density corresponding to inner membrane.

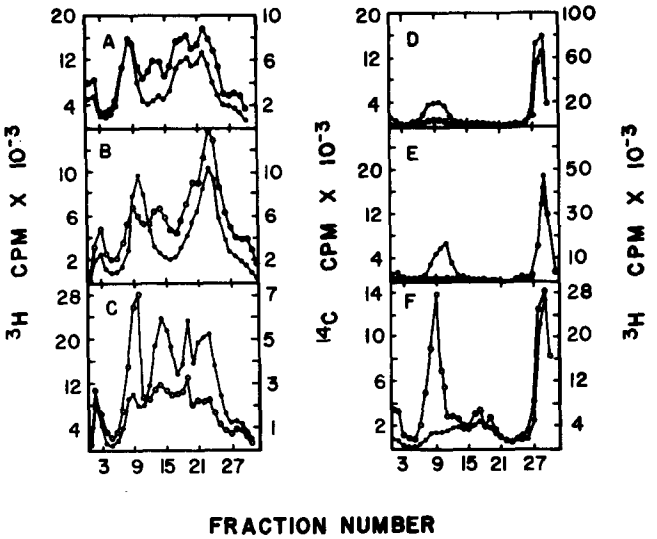

Fig. 1. Separation of membrane proteins from infected cells. An overnight culture of *E. coli* B was diluted 1 : 200 into 150 ml of F medium supplemented with 0.05 μCi of L-[^{14}C]leucine/ml and grown at 37°C to a concentration of $4 \cdot 10^8$ cells/ml. Cells were harvested and resuspended in 150 ml of pre-warmed F medium supplemented with 50 μg L-tryptophan/ml. The culture was divided into three equal portions and each portion was infected with 7.5 $T4D_0$/cell. Each portion was pulse-labeled with 4.0 μCi L-[^{3}H]-leucine/ml for a different consecutive 4-min interval beginning 1 min after infection. At the end of the pulse period, the infection was terminated by pouring the sample over 0.5 vol. of crushed, frozen F medium containing 150 μg chloramphenicol/ml. Isolation and fractionation of membrane was then carried out as described in Materials and Methods. Prior to layering on the gradient, half of each sample was treated with Sarkosyl and incubated for 20 min at 23°C. The top of the gradient is on the right. Results of labeling with L-[^{3}H]leucine 1—5, 5—9 and 9—13 min after infection are shown in panels A, B, and C, respectively. Corresponding samples treated with Sarkosyl are shown in panels D, E, and F. Symbols: •, L-[^{14}C]leucine label; ○, L-[^{3}H]leucine label.

Detergent solubilization of host outer membrane proteins

Outer membrane isolated from uninfected *E. coli* B cells is resistant to solubilization by the ionic detergent Sarkosyl [24] and by the non-ionic detergent Triton X-100 in the presence of Mg^{2+} [28]. Outer membrane from cells infected with T4*am*N82 exhibits the same resistance to solubilization as that from uninfected cells [20]. However, as shown in Fig. 1D—F, outer membrane from cells infected with $T4D_0$ for 5 or 9 min exhibits partial sensitivity to Sarkosyl and, after 13 min of $T4D_0$ infection, virtually all of the pre-existing protein of outer membrane has become sensitive to disruption by Sarkosyl. Pre-existing outer membrane proteins from cells infected with wild-type T4 also exhibit an increase in sensitivity to the detergent Triton X-100 in the presence of Mg^{2+} (Fig. 3).

Alterations in the protein composition of the outer membrane after infection

A comparison of membrane proteins in uninfected cells with host proteins present in membrane preparations isolated from cells late in T4 infection revealed the presence of an additional polypeptide in the latter (Fig. 4). This polypeptide was not seen in the studies with the DNA-negative mutant T4*am*N82 [20], but late protein synthesis is apparently not necessary for its

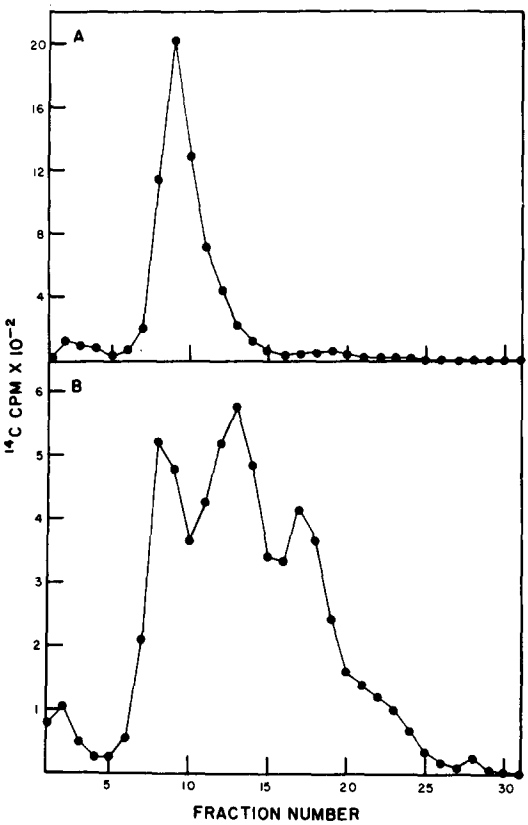

Fig. 2. Distribution of host lipopolysaccharide after infection. An overnight culture of *E. coli* PL-2 was diluted 1 : 200 into 100 ml HB medium supplemented with 0.1 mg threonine/ml, 0.1 mg leucine/ml, 1 μg thiamine/ml, 43 μg galactose/ml and 0.125 μCi [^{14}C]galactose/ml and grown at 37° C to a concentration of $3 \cdot 10^8$ cells/ml. Cells were harvested and resuspended in 100 ml of prewarmed HB medium supplemented as above except 50 μg L-tryptophan/ml was added and radioactive galactose was omitted. The culture was divided into two equal portions and each was infected with 10 $T4D_0$/cell. Infection was terminated at 5 or 13 min by pouring the sample over 0.5 vol. of crushed, frozen HB medium containing 150 μg/ml chloramphenicol. Isolation and fractionation of membrane was then carried out as described in Materials and Methods. The bottom of the gradient is on the left. Results of 5 and 13 min of infection are shown in panels A and B, respectively. •, [^{14}C]galactose.

appearance as this protein was present in membrane as early as five minutes after infection. Inspection of outer, middle, and inner membrane for the appearance of this 54 000 dalton protein showed that it appears predominantly in the outer membrane and that it does not appear to exist in appreciable quantitites in any membrane species prior to infection (Fig. 5). Host outer membrane proteins become sensitive to detergent treatment late in T4 infection (Figs. 1F and 3C); 54 000 and outer membrane proteins of uninfected cells exhibited identical patterns of detergent sensitivity. Fig. 5 also shows that there are some other changes in the protein composition of these membranes such as the loss of a 62 000 dalton protein from outer membrane and the mixed membranes.

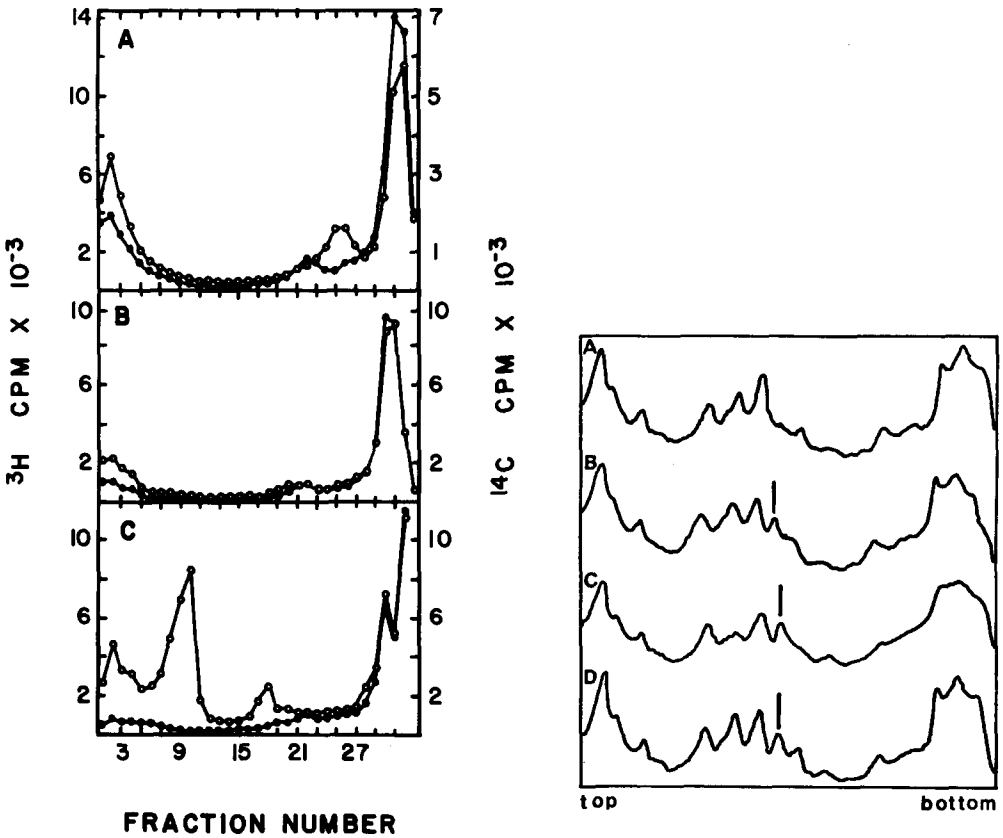

Fig. 3. Effect of Triton X-100 on membrane preparations from infected cells. *E. coli* B cultures were grown and infected as described in Fig. 1, except that the medium was T medium supplemented with 0.4% glucose, 10 μg casamino acids/ml, 10^{-4} M Na_2SO_4, and 0.025 μCi L-[^{14}C]leucine/ml and that pulse-labeling employed 2.0 μCi L-[^{3}H]leucine/ml. Results of pulse-labeling for 1—5, 5—9, and 9—13 min after infection are shown in panels A, B and C, respectively. •, L-[^{14}C]leucine label; ○, L-[^{3}H]leucine label.

Fig. 4. Densitometer tracings of autoradiograms of host proteins with molecular weights ranging from 30 000 to 90 000 in uninfected cultures. Samples for slab gel electrophoresis were prepared from cultures of *E. coli* B grown and infected as described for Fig. 1, except that T medium supplemented with 0.4% glucose, 10 μg casamino acids/ml, 10^{-4} M Na_2SO_4 and 17 μCi $H_2{}^{35}SO_4$/ml was used. Samples are total membrane: A, uninfected; B, after 5 min of infection; C, after 9 min of infection; D, after 13 min of infection. Vertical line indicates position of 54 000 dalton protein.

Examination of the newly synthesized proteins appearing in the gradient region corresponding to outer membrane

Much of the protein synthesized late in T4 infection appears in the region of the gradient corresponding to outer membrane and is not solubilized by detergent treatment (Figs. 1F and 3C). The distribution of proteins synthesized after infection was examined using T4*am*B17, a mutant which is defective in the major structural protein of the T4 head. Fig. 6 shows a decrease in the amount of newly-synthesized protein in the region of the gradient corresponding to outer membrane compared to that seen after wild-type T4 infection (Figs. 1C and 1F). This suggested that a structural intermediate in T4 head assembly was

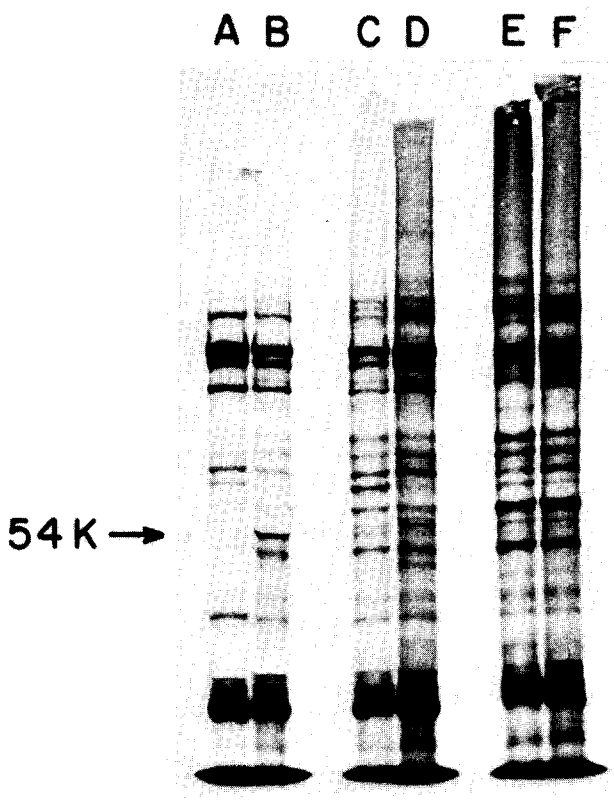

Fig. 5. Host membrane proteins after T4 infection. Cultures of *E. coli* B were grown and infected as described for Fig. 4. Infection was terminated after 13 min of infection by pouring the culture over 0.5 vol. of crushed frozen T medium plus 150 μg chloramphenicol/ml. Isolation of the several membrane fractions was then carried out. All samples were resuspended in 0.1 ml of Laemmli solubilization buffer prior to discontinuous polyacrylamide gel electrophoresis. Autoradiography was performed as described in Materials and Methods. Samples are: A, uninfected outer membrane; B, outer membrane after infection; C, uninfected middle fraction of membrane; D, middle fraction of membrane after infection; E, uninfected inner membrane; F, inner membrane after infection.

responsible for the peak of detergent-resistant T4 protein observed at the density of outer membrane late in infection. Accordingly, newly-synthesized proteins in this detergent-resistant fraction which appear in the region of the gradient corresponding to outer membrane were compared to proteins of the mature T4 particle (Fig. 7). The predominant peak in both preparations has a molecular weight of 45 000 and corresponds to the major head protein of T4 (P23*). Other head proteins tentatively identified in this detergent resistant material are P20, P23, P24*, P22, IP III and IP III*. This detergent-resistant material which contains proteins which appear in T4 head morphogenetic intermediates is a fortuitous contaminant of our total membrane preparations and there is apparently no real association between outer membrane and these T4 head structures (Fig. 8). Centrifugation for shorter or longer times than the usual 16 h period revealed that the peak of protein synthesized late in T4 infection migrates independently of the outer membrane material.

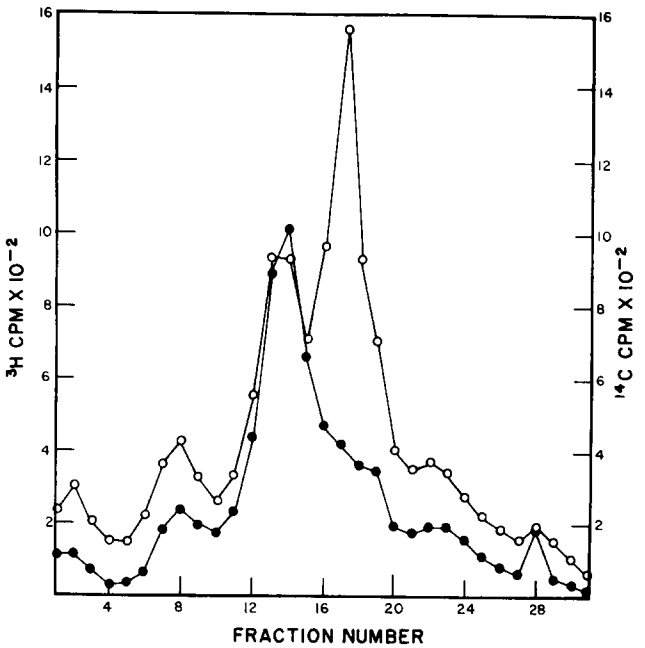

Fig. 6. Separation of membranes from cultures infected with T4*am*B17. Overnight culture *E. coli* B was diluted 1 : 200 in 50 ml of F medium supplemented with 6 μg glycerol/ml, 0.06 mg galactose/ml, and 0.15 μCi [^{3}H]glycerol/ml and grown at 37°C to a concentration of $3 \cdot 10^{8}$ cells/ml. Cells were harvested and resuspended in pre-warmed supplemented F medium containing no [^{3}H]glycerol and with 50 μg L-tryptophan/ml. The culture was infected with 7.5 T4*am*B17/cell and pulse-labeled with 0.1 μCi L-[^{14}C]leucine 9—13 min after infection. The infection was terminated by pouring the sample over 0.5 vol. of crushed frozen F medium containing 150 μg chloramphenicol/ml. Isolation and fractionation of membrane was then carried as described in Materials and Methods. •, [^{3}H]glycerol; ○, L-[^{14}C]leucine label.

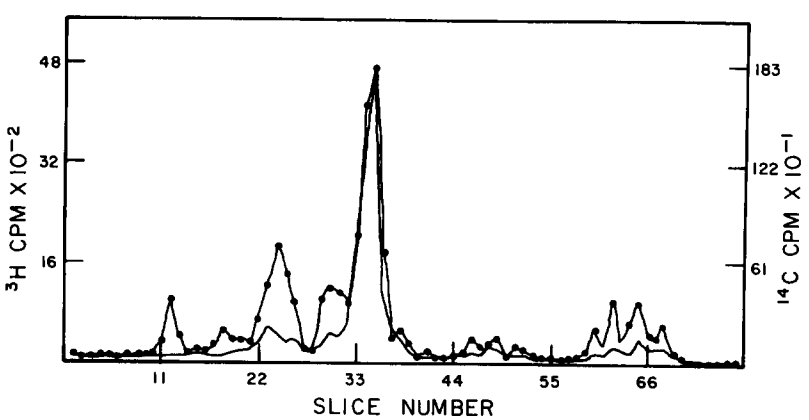

Fig. 7. Coelectrophoresis of newly synthesized Sarkosyl-resistant proteins from the outer membrane region of the gradient with mature T4 particles. Membrane preparations were obtained as described for Fig. 3 except that the cells were unlabeled before infection and 3 μCi L-[^{3}H]leucine/ml was used for the pulse-labeling. After gradient fractionation, fractions 7—10 were collected as described in Materials and Methods. This pellet was resuspended in 1 ml 25% sucrose, 5 mM EDTA and treated with 0.1 ml 5% Sarkosyl for 20 min at 23°C prior to sucrose gradient centrifugation. Gradient fractions 7—10 were again collected and then resuspended in 0.2 ml of Laemmli solubilization buffer. 0.05 ml of this sample was mixed with 0.05 ml L-[^{14}C]leucine-labeled T4D_0 and coelectrophoresed on cylindrical gels as described in Materials and Methods. Labels: ———, L-[^{3}H]leucine; •———•, L-[^{14}C]leucine.

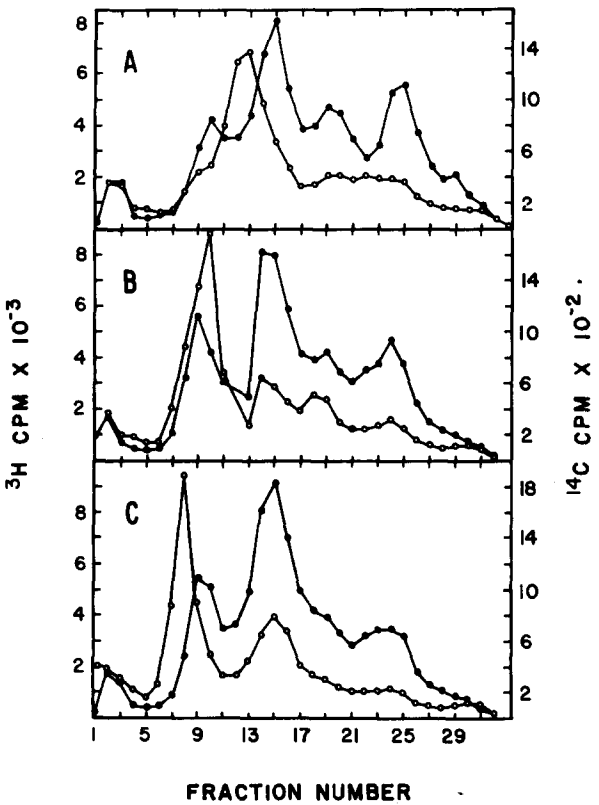

Fig. 8. Effect of length of centrifugation on the distribution of phage and host proteins. *E. coli* B cultures were grown as described in Fig. 1, except cells were labeled with 0.025 μCi L-[^{14}C]leucine/ml and pulse-labeled nine to thirteen minutes after T4D$_0$ infection with 2.0 μCi L-[^{3}H]leucine/ml. Isolation and fractionation of membrane was carried out as described in Materials and Methods except that centrifugation was terminated at ten hours for A, sixteen hours for B, and twenty-two hours for C. •, L-[^{14}C]leucine; ○, L-[^{3}H]leucine.

Changes in NADH oxidase activity

Membrane-bound NADH oxidase is localized in the cytoplasmic membrane of uninfected cells [23]. This enzyme was examined for changes in its activity and in its localization which might indicate T4-induced alterations of the inner

TABLE I

EFFECT OF INFECTION ON MEMBRANE-BOUND NADH OXIDASE ACTIVITY IN TOTAL MEMBRANE PREPARATIONS

Cultures of *E. coli* B growing exponentially in broth were infected with a multiplicity of infection of 7.5 when they reached a concentration of 4 · 10^{8} cells/ml. n.t., not tested.

Phage	Time (min) after infection			
	0	10	20	30
T4D$_0$	0.451	0.359	0.203	0.217
T4*am*H26	0.293	0.237	0.222	0.174
T4*amt*A3	0.361	0.406	0.319	0.229
T4*am*N82	0.269	n.t.	n.t.	0.193

TABLE II

COMPARISON OF THE SPECIFIC ACTIVITIES OF NADH OXIDASE ISOLATED FROM UNINFECTED AND T4*rII*187-INFECTED CELLS BY TWO MEMBRANE ISOLATION PROCEDURES

Infection was allowed to proceed until lysis was detectable (approximately 30 min).

Sample	Specific activity	Percentage of uninfected activity
Uninfected *	0.105	100.0%
Infected *	0.027	25.7%
Uninfected **	0.025	100.0%
Infected **	0.013	52.0%

* Membrane preparation (total) isolated by the procedure of Osborn et al. [23].
** Membrane preparation isolated by the procedure of Inouye and Guthrie [26].

membrane. The results of studies with total membrane preparations from cultures infected with various T4 strains is shown in Table I. A reduction in activity with time is observed after all infections; the activity after 30 min of infection is least in membrane preparations from T4D_0-infected cultures and greatest in preparations from cells infected with T4*am*N82, where no late protein synthesis occurred. Approximately the same pattern of activity loss is seen with all four phage tested. Also, different procedures for isolating total membrane give different specific activities of NADH oxidase (Table II). The enzyme activities obtained from total membrane isolated by the procedure [23] used for the experiments shown in Table I are more than four times those obtained with another procedure [26].

The effect of infection by T4*amt*A3, T4*am*H26 and T4*am*N82 on localization of NADH oxidase activity was also examined. Isolated outer, middle and inner membrane species were obtained from gradients and assayed for NADH oxidase (Table III). For all three mutants, there was a loss of NADH oxidase activity from the cytoplasmic membrane and an increase in activity in the regions of the gradient corresponding to middle and outer membrane.

TABLE III

EFFECT OF INFECTION ON MEMBRANE-BOUND NADH OXIDASE ACTIVITY IN ISOLATED MEMBRANE SPECIES

n.d., not detectable; n.t., not tested.

Phage	Membrane species	Time (min) after infection			
		0	10	20	30
T4*am*H26	Outer	0.004	0.005	0.012	0.017
	Middle	0.032	0.044	0.075	0.109
	Inner	0.357	0.495	0.228	0.127
T4*amt*A3	Outer	n.d.	0.011	0.017	0.020
	Middle	0.026	0.048	0.093	0.085
	Inner	0.733	0.472	0.224	0.258
T4*am*N82	Outer	n.d.	n.t.	n.t.	0.019
	Middle	0.037	n.t.	n.t.	0.064
	Inner	0.538	n.t.	n.t.	0.351

Discussion

This work has concentrated principally on modifications in bacterial constituents of the envelope which occur late after T4 infection. Evidence that both the outer and inner membrane are modified is presented. The most obvious envelope alteration is the inability to separate cleanly cytoplasmic and outer membrane species by isopycnic sucrose density gradient centrifugation. This phenomenon, which takes place only after T4 late protein synthesis starts, has been noted previously [29,30]. Two specific changes in the outer membranes are documented here. (i) It becomes sensitive to detergent (Sarkosyl and Triton X-100) disruption. This not only indicates that a major alteration in the structural interrelationships among components of the outer membrane has occurred, but also precludes use of membrane separation procedures [24,28] involving these detergents. To our knowledge, there is no method which yields a good separation of inner and outer membrane from cells in which T4 late protein synthesis has begun. (ii) The displacement of lipopolysaccharide from outer membrane to membrane fractions with lighter densities is also indicative of outer membrane alterations. Studies on NADH oxidase demonstrate that the cytoplasmic membrane has also been modified. The specific activity of NADH oxidase decreases with increasing time of infection and enzyme activity is partially delocalized.

All of the observed modifications suggest that some late function(s) of T4 alters the cellular envelope so that a significant amount of hybridization of inner and outer membrane occurs during the membrane fractionation procedures. The basis for the envelope alterations is not known but several obvious possibilities exist. (i) Adhesion zones [2,3], which link inner and outer membrane, are believed to play a role in several aspects of T4 physiology [3,31,32] and also to function in lipopolysaccharide deposition [33]. Phage-directed alterations in these junctions could thus significantly affect outer membrane stability. (ii) Phospholipases, either phage- or host-specified, may be responsible. (The presumed phospholipase product of gene *t* is not involved, however.) (iii) A late effect of T4 infection which causes fragility of the cytoplasmic membrane in lysis-inhibited cells has recently been described [34]. The present observations may be related to this phenomenon but in no case did we deliberately superinfect cultures and the alterations we have described occur earlier in the infective cycle than the modification in the permeability barrier. To elucidate fully the basis for the envelope changes which occur late after T4 infection will probably require studies with multiple phage and bacterial mutants, as it seems likely that a number of functions are involved.

Another modification in existing host membrane constituents is the infection-induced association of a 54 000 dalton polypeptide with outer membrane. Others [35] have reported that there are no major changes in host membrane proteins following infections. However, examination of their data (Ref. 35, Fig. 6) does reveal the presence of additional protein of 54 000 daltons. These workers studied total membrane proteins and used 12.5% polyacrylamide gels so the additional protein is not as readily apparent as it is in the present work. That the association of 54 000 dalton protein with membrane does not occur following infection with the DNA-negative mutant T4*am*N82

indicates that phage genes affect the binding. That it occurs early (5 min) after $T4D_0$ infection and is affected by the state of template DNA suggests that the required phage function is coded for by delayed-early RNA [36]. The significance of the association is not known; the only precedent is the transient association of DNA polymerase I with membrane [14]. 54 000 dalton protein was not detected in mature phage particles (data not shown) and it was not present with the phage morphogenetic structure found at the position of outer membrane (Fig. 6). Aebi et al. [37] have reported the presence of a 36 000 dalton host protein in the mature phage head. It is not known if there is some precursor-product relationship between 54 000 dalton protein and the smaller polypeptide.

Lastly, this report describes the adventitious appearance of an intermediate in T4 head assembly in membrane preparations. This difficulty can be circumvented by the use of appropriate T4 mutants, such as T4*am*B17.

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