JOURNAL OF VIROLOGY, Mar. 1974, p. 631-641

Vol. 13, No. 3
Printed in U.S.A.

# Bacteriophage T4-Mediated Release of Envelope Components from *Escherichia coli*

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Received for publication 17 December 1973

When *Escherichia coli* B, labeled by prior growth in $^{14}$C-glucose, are infected with T4 phage there is a rapid release of $^{14}$C-nondialyzable material into the medium. About half of this material is derived from the cell envelope as evidenced by its content of phospholipid and lipopolysaccharide and its buoyant density upon isopycnic ultracentrifugation of 1.19 g/cm$^3$. It is similar in its gross chemical and physical properties to envelope material released at a lower rate from growing uninfected cells or from cells whose protein synthesis is inhibited by chloramphenicol (22). The rate of release of this envelope material at a multiplicity of infection (MOI) of 10 is greatest in the first minute after infection, and release is completed by 4 min. The rate of its release, as a function of MOI at 2 min after infection, is greatest at low MOI (e.g., MOI 2 and 4); in addition, the release does not continue above MOI 30. The main conclusion derived from the data is that phage, as part of the process of adsorption and injection of DNA, cause an increased release of envelope substance from the cells. With the assumption that all of the envelope material released is derived from the outer envelope, it is estimated that uninfected cells release 20 to 30% of their outer envelope per hour, whereas infected cells release 30% in 2 min at MOI 30. Further, because release does not continue at high MOI, this phenomenon is not considered to be a direct cause of lysis from without. Data are also presented on the amounts of other non-dialyzable $^{14}$C-components released and on the differences in the kinetics of release from chloramphenicol-treated cells compared to phage-infected cells. To avoid the possibility that the release is due to phage lysozyme which is an adventitious "contaminant" of wild-type phage, a phage mutant (T4BeG59s) devoid of this enzyme was used in these experiments.

The mechanism by which a T-even phage attaches to its host cell and injects its DNA through the cell envelope is complex. Studies with the electron microscope (27) have shown that infection first involves attachment of the long tail fibers of the phage to the cell envelope, followed by attachment of the short tail pins of the base plate, contraction of the sheath, and injection of the DNA through the tail tip into the cell. During the latter process the tip of the tail appears to penetrate the cell envelope. Other electron microscope studies show that the phage adsorbs at sites where inner membrane and outer membrane adhere to each other, i.e., membrane adhesion sites (4).

Little is known of the biochemistry of this process. The first step (29), the attachment of the long tail fibers of the phage to the lipopolysaccharide of the cell envelope, has been demonstrated in vitro (31). More recently, evidence for a role of dihydrofolate reductase, a component of phage tail plates, in the adsorption and infection process has appeared (15). However, the details of these reactions, as well as the chemistry of the succeeding steps, remain to be explored.

Previous biochemical studies on other aspects of this process have shown that T-even phage has a profound effect upon the *Escherichia coli* cell envelope. Kozloff and co-workers (3, 7) demonstrated the release of isotope from $^{15}$N-, $^{14}$C-, or $^{32}$P-labeled cell wall preparations incubated with disrupted phage, and postulated that phage contain a lytic activity. Puck and Lee (19, 20) noted the leakage of small molecules from cells after phage infection and postulated that the phage punches a hole in the cell envelope which is later sealed. Silver demonstrated that phage-infected cells become more permeable to acridines and that this change in

envelope function is controlled by phage genes *ac* and *q* (24). He also confirmed the findings of Puck and Lee on leakage of inorganic ions and the subsequent sealing reactions (25). There are also other special properties of T-even phage that are envelope-related: the occurrence of lysis from without at high multiplicities, and the development, after infection, of lysis inhibition, of immunity to superinfection and of resistance to lysis from without (16).

The presence of two lytic activities in the phage particles themselves has been demonstrated (10, 30, 32). One of these is the well-characterized phage lysozyme, the e gene product. As recently shown (10), however, this enzyme, contrary to earlier belief, is not necessary for the initial steps in infection, and its appearance in phage is apparently adventitious. Phage lacking this enzyme can readily infect cells and cause lysis from without. The other activity, a recent discovery (10, 32), is not well characterized; its actual substrate, its mechanism of action, its role in infection, and whether it is a phage gene product are not known.

In this communication the biochemical nature of the interaction of T-even phage with *E. coli* during adsorption and injection of DNA is further investigated. To avoid the possibility that the results obtained might be due to the very active phage lysozyme present adventitiously in wild-type phage, a mutant free of this lytic activity is used. I find that when cells labeled by growth in $^{14}$C-glucose (uniformly labeled) are infected with these phage, nondialyzable $^{14}$C-cell material is rapidly released into the medium. The chemical nature of this material and the kinetics of its release lead me to conclude that phage in the course of adsorption cause the release of envelope material from the cell. A preliminary report of this work has appeared (M. R. Loeb, Abstr. Annu. Meet. Amer. Soc. Microbiol., 1972).

## MATERIALS AND METHODS

**Cells and phage.** *E. coli* B was obtained from L. Simon. T4BeG59s phage was obtained from J. Emrich. It is referred to as T4Bes in the text. This phage has an extensive deletion in the lysozyme gene, designated eG59, which assures the production of lysozyme-free phage, and an additional mutation in the *s* gene, which enables infected cells to release phages at the end of the lytic cycle although phage lysozyme is absent. There is evidence that the s gene is involved in repair of damage done to the cell envelope upon phage infection. The biology of infection with this phage and means of assay have been described in detail (9). This phage mutant, as obtained from J. Emrich, also contains markers in genes *ac* and *q*.

**Medium.** Minimal medium was Adams' M9 (1) with 0.5 to 1 mg of glucose per ml. The glucose was sterilized by filtration through a Millipore filter (0.22 $\mu$m diameter). Broth was 10 g of tryptone (Difco), 5 g of NaCl made up to 1 liter.

**Phage stocks.** It is difficult to make consistently high titer phage stocks of T4Bes in liquid culture. However, by adapting the plate lysate method described in Adams (1), it is possible to obtain good titers of phage. About $10^5$ phage in 0.1 ml, an amount sufficient to produce almost confluent lysis, were added to 2.5 ml of regular top agar containing 0.5 ml of *E. coli* B (taken from a freshly grown broth culture with about $4 \times 10^8$ cells/ml) and poured onto regular bottom-agar plates. After incubation overnight at 37 C, 5 ml of broth were added to each of the plates. The top agar was broken up and collected with a pipet and a few milliliters of chloroform were added. After 30 to 60 min at room temperature, the lysate was made 5 mM in $MgCl_2$, a crystal of DNase was added, and the mixture was incubated for 30 min at 37 C. The phage were collected by two cycles of differential centrifugation (1) followed by a low-speed centrifugation. The phage pellets obtained after each high-speed centrifugation were layered with M9 medium (no glucose) overnight and then suspended gently with a pipet. The lysozyme-negative character of these phage preparations was checked by assaying sonicates of cells 15 min after infection (multiplicity of infection [MOI] 10) by the method of Sekiguchi and Cohen (23).

**General experimental methodology: growth and labeling of cells, infection, collection of supernate and dialysis.** The same basic protocol was used in all experiments. A 20- to 60-ml amount of M9 containing 0.5 mg of uniformly labeled $^{14}$C-glucose per ml (specific activity: $4 \times 10^6$ to $4 \times 10^7$ counts per min per mg) was inoculated with *E. coli* B obtained from overnight growth in M9 containing 1 mg of glucose per ml. Sufficient inoculum was used to give a starting concentration of $3 \times 10^7$ cells/ml. The cultures were aerated at 37 C and grown to a concentration of $5 \times 10^8$ cells/ml, as determined turbidimetrically with a Klett colorimeter. The culture was then added to one-half volume of ice-cold M9 (minus glucose), centrifuged at 4 C at 7,000 $\times$ *g* for 15 min, and the cells were collected and washed by a centrifugation with cold M9 three times. The cells were resuspended at a concentration of $5 \times 10^8$ cells per ml in M9 containing 0.5 mg of unlabeled glucose per ml, and were aerated at 37 C for about 15 min. The purpose of this growth in unlabeled medium was to remove the background of small labeled molecules, and to allow cells to establish steady state growth conditions after washing in the cold. The cell suspension was distributed among a suitable number of aeration tubes. One portion of the cells served as control and the other(s), except where otherwise stated, was infected with T4 Bes after the addition of 25 $\mu$g of L-tryptophan per ml of culture. The time of infection is always denoted as zero time in these experiments. At stated times after infection, portions of the culture were added to one-half volume of cold M9 (minus glucose) and centrifuged in the cold as follows: two times at 7,000 $\times$ *g* for 10 min and once at 13,000 $\times$ *g* for 1 h. The last

centrifugation would insure that cells, lysed cells, and phage carrying cell debris were removed from the supernate. In general, little additional radioactivity pelleted in this last centrifugation. The supernates were dialyzed against 40 volumes of cold water for 40 to 48 h with several changes of water. These nondialyzable supernates are the subject of this communication.

**Determination of total $^{14}$C-incorporation into cells.** The amount of radioactivity in the acid-precipitable fraction was used as a measure of total $^{14}$C-incorporated into the cells. A 0.05-ml amount of cells, obtained just before addition of phage, was added to 1 ml of cold 10% trichloroacetic acid; the precipitate was collected on a membrane filter (Millipore Corp.) 30 min later, washed with cold trichloroacetic acid, dried, and counted.

**Radioactivity determinations.** All aqueous solutions were counted in a Beckman LS-230 counter by using 10 ml of a scintillation fluid consisting of 42 ml of concentrated Liquifluor (New England Nuclear Corp., Boston, Mass.) and 60 g of naphthalene per liter of dioxane. To completely solubilize portions from the sucrose gradients, 1 to 2 ml of water was also added to each 10 ml of scintillation fluid. Filters were counted in 5 ml of Liquifluor diluted with toluene according to the manufacturer's instructions. Petroleum ether and chloroform extracts were counted by drying a sample on the bottom of a scintillation vial and adding 5 ml of Liquifluor. Silica gel thin-layer plastic sheets were counted by cutting strips 0.5 cm by 1 cm wide and placing them in 5 ml of Liquifluor.

**Sources of materials.** Beta-hydroxymyristic acid was a gift from Howard Goldfine. U-[$^{14}$C]glucose was purchased from Amersham/Searle Corp., Arlington Hts., Ill.; dialysis tubing (5/8 inch diameter) was from A. H. Thomas, Philadelphia, Pa. cleaned by boiling for 20 min in 5% $NaHCO_3$, bovine pancreatic DNase (crystalline), and egg white lysozyme (two times crystallized) from Worthington Biochemical Corp., Freehold, N.J. Silica gel-coated plastic sheets (IB2) for thin-layer chromatography was from J. T. Baker Chemical Co., Phillipsburg, N.J.; chloramphenicol was from Sigma Chemical Co., St. Louis, Mo.

## RESULTS

In these experiments the basic protocol described in Materials and Methods was followed. The data are usually normalized to counts per minute per milliliter of cells, with 1 ml containing $5 \times 10^8$ cells.

**Release of nondialyzable material from uninfected and infected cells.** When *E. coli* B cells, labeled by prior growth in $^{14}$C-glucose, are resuspended in unlabeled M9-glucose media, non-dialyzable $^{14}$C-containing material is released into the medium. This is shown in Fig. 1a. Although this figure presents data for a short time span, other experiments (see Fig. 3) show that release of such material continues at a linear rate of about 0.1% of the total initial $^{14}$C content per minute for at least 90 min. There is, however, a rapid and considerable increase in the rate of release of such material if the cells are infected with T4Bes (MOI 10), as shown in Fig. 1a. This release depends upon phage adsorption since it does not occur unless L-tryptophan, an adsorption cofactor, is added.

It is apparent from Fig. 1a that the nondialyzable material obtained from infected cells at any particular time point contains as background considerable amounts of material from uninfected cells. This is a consequence of the

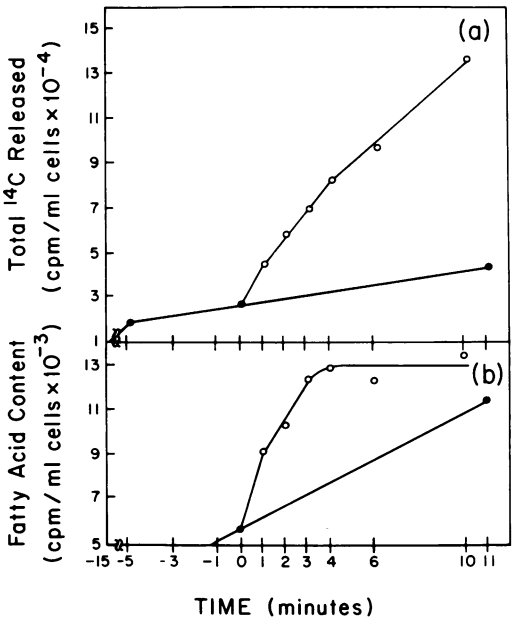


FIG. 1. *Release of non-dialyzable $^{14}$C-material from uninfected and infected cells (MOI 10). The basic protocol described in Materials and Methods was followed. $^{14}$C-labeled cells were washed, resuspended, and subsequently divided into two portions. One served as control (uninfected cells); the other was infected with T4Bes (MOI 10) after addition of L-tryptophan. Portions were removed from each of the cultures at specified times, centrifuged, and dialyzed. The negative numbers on the abscissa refer to the 15-min period of growth in unlabeled media after resuspension and prior to phage infection. Zero marks the time of phage infection. a, Total non-dialyzable $^{14}$C-material released; b, $^{14}$C-covalently bound fatty acid content of the released $^{14}$C-material. For determination of fatty acids, duplicate samples were hydrolyzed and extracted into petroleum ether as described (8). These extracts contained at least 95% of their $^{14}$C as fatty acids as determined by thin-layer chromatography in petroleum ether: acetic acid: $H_2O$ (70:30:2). Symbols: ●, uninfected cells; ○, infected cells.*

experimental design (see Materials and Methods), which was specifically chosen to insure that the washed prelabeled cells were in a physiological steady state. Such a precaution may be necessary in a study of the rapidly occurring events involved in cell surface phage interactions. Although this background is a disadvantage, especially in the subsequent comparison of the chemistry of materials released by uninfected and infected cells, it does not in any way detract from the main findings and conclusions presented. However, in evaluating the data it is important to be aware of this situation. For example, the non-dialyzable material obtained from cells 2 min after infection at MOI 10 contains essentially two fractions present in about equal amounts. One is the material released by resuspended uninfected cells over the relatively long 15-min period prior to infection, whereas the other is the material released after phage infection over the much shorter time span of 2 min.

**General nature of released material.** A number of observations suggested that the released material contained lipid. (i) The material tended to stick to glass upon storage in the cold. (ii) After lyophilization, much of the material would not redissolve in water, buffers, or in dilute acid or alkali, but could be recovered by dissolution into 1% SDS. (iii) When applied to Sephadex columns, even in the presence of high salt (M NaCl), 60% of the material adhered to the gel and the remaining 40% eluted in a broad band extending beyond the end point of the column. The radioactivity which adhered to the gel could be recovered with 1% SDS.

Since both the major (phospholipid) and minor (lipopolysaccharide and lipoprotein of the peptidoglycan) lipid components of *E. coli* contain fatty acids, the presence of covalently bound fatty acids would be a reliable indication of lipids in the released material. Consequently, these were assayed for by the standard procedures of hydrolysis with ethanolic KOH followed by extraction into petroleum ether (8). The radioactivity in the petroleum ether extracts was determined and the results appear in Fig. 1b after normalization to counts per minute per milliliter of cells. The material released from both uninfected and infected cells contained fatty acids. However, whereas uninfected cells release fatty acid-containing material at a low rate for an extended period of time (see also Fig. 3b), phage-infected cells greatly increase their rate of release immediately upon infection and this release stops by 4 min after infection. Thus, material being released by infected cells after 4 min does not contain fatty acids and the release of lipid material due to phage is an early event.

When the released material derived either from uninfected cells at zero time or from cells at 2, 4, or 6 min after infection is subjected to isopycnic centrifugation in a sucrose gradient, the results shown in Fig. 2 are obtained. Qualitatively, there are considerable similarities in the behavior on the gradient of material released from uninfected or infected cells. The material which appears as a peak at fractions 9 to 10 is present in all samples, and it is this material which is primarily considered in this paper. It has reached its equilibrium position in the gradient at a buoyant density of 1.19 g/cm$^3$ which is characteristic of the *E. coli* outer envelope (18). Analysis of this material for fatty acids by the alkaline hydrolysis procedure of Mindich (17) shows that at least 90% of the total fatty acid content of the released material is

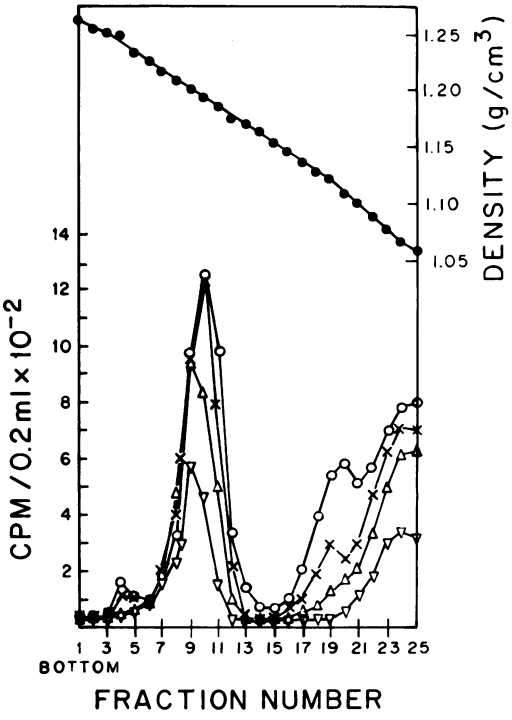


FIG. 2. *Isopycnic centrifugation of non-dialyzable $^{14}$C-material released into the media by uninfected and infected cells (MOI 10). A 2-ml amount of dialyzed released material obtained at various times after infection as described in the legend to Fig. 1 was applied to a 36-ml gradient of 20 to 55% (wt/wt) sucrose (in water) and centrifuged for 67 h at 27,000 rpm at 4 C. Fractions (40 drops each) were collected from a hole punctured in the bottom of the tube and the radioactivity assayed. Symbols: ▽, zero time; △, 2 min; ×, 4 min; ○, 6 min.*

accounted for in this fraction of the gradient. Because of its buoyant density, its content of fatty acid, and also as a result of some chemical determinations described below, it is concluded that this fraction of the released material is derived from the outer envelope of *E. coli*. The measurement of fatty acids shown in Fig. 1b is essentially an assay for this fraction. Figures 1b and 2 both show a dramatic and rapid increase in the rate of release of this material upon phage infection and also the cessation of this release 4 min after infection.

As for that part of the released material which does not band as envelope, it can be seen that radioactivity also appears at the top of the gradients of material from both uninfected and infected cells and in fractions 17 to 21 in the gradient of material from infected cells only. The material in both of these fractions increases with time after infection, and unlike the envelope material, continues to appear during the time period studied. Although the nature of these substances and their significance in relation to the infection process are currently under investigation, they are not considered further in this communication.

Certain other features of the released material and the nature of its release should be noted. First, it contains little if any free fatty acids. Thus, acidification of the material followed by petroleum ether extraction yields 2% at most of the released radioactivity as petroleum ether-soluble substance. Also, little if any fatty acid-containing material is lost upon dialysis. Second, the data indicate that the release is stopped by chilling and that this phenomenon is therefore temperature dependent, at least in part. (If chilling did not stop the reaction there would be no difference in the amount of material released at 1 min as compared to 4 min since all samples were subsequently centrifuged at the same time.) Third, the rate-limiting step in the release of envelope material is probably not the rate of phage adsorption since the phage adsorb more rapidly than the material is released. At all multiplicities used in these experiments at least 70% of the phage adsorbed in the first minute and at least 85% by the second minute. Fourth, the release of material by phage is not adversely affected by the treatment in the cold of labeled cells prior to resuspension. An alternate procedure of washing the cells on a membrane filter (Millipore Corp.) at room temperature followed by resuspension and growth and infection does not appreciably alter the results.

In summary, the data presented in Fig. 1 and 2 demonstrate: (i) the release of envelope material (as well as other unknown cell components) from uninfected cells; (ii) the increased rate of release of envelope material and other substances rapidly upon phage infection; (iii) the discontinued release of envelope material, but not of other substances, from infected cells by 4 min after infection. Table 1 presents a summary of these findings in more quantitative terms. The kinetics of release of lipid-containing material are consistent with the hypothesis that an initial step in phage infection involves an attack upon the cell envelope resulting in release into the medium of cell envelope substance. The cessation of release at 4 min is due to an unknown mechanism, possibly the postulated sealing reaction, and is considered in the discussion section.

**Further chemical characterization of released material.** In addition to determining the fatty acid content of released material, the lipid components in which these fatty acids might be expected to appear, namely phospholipid and lipopolysaccharide, were also assayed for. These analyses were performed on released nondialyzable material collected from uninfected cells at zero time and from infected cells (MOI 10) 2 min after infection. As described above, these latter samples contain a considerable background of material released from uninfected cells. Phospholipids were extracted from the released material by the Bligh and Dyer procedure (5) and were subjected to thin-layer

TABLE 1. *Summary of data on non-dialyzable materials released from uninfected and infected cells*

| Time (min) | % $^{14}C$ content of cells ($1.7 \times 10^6$ counts per min per ml of cells) | | Envelope material in released material (%)[a] | Fatty acids in released material (%) |
|---|---|---|---|---|
| | In released material | In fatty acid of released material | | |
| Uninfected cells | | | | |
| −5 | 1.1 | | | |
| 0 | 1.6 | 0.33 | 45 | 21 |
| 11 | 2.6 | 0.67 | | 26 |
| Infected cells | | | | |
| 1 | 2.7 | 0.53 | | 20 |
| 2 | 3.5 | 0.59 | 46 | 18 |
| 3 | 4.2 | 0.73 | | 17 |
| 4 | 4.9 | 0.75 | 48 | 15 |
| 6 | 5.8 | 0.76 | 40 | 13 |
| 10 | 8.1 | 0.78 | | 10 |

[a] Determined as counts per minute in envelope peak of gradient divided by total counts per minute of gradient.

chromatography in a solvent mixture of chloroform-methanol-$H_2O$ (65:25:4) (2), using phospholipids extracted from *E. coli* as standards. The distribution of radioactivity on the chromatograms was determined and the results indicated that about 12% of the material released, either from uninfected or infected cells, is phospholipid with the majority of the radioactivity appearing as phosphatidyl ethanolamine.

The presence of lipopolysaccharide was indicated by the appearance of β-hydroxymyristic acid in the fatty acid hydrolysates. This fatty acid is specific for lipopolysaccharide (14), and by chromatography in the appropriate solvent (6) can be separated from the other fatty acids of *E. coli*. Thin-layer chromatography of the petroleum ether-soluble material extracted from KOH hydrolysates revealed 6 to 15% of the radioactivity migrating with an authentic sample of β-hydroxymyristic acid. It should be noted that variable yields with this fatty acid are common since (i) it is more difficult to obtain it by hydrolysis because it is present in an amide linkage rather than an ester linkage; and (ii) it tends to be converted to other fatty acid derivatives upon hydrolysis (21). Thus, the 6 to 15% values are probably low estimates.

However, the presence of lipopolysaccharide was also indicated by examining a fraction of released material which would be expected to be enriched in lipopolysaccharide, namely the material remaining at the interphase after removal of phospholipids by the Bligh and Dyer procedure (5). To do this experiment, 3 mg (wet weight) of *E. coli* cells were used as carrier in the Bligh and Dyer extraction. The carrier cells were needed to allow a tangible interphase to form. After removal of the chloroform, i.e., phospholipid layer, the interphase material was collected and hydrolyzed with KOH, and the petroleum ether extract of fatty acids was obtained and subjected to thin-layer chromatography. In this case, material migrating as β-hydroxymyristic acid accounted for at least 28% of the fatty acids present at the interphase. This is within the range reported for the proportion of this fatty acid in the total fatty acid population of lipopolysaccharide (14). This finding of a fraction enriched in β-hydroxymyristic acid provides additional evidence for the presence of lipopolysaccharide in the released material. To determine the actual content of lipopolysaccharide further analyses are being performed.

Some analyses of material obtained from the sucrose gradient were also conducted and, as mentioned above, fatty acid-containing substance was found almost exclusively in the material banding at 1.19 g/cm$^3$. About 30% of the radioactivity in this envelope band from uninfected or infected cells can be extracted as phospholipid and again the fatty acids contained in the material remaining after removal of phospholipids are enriched in β-hydroxymyristic acid.

In summary, the gross lipid content of material obtained from the same number of growing cells or infected cells are, though different in absolute amounts, proportionately similar: fatty acid content, 20%; phospholipid content, 12%; envelope fraction, 45%; phospholipid content of envelope fraction, 30%. In addition, there is evidence for lipopolysaccharide because of the presence of β-hydroxymyristic acid. Thus, the major difference noted so far in the lipid-containing material released from uninfected as compared to infected cells is in the greater rate of release from the latter. However, the possibility of less obvious differences, physical or chemical, is currently under investigation. Also being studied are the possible differences in those substances appearing at or near the top of the gradient: some of these are also released very soon (within 2 min) after infection, and their appearance may be directly related to the envelope phenomenon.

**Is release of envelope material by phage similar to chloramphenicol-induced release of envelope material?** The observed release of envelope material by normally growing uninfected cells is essentially a corroboration of the data of Rothfield and Pearlman-Kothencz (22). In addition to demonstrating this phenomenon, these authors also showed an enhanced release of such material, composed of phospholipid (50%), lipopolysaccharide (35%), and protein (15%), upon inhibition of protein synthesis. They suggested that the inhibited cells have a relatively relaxed synthesis of outer membrane material and that these nongrowing cells, although producing decreased amounts of envelope, still do make an excess which is then excreted into the medium. Since phage infection results in a rapid inhibition of host protein synthesis, and hence in a shut-down of the supply of envelope proteins coded for by host DNA, it was suggested that the phage-induced release of envelope may also be due to this phenomenon rather than to the attack of the phage upon the envelope. Actually, the kinetics of release (Fig. 1b), showing a rapid effect without a lag, argue against this suggestion. For although it has been demonstrated that phage infection rapidly inhibits translation of host messenger RNA by an unknown mechanism (13), those proteins being synthesized at the

time of infection will be completed and these plus the precursor proteins which have already been made will be available for a short time. Thus, one would expect a lag of at least 2 min before an effect caused by inhibition of protein synthesis on increasing the rate of release of envelope substance would be seen.

Nevertheless, the possibility that the results described in Fig. 1 and 2 were due to inhibition of host protein synthesis was tested experimentally by comparing the release of material by phage-infected cells and chloramphenicol-treated cells. The results appear in Fig. 3a where total $^{14}C$ released is shown, and in Fig. 3b where the fatty acid content of this released material is shown. They demonstrate a distinct difference in the kinetics of release for these two cell populations. The chloramphenicol-treated cells release envelope material at the same rate as untreated cells for at least 4 min. However, in this same time period the infected cells have

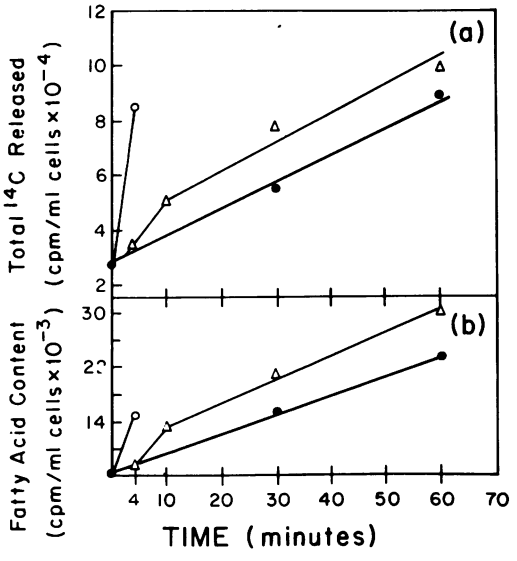


FIG. 3. *Comparison of release of $^{14}C$-non-dialyzable material by uninfected cells, infected cells, and chloramphenicol-treated cells. Labeled cells (total $^{14}C$-incorporated, 1.8 × $10^6$ counts per min per ml of cells), after resuspension in non-radioactive media for 15 min, were divided into three portions. One (uninfected) served as control. At the point denoted as zero time, a second portion was infected with T4Bes (MOI 10) after addition of L-tryptophan, whereas the third portion was treated with chloramphenicol (final concentration 140 μg/ml). At specific time intervals portions were removed from each of these cultures, chilled, centrifuged, and dialyzed. a, Total $^{14}C$-material released; b, $^{14}C$-fatty acid content of released material. Assays for fatty acids performed as in Fig. 1b. Symbols: ●, uninfected cells; ○, infected cells; △, chloramphenicol-treated cells.*

released all the envelope material they are going to release (Fig. 1b and 3b). Somewhere between 4 and 10 min the chloramphenicol-treated cells exhibit a burst of envelope release, and this is then followed by a linear rate of release which is perhaps slightly greater than that of uninfected cells during the time span of 60 min. These results indicate that it is unlikely that the phage-induced release is due to inhibition of host protein synthesis. They also indicate a difference in envelope metabolism between infected cells and chloramphenicol-treated cells. Thus, the former discontinue release of envelope substance even though host protein synthesis is inhibited while the latter continue to release fatty acid-containing material from these prelabeled cells for at least 60 min. Further study of the synthesis of envelope and its control, especially in infected cells, will be required in order to understand this result.

**Release of material as a function of MOI.** If each phage which adsorbs to a host cell attacks the envelope in the process of infection, and if the release of envelope material is a result of this process, then the amount of such material appearing in the medium should be directly related to the MOI. An experiment designed to test this hypothesis was performed. The results, showing the relationship of MOI upon release of material, appear in Fig. 4a and b and 5.

Figure 4a shows that as the MOI increases, the amount of $^{14}C$ material released measured at 2 min after infection also increases. Similar results are found if envelope material itself, measured as fatty acids obtained upon hydrolysis, is assayed (Fig. 4b). However, there is a limit to the amount of fatty acid-containing material released: above MOI 30 no further increase in this fraction occurs, although other $^{14}C$-containing material continues to be released (Fig. 4a). The results of isopycnic centrifugation of material, released 2 min after infection at varying MOI are shown in Fig. 5. There is, with increasing MOI, an increased amount of envelope material (fractions 8 to 12) which bands at a buoyant density of 1.19 g/cm$^3$, and there are also increasing amounts of other compounds which are near or at the top of the gradient. Fatty acid-containing materials appear primarily in fractions from the envelope band, as determined by analysis of material across the gradient. The free fatty acid content of released material is negligible.

Although there is a relationship between the amount of envelope material released and MOI, the simple hypothesis stated above does not explain the discontinued release of envelope above MOI 30. There actually appears to be a

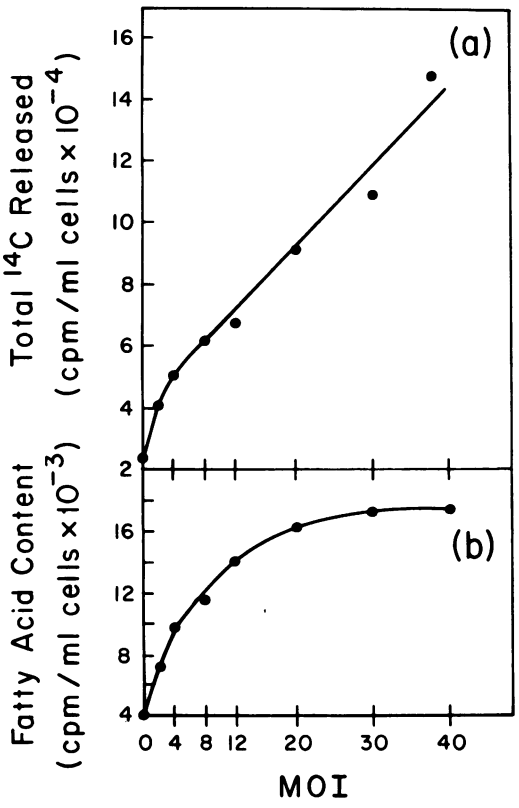


FIG. 4. *Release of $^{14}C$-non-dialyzable material from infected cells as a function of MOI. A 40-ml amount of labeled cells after resuspension (total $^{14}C$-incorporated, 1.6 × $10^8$ counts per min per ml of cells), and growth for 12 min were divided into eight 5-ml portions. After addition of* L-*tryptophan, 0.7 ml of appropriate dilutions of T4Bes phage was added to provide varying MOI. Two minutes after infection, samples were collected. a, Total non-dialyzable $^{14}C$-material released; b, $^{14}C$-Fatty acid content of released material.*

limit to the amount of substrate available to the phage. This is further indicated in Fig. 6 where a plot of log percent substrate (i.e., fatty acid) released as a function of MOI yields a straight line. Thus, the data suggest that the first several phage to adsorb cause release of envelope material, but that phage subsequently adsorbed do not result in such release because effectively there is no substrate remaining. It remains to be seen whether the limited amount of available substrate represents the actual situation as it exists at the time of infection or whether it is a rapid consequence of the results of infection, e.g., the sealing reaction of Puck and Lee (19, 20). It would be of interest to know if one phage, given enough time, could release the same amount of envelope as 30 phage do within 2 min, that is, is each phage restricted to act locally or can its action be spread over the entire surface. Experiments at MOI 3 (data not shown) indicate that the action is local, i.e., cells infected at MOI 3 release considerably less material, about one third as much, compared to MOI 10, even at 10 min. However, this result could be due to the sealing reaction so that even though a phage might have the potential to carry out a spreading reaction, the sealing mechanism prevents its expression; thus the question raised is not yet answered.

Because of its buoyant density of 1.19 g/cm$^3$ and the presence of lipopolysaccharide, it is quite probable that the released envelope material is derived largely from the outer membrane of *E. coli* rather than from the entire complex of inner and outer membrane. Since the outer membrane is about 10% of the dry weight of *E. coli* (14) and since the content of envelope material released in these experiments is known, the following calculations can be made.

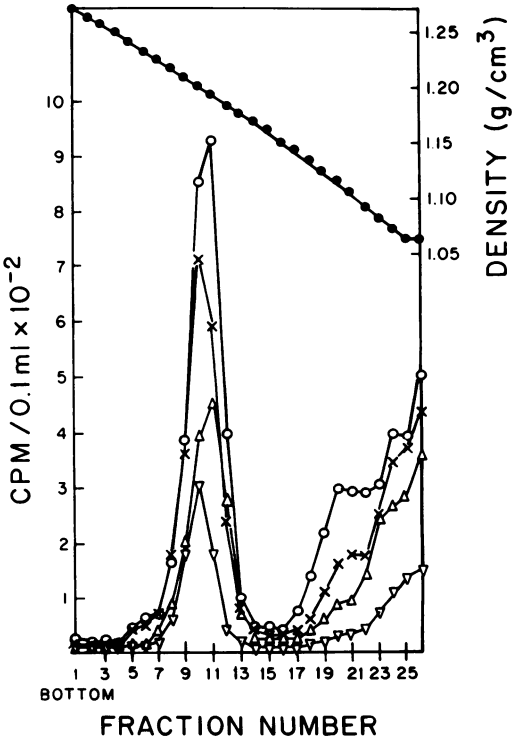


FIG. 5. *Isopycnic centrifugation of $^{14}C$-non-dialyzable material released from cells infected at various MOI. Material obtained from the experiment described in the legend to Fig. 4 was applied to a sucrose gradient. The gradient, conditions of centrifugation, etc., are described in the legend to Fig. 2. Symbols: ▽, MOI 0; △, MOI 8; ×, MOI 20; ○, MOI 30.*

Growing uninfected cells release 20 to 30% of their prelabeled outer membrane per hour. Cells infected with phage (MOI 30) release about 30% of their outer membrane in 2 min. This latter value has been corrected for the background due to uninfected cells, and it represents the maximum amount of releasable material noted above.

## DISCUSSION

The aim of this investigation was to gain information on the reactions which occur when T4 phage adsorbs to the *E. coli* B envelope and injects its DNA into the cell. This communication presents data on one aspect of this interaction: the increased release, upon infection, of non-dialyzable cell envelope material and other unknown cellular substances into the medium. For several reasons, but primarily because the release of envelope material occurs rapidly after infection, it is concluded that the release results from some event in the complex process of phage adsorption to the cell, and that it is not a consequence of what happens after DNA injection such as inhibition of host protein synthesis.

Since the initial interaction of T4 phage with *E. coli* is so complex and may consist of reactions sequentially dependent upon each other, I purposely chose to begin this study by using a crude system, i.e., whole cells and whole phage, although the ultimate goal is to reconstruct the system in vitro. One of the problems with the crude in vivo system is the possibility that the released material represents cell contents arising from a variety of causes. Thus, at moderate MOI (e.g., 8 to 20), some release may be a result of lysis from without (LWO) of a few cells, and at higher MOI (20 to 40), of LWO of considerably more cells in the culture. Further, material found at later times in infection may arise from mechanisms totally unrelated to the adsorption phenomenon. However, the data which we feel support our conclusion are those derived from experiments using low MOI (i.e., up to 12) and early times of sampling (i.e., 2 min). These data are clearly not due to the other causes just mentioned because: (i) at low MOI (MOI 2 and 4) LWO is unlikely, especially since the cells were grown under conditions which minimize that possibility (12); (ii) if the release of envelope were a result of LWO, one would not expect the amount released per phage to decrease as the MOI increases; (iii) the rate of release of envelope material is greatest in the first minute after infection. This is also unexpected if the phenomenon is a result of LWO.

Thus, it is incompatible with the data that the release of envelope material, at least at low MOI and very early in infection, is a result of LWO. It is, however, interesting to consider the release as a cause of the LWO phenomenon since it represents damage to the cell envelope. There is, however, an aspect of the data which disputes this possibility; namely, the finding that above MOI 30 there is a cessation of release of envelope substance, although LWO is known to occur most effectively when cells are infected at large MOI (e.g., 100 to 200). Thus, the release of envelope material as observed herein is not a direct cause of LWO. Puck and Lee (19, 20) proposed that LWO results when the cell, attacked by many phage, cannot repair the

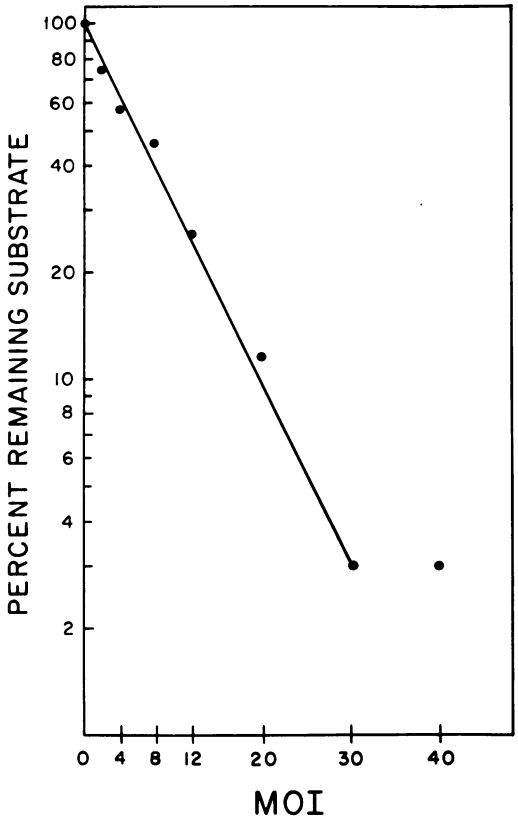


FIG. 6. *Plot of log percent substrate remaining as a function of MOI. The release of fatty acid-containing material due to phage alone was calculated by subtracting the fatty acid content of material released by uninfected cells at zero time from that released by phage at each MOI. The value obtained for MOI 30 was set equal to 97% release of total available substrate and subsequent calculations were based on that figure. The data presented in this figure are based on the assumption that the fatty acid content of released material is a direct measure of the amount of substrate released by the phage.*

envelope damage fast enough; consequently, the higher the MOI the more LWO and the more rapidly it occurs. The results described herein do not support this hypothesis; nor do they disprove it, since damage to the cell surface could be done in a way that is not manifested by release of material. However, the results do support another mechanism of LWO, proposed by Watson (28) on the basis of his experimental data. He suggested that the phage which cause LWO are those which do not carry out the entire process of adsorption but are, for unknown reasons, prevented from doing so. The results presented in this paper viewed in light of his model would suggest that the first phage to adsorb and infect cause release of envelope material while additionally adsorbed phage cannot carry out the entire set of adsorption-infection steps, possibly as a result of the sealing reaction or exhaustion of substrate. Sufficient numbers of these phage, however, attached to the cell envelope, could conceivably interfere with its proper functioning and this could result in LWO.

Another aspect of the data which merits comment is the shut-off of release of envelope material by 4 min after infection. In this regard phage-infected cells obviously differ from growing uninfected cells as well as from uninfected cells whose protein synthesis is inhibited, since in these two cases envelope material is continually released into the medium. The shut-off may be due to (i) the sealing process which is not under the influence of phage genes (25), or (ii) a phage gene which could be responsible for other membrane-related phage functions such as the development of immunity. It is also possible that the shut-off observed herein may be the result of lesions in the genes present in the T4Bes phage used in these experiments, in particular the *s*, *ac*, and *q* genes. These possibilities are all under current investigation.

Although we have noted release of envelope material upon phage infection, we have no information as to the mechanism of this release. It may be due to enzyme(s) in the phage, and/or enzyme(s) in the cell activated by phage attachment, and/or enzymatic interactions between phage and cell. The more recent reports on lytic activities in phage (10, 32) provide no information on their mechanisms. In one report (10) phage disrupted by freeze-thawing and known to be free of phage lysozyme caused a decrease in optical density of suspensions of freeze-dried *E. coli* near neutral pH. In the other report (32) similar phage preparations caused release of $^{14}C$ from a cell envelope preparation, with pH optima at pH 3 and pH 5, but no activity at pH 7 and no activity on chloroform-treated *E. coli* or intact *Micrococcus lysodeikticus*. Thus, the activities measured in those two systems may not be identical and in addition may be due either to enzymes in the phage preparation or to activation of host enzymes by components of the phage preparation, or to non-enzymatic interactions between the reaction components. Because the envelope material released by uninfected and infected cells in the experiments described in this paper is similar in its gross chemical and physical properties, it is possible that the release caused by phage is due to an enhancement of the release by normal cells. The mechanism of release by normal cells itself is not known, but it could result from the balance that must exist during cell growth between enzymes which make breaks in the envelope to allow for insertion of new material and enzymes which catalyze these insertions. Possibly the phage inhibit the insertion enzymes and thereby allow the breaking enzymes to express themselves by the increased release of material.

Although it was necessary to use lysozyme-free phage in these experiments to avoid the possibility that the results might be due (albeit indirectly, since we are measuring envelope material and not rigid layer material) to lysozyme action, results with wild-type phage indicate that this precaution may not be necessary. Wild-type infection causes release of material which, on the basis of preliminary chemical and physical criteria, resembles that released by T4Bes.

Finally, we would like to suggest (i) that release of envelope material upon phage infection may be responsible for the observation that increasing the MOI of T4 from 2 to 20 results in fewer infective centers and decreased phage produced per infective center (11); (ii) that the type of lethal zygosis (26) which occurs when female *E. coli* are mated with male *E. coli* at multiplicities of 10 to 25 may perhaps involve release of envelope material, possibly as part of the normal mating event; and (iii) that the well-documented early effects of phage infection such as leakage of ions and increased permeability to certain dyes may also be a result of the damage to the cell brought about by release of envelope substance.

## ACKNOWLEDGMENTS

I thank Joyce Emrich for supplying the phage mutant T4BeG59s and Howard Goldfine for his generous supply of β-hydroxymyristic acid. I appreciate the helpful suggestions of Leonard H. Cohen, Robert P. Perry, Betsy Ohlssen-Wilhelm, Lewis I. Pizer, and Irwin A. Rose, and I also thank Thomas F. Anderson in whose laboratory these experiments were performed.

This investigation was supported by a grant from the National Science Foundation (GB-29291X1), to Thomas F. Anderson, by Public Health Service grant RR-05539 from the

National Institute of Research Resources, and by an appropriation from the Commonwealth of Pennsylvania to The Institute for Cancer Research.

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