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Enlargement of *Escherichia coli* After Bacteriophage Infection

II. Proposed Mechanism

M. L. FREEDMAN[1] AND R. E. KRISCH

Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439

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Division of *Escherichia coli* was stopped and mean cellular volume was increased after infection with T-even phage. This host cell enlargement was temperature-dependent, cyanide-sensitive, and stable in the presence of hypertonic medium. Enlargement ceased at about the same time that energy metabolism ceased. Initially, enlargement was accompanied by a decrease in mean cell density. Tritiated 2,6-diaminopimelic acid was accumulated and incorporated into cold acid-insoluble material at the preinfection rate. These findings suggest that the effect on host cell size is only in part an osmotic phenomenon and that it also reflects continued growth of the surface of the infected cell in the absence of cell division.

In the preceding paper (11), we have demonstrated electronically that cells of *Escherichia coli* B/r and B_{s-1} stop dividing and increase in mean cell volume soon after infection with T-even bacteriophage. This finding confirms and extends earlier reports of host cell enlargement after bacteriophage infection of *E. coli* and *Bacillus megaterium* (4, 6, 14).

A number of reports suggest that the increase in volume of infected cells may result from osmotic effects. These include the postinfection loss from the cell of cell-associated ^{15}N, ^{35}S, and ^{32}P label (5, 21); intracellular polycationic putrescine (1); the metabolic cofactor nicotinamide adenine dinucleotide (NAD; reference 24); and intracellular $^{28}Mg^{2+}$ and $^{42}K^{+}$ ions (26). The transient decrease in turbidity of *E. coli* cultures infected with T-even phages (8) also suggests an osmotic effect. Similar decreases in optical density with corresponding increases in particle size have been equated with changes in internal osmotic pressure when suspensions of mitochondria are exposed to hypotonic medium (28). In several instances, the term "swelling," as opposed to "growth," has been applied to the phage-induced enlargement of bacterial cells (4, 7, 14).

On the other hand, none of the work cited above rules out the possibility that postinfection cellular enlargement is partially or wholly accounted for by continued host cell metabolism, i.e., by cell "growth" in the absence of cell division. Mukai et al. (18) have reported that exposure to T4 phage does not interfere with bacterial oxygen uptake until about 30 min after infection.

In the present report, we have attempted to elucidate the mechanism of the observed postinfection increase in host cell volume. We distinguish between the two mechanisms mentioned above: (i) an osmotic equilibration between the host cell and its environment, caused by penetration of the cell wall and penetration or alteration of the cell membrane by the phage needle, and characterized by an egress of cellular ions and an ingress of water; and (ii) continued net intracellular macromolecular synthesis or continued synthesis and growth of the cell surface in the absence of cell division, or both.

MATERIALS AND METHODS

Strains of phage and bacteria. The *E. coli* stocks and the bacteriophage used as well as the culture media and growth conditions have been described (11). Since our previous findings and the reports of others (17) indicate that T-even phage cause marked effects on host cell size and metabolism, we have limited this investigation to these viruses. For our experiments on cell wall synthesis, *E. coli* K-12 requiring diaminopimelic acid (DAP^-) was grown in the standard medium supplemented with DAP and L-lysine (5×10^{-5} M each), and *E. coli* B/r was grown in M9 medium supplemented only with DAP, L-lysine (5×10^{-5} M each), and glucose (0.1%).

Physical and biochemical measurements. A modified Coulter counter-multichannel analyzer was used to measure cell size distributions and cell titers

[1] Present address: The University of Connecticut Health Center, Department of Oral Radiology, McCook Hospital, Hartford, Conn. 06112.

(11, 13). The optical densities (OD) of uninfected control and of phage-infected cultures were followed at 450 nm in glass cuvettes (1-cm pathlength) with a Zeiss PMQ II spectrophotometer. The relationship of the dry weight of cell material to the OD was determined by measuring the OD of serially diluted *E. coli* B/r growing exponentially in the standard medium. Concentrated, washed samples of the same cell batch were placed on tared aluminum planchettes and dried in vacuo at 80 C to a constant weight. A plot of OD versus dry weight established a relationship of 1 OD unit = 0.18 mg of cell material.

For dry weight determinations, shake cultures in balanced growth were used to innoculate 500 ml of standard medium (11) in graduated cylinders. These cultures at 37 C were stirred magnetically, sparged with water-saturated air, and maintained at titers below 3×10^7 cells/ml in unlimited balanced growth. At intervals after infection, 50-ml samples were trapped on preweighed HA membrane filters (Millipore Corp.; mean pore size, 0.45 μm; diameter, 2 inches) and washed twice with 25-ml volumes of distilled water to remove external growth medium. The filters were placed in tared aluminum planchettes and dried in vacuo at 80 C to a constant weight as determined with a Mettler MicroGram-atic M-5 S/A balance.

Cell wall synthesis was measured by the incorporation into cold 5% trichloroacetic acid-insoluble material of tritiated 2,6-diaminopimelic acid dihydrochloride (15, 23) obtained from the Radiochemical Centre, Amersham, England. Samples (1 ml) from 10-ml shake cultures of infected and of uninfected control cells were exposed to DAP (final specific activity, 195 μCi/μmole for *E. coli* B/r and 133 μCi/μmole for *E. coli* K-12 DAP$^-$) for 5 min, brought down on HA membrane filters (Millipore Corp.; mean pore size, 0.45 μm; diameter, 1 inch), washed three times with 5-ml of ice-cold 5% trichloroacetic acid, and rinsed with distilled water. The filters used had previously been soaked in growth medium containing excess carrier DAP to reduce adventitious retention of the label. The filters with trapped, washed cells were wrapped in squares of Whatman no. 1 filter paper to moderate combustion and were oxidized in a Packard tritium oxidizer. The radioactivity was determined with a Beckman CPM-100 liquid scintillation spectrometer.

RESULTS

Averaged data from two dry weight experiments are presented in Fig. 1a and b. The relative mean cell volume (MCV) of *E. coli* B/r infected with viable rapid-lysis T2r phage increased by 80% (Fig. 1a). The relative mean dry weight per cell (MCW) also increased after infection, reflecting the intracellular synthesis of progeny

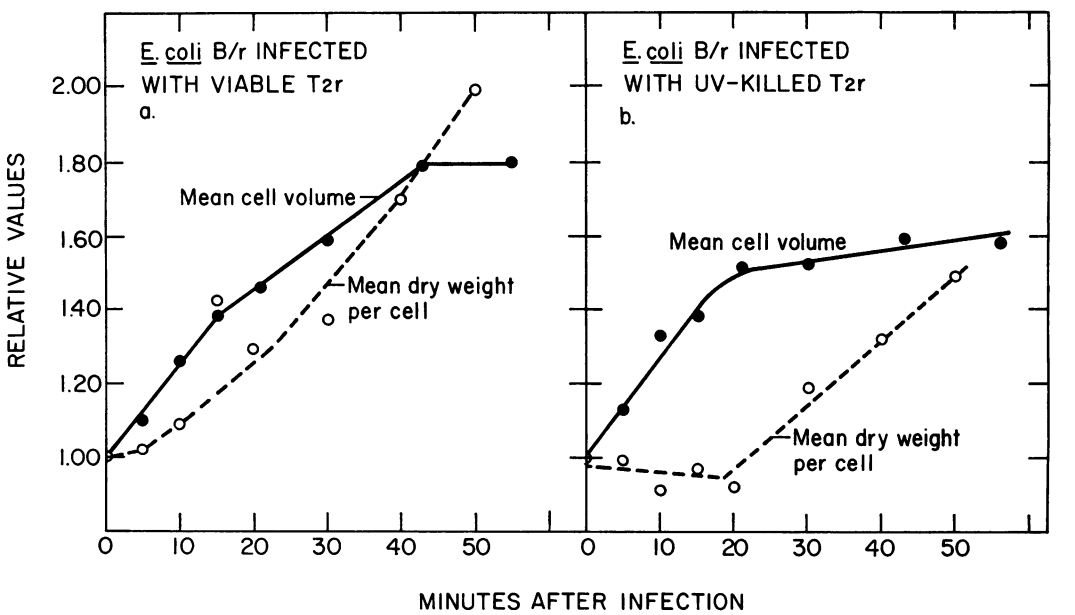

FIG. 1. *Effect of viable phage infection (a) and ultraviolet light-killed phage infection (b) on mean cell volume and mean cell dry weight. Relative values are derived by dividing values at times after infection by the values at the time of addition of phage at MOI values of 4. Mean cell volumes are determined by numerical integration of frequency distributions for cell volume from a Coulter counter-multichannel analyzer. Mean cell dry weights are the quotients of the weights of 50-ml samples of infected cultures, washed and dried at 80 C in vacuo to constant weight, divided by the electronically determined cell titer. Average dry weight per cell immediately after infection ($t = 0$) was $3.39 \pm 0.34 \times 10^{-13}$ g.*

phage. Relative values are obtained by dividing the MCV or cell mass after infection by the value at the time of infection. Relative values < 1 reflect decreases in the parameter under study, whereas values > 1 reflect increases. The cell dry weight initially did not keep pace with the cell volume. If ultraviolet (UV) light-killed phage were used (Fig. 1b), MCV increased by about 60% in the first 20 min with no increase in MCW. Thus, the increase in cell volume was occurring in the absence of any net intracellular macromolecular synthesis. After 20 min, MCW increased and infected cells enlarged more slowly. Our previous findings (11) revealed no drop in titer after cells were infected with UV-killed phage.

The change in relative mean cell density (MCD), taken as the quotient of MCW divided by MCV from data presented in Fig. 1a and b, is presented in Fig. 2a. There was a prolonged decrease in MCD of 10 to 30% after phage infection. The effect on cell density was greater when "dead" virus was used, presumably because there was no increase in MCW from progeny

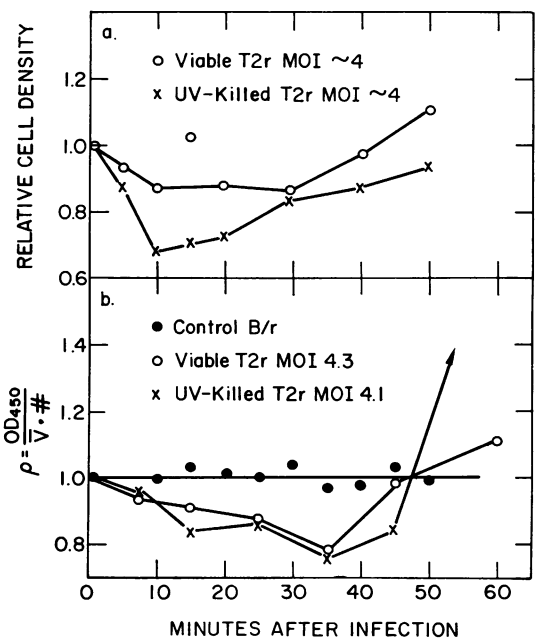

FIG. 2. *(a) Relative cell density equals mean cell dry weight divided by mean cell volume from Fig. 1a and b. Values have been normalized to 1 at t = 0 min after infection. (b) Density, (ρ) equals optical density (OD) at 450 nm, measured with a spectrophotometer in which 1 OD unit = 0.18 mg (dry weight) of cell material, divided by the mean cell volume ($\bar{V}$) times the cell titer (#) determined electronically with a Coulter counter-multichannel analyzer.*

phage protein or deoxyribonucleic acid (DNA). In Fig. 2b, MCD is derived from OD measurements (taken as an approximate measure of the dry weight of cell material per milliliter of infected culture) divided by corresponding MCV times cell titers (reflecting the total cell volume per milliliter of culture). This method provided an independent measurement of infected cell density. Uninfected control cells maintained a constant MCD. Infection with viable or with UV-killed viruses, on the other hand, resulted in a drop in MCD of approximately 20%. Although the patterns of decrease in infected cell density differ slightly between Fig. 2a and b, the magnitudes and durations of the MCD changes are similar. Both methods reveal that phage adsorption and penetration initially cause the host cell to decrease in density, at the time that cell division is terminating and cell volume is increasing. The later recovery of MCD after infection by viable phage probably reflects the intracellular formation of progeny phage and a reduction of cell titer from lysis, whereas recovery of the MCD after infection by UV-killed phage probably reflects resumption of synthesis by the host cell.

To determine if the increase in MCV and the concomitant decrease in MCD initially observed after phage infection were exclusively osmotic phenomena, infected cells were exposed to hypertonic sucrose solutions. The enlargement, if osmotic, should be reversible. *E. coli* B/r grown in standard growth medium (Fig. 3a and b) was infected with T4 phage, and samples from the infected culture and from an uninfected control culture were electronically counted and sized, also in standard growth medium rather than HCl, and in growth medium supplemented with 10% sucrose (w/v). Bayer (3) reported this sucrose concentration to be sufficient to cause abrupt but reversible plasmolysis of *E. coli*. The relative cell concentration of uninfected control cultures increased exponentially, and the division of infected cells was strongly inhibited (Fig. 3a). Sucrose in the counting fluid slightly reduced the counting efficiency of the Coulter apparatus. The change in MCV of *E. coli* B/r after infection by T4 phage was stable in the presence of hypertonic sucrose (Fig. 3b); enlargement of infected cells was not reversed. This indicates that passive osmotic swelling from intracellular turgor, with resultant stretching of the bacterial wall, is not the sole mechanism responsible for enlargement.

In related experiments (*unpublished data*), *E. coli* B/r cells were grown and infected with phage in standard medium supplemented with 10% sucrose, a concentration initially sufficient to cause plasmolysis. These cells were rapidly

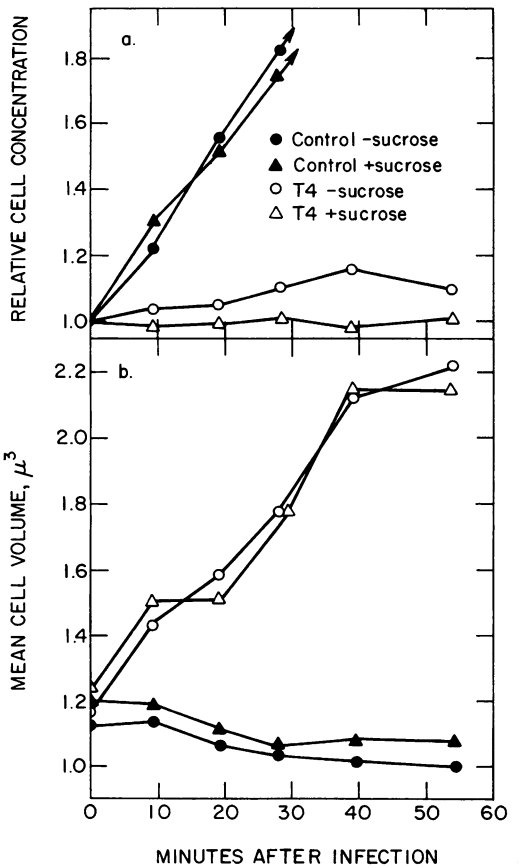

FIG. 3. *Division arrest (a) and enlargement (b) of E. coli B/r infected at an MOI of 10 with viable T4 phage. Cells were in balanced growth in standard medium at the time of infection. Samples (0.5 ml) of culture were withdrawn at intervals and diluted 20-fold into fresh growth medium, with or without 10% sucrose, for determination of mean cell volume and cell titer.*

diluted 20-fold into 0.1 N HCl counting solution in the presence or absence of 10% sucrose. Both the infected and uninfected cells diluted into sucrose-free medium did not show an increase in MCV or a decrease in titer relative to corresponding cells diluted into 10% sucrose medium. Cell enlargement and inhibition of cell division developed with approximately the same kinetics as in Fig. 3 regardless of counting medium. These findings further suggest that some mechanism other than osmosis plays an important role in causing cell enlargement.

If the increase in MCV due to phage infection were primarily osmotic, the rate of increase should show little if any temperature dependence within the range of 22 to 37 C. Table 1 presents, for four different temperatures, the doubling time for MCV of *E. coli* B/r infected with T2r rapid-lysis phage, as well as the doubling time for the culture titer of uninfected controls. The temperature dependence of both doubling times was measured by the factor of decrease effected by a 10 C temperature increase (Q_{10}). Contrary to the predictions for an osmotic effect, the doubling time for MCV of infected cells showed the same strong temperature dependence as did the doubling time for culture titer of uninfected cells. Each has an average Q_{10} of 2.4.

TABLE 1. *Effect of increasing temperature on the doubling times of mean cell volume (MCV) for infected cells and cell number for uninfected cells*

Temp[a] (C)	Phage-infected cells[b]		Uninfected control cells	
	MCV doubling time (min)[c]	MCV Q_{10}[d]	Cell no. doubling time (min)	No. Q_{10}[d]
22.5	176		89	
		2.6		2.5
32	67		36	
27	82		56	
		2.2		2.3
27	37		24	

[a] Shake cultures (10 ml) of *E. coli* B/r in balanced growth were maintained at each temperature throughout the experiments.

[b] Infected with rapid-lysis T2r phage at MOI values from 6 to 10.

[c] Based on the rate of increase in MCV during the first 20 min after phage addition.

[d] Average, 2.4. The temperature dependence of both doubling times was measured by the factor of decrease effected by a 10 C temperature increase.

Doermann (9) reported that phage adsorption to and penetration of the semipermeable bacterial wall and membrane are not prevented by the metabolic poison NaCN. Figure 4a shows that the relative cell concentration increased exponentially in a control culture lacking phage T2 and NaCN, whereas cultures exposed to phage, to cyanide at a final concentration of 2×10^{-3} M, or to both agents together ceased cell division for as long as 35 min, at which time the cells were washed and resuspended in fresh growth medium. Figure 4a shows that cyanide blockage of cell division was freely reversible, except where phage infection had occurred. Figure 4b shows that the control culture in balanced growth maintained a near-constant MCV, whereas the culture infected with phage increased in MCV by 60%. Cyanide greatly inhibited the host cell volume increase, as shown by the culture exposed

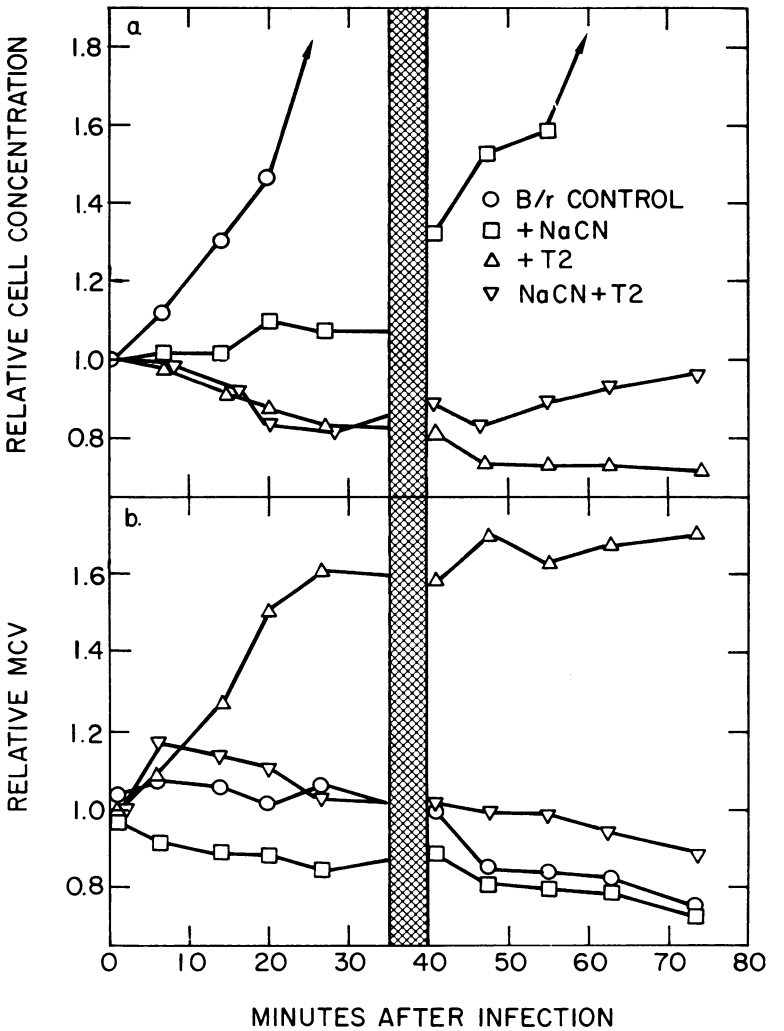

FIG. 4. *Effects of NaCN or T2 phage, or both, on cell division (a) and on cell enlargement (b). E. coli B/r cells in balanced growth were exposed to NaCN at a final concentration of 2 × 10⁻³ M to bacteriophage at an MOI of 2 or to both. Culture samples (0.5 ml) were withdrawn at intervals and diluted 20-fold into 0.1 N HCl, and the cells were counted and sized. Between 35 and 40 min, the cultures were centrifuged at 37 C (3 min at 2,500 × g) to pellet the cells, and the supernatant fluids were discarded. The pellets were resuspended in equal volumes of prewarmed growth medium which had been used to grow cells to a comparable titer and then were filter-sterilized.*

for 35 min to both NaCN and phage T2; there was an increase in MCV of less than 20% not blocked by cyanide. Removal of the metabolic inhibitor between 35 and 40 min did not result in abrupt cell enlargement as might be expected if the poison somehow interfered with a rapid physical phenomenon like osmotic swelling, as opposed to a more slowly developing, temperature-dependent, biochemical process such as macromolecular synthesis. The observed dependence of rate of enlargement on temperature and the arrest of most infected cell enlargement by NaCN suggest that the enlargement effect has biochemical components requiring energy.

To investigate the possibility that continued synthesis of the cell wall after infection would explain our results, bacteria were pulse-labeled with ^{3}H-DAP for 5-min periods after phage infection. Figures 5a to c and 6a to c present the averaged data from experiments with two bacterial strains. Figure 5a shows that uninfected control *E. coli* K-12 DAP$^-$ cells contained to divide, whereas the division of cells infected by

T2 phage was slowed and finally blocked after 30 min. The relative MCV of the control cells remained constant, but the MCV of infected cells increased by 30% (Fig. 5b). The counts per minute per cell for both control and infected cultures were similar, indicating that DAP uptake and incorporation into cold trichloroacetic acid-insoluble material, assumed to be cell wall, continued at the preinfection rate. L-Lysine was present during exposure to tritiated DAP to inhibit conversion of DAP into protein precursors (20, 27, 29, 30). It should be noted that the doubling time of the K-12 strain was 45 min, which is almost twice that of similarly cultured B/r cells. This may account for the slower response of cell division and MCV to phage infection for *E. coli* K-12 DAP⁻ than that shown previously for *E. coli* B/r. When non-DAP-requiring *E. coli* B/r was infected with T2r phage, control cultures increased in number exponentially and maintained constant MCV values; phage-infected cultures showed inhibition of division and enlarging MCV values (Fig. 6a and b). DAP uptake and incorporation into cold acid-insoluble material was similar in the uninfected controls and in the phage-infected cultures (Fig. 6c). Both *E.coli* K-12 DAP⁻ and *E. coli* B/r continued to incorporate DAP after phage infection at the preinfection rate. This is consistent with a continuation of cell wall synthesis at a normal or near-normal rate during the period of host cell enlargement and inhibited cell division after T-even phage infection.

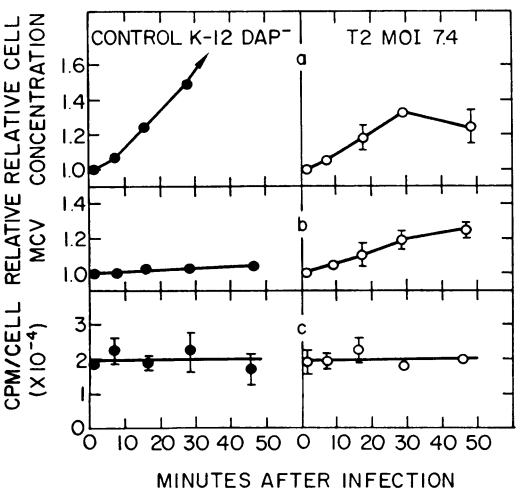

FIG. 5. *Effect of phage infection on cell division (a), on mean cell volume (b), and on incorporation of ^{3}H-diaminopimelic acid (DAP) into cold trichloroacetic acid-insoluble material after a 5-min pulse exposure to the isotope (c), E. coli K12 DAP⁻, and phage T2. Cells were grown in standard medium supplemented with 5 × 10⁻⁵ M each of DAP and L-lysine. Samples (1 ml) from phage-infected cultures, as well as from uninfected control cultures in balanced growth, were exposed to isotope at a final specific activity of 133 μCi/μmole. After 5 min of exposure at 37 C, the cells were iced, trapped on HA membrane filters (0.45-μm mean pore size; Millipore Corp.) presoaked in carrier DAP, and then washed with three 5-ml portions of ice-cold 5% trichloroacetic acid and one 5-ml portion of water. Filters with trapped extracted cells were oxidized and counted in a liquid scintillation spectrometer. At the start of each isotope incubation, a separate 0.5-ml culture sample was diluted 20-fold into 0.1 N HCl and sized and counted electronically. Bars represent the range of values in two experiments with MOI values of 4.1 and 10.7.*

DISCUSSION

Because MCW does not keep pace with the increase in MCV after infection with T-even phage, there is a reduction in MCD. Such a change in density after the phage needle penetrates a sensitive host could result from passive swelling or from active phenomena such as altered cellular syntheses in the presence of division blockage or from both. For example, if cellular macromolecular synthesis continued at the preinfection rate while cell division was blocked by infection, cell density should not change. If, on the other hand, synthesis of one

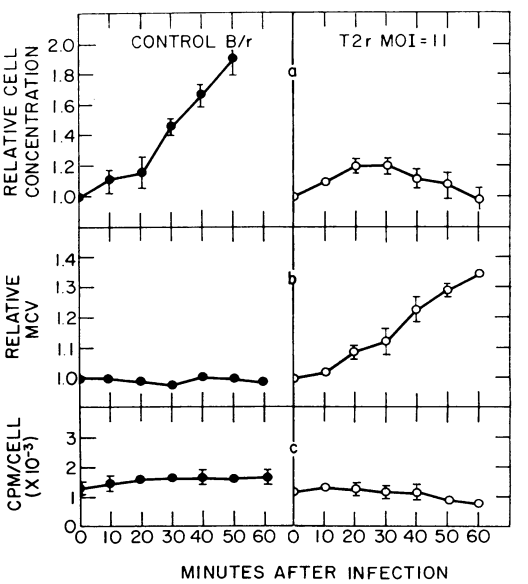

FIG. 6. *The same as Fig. 5a to c but employing E. coli B/r and rapid lysis phage T2r. The final specific activity of ^{3}H-diaminopimelic acid in these experiments was 195 μCi/μmole. The bars on the points represent the range of values from two similar experiments, both with MOI values of 11.*

or more cell components were inhibited by infection, a decrease in density would result from the increase in MCV. Evidence exists that T-even phage infection terminates host cell DNA, ribosome, and protein synthesis (11); thus, it is plausible either that the enlargement effect is wholly or partially osmotic with distortion of the cell surface (19) caused by intracellular turgor, which has been reported at 5 to 30 kg/cm^2 (15), or that only the cell surface continues to grow, creating larger, "unfilled" bacteria. If phage infection resulted in a local weakening or dissolution of the cell wall followed by repair, as suggested by some workers (22, 25), then the volume increase might be expected to be a temporary phenomenon and to be dependent on multiplicity of infection (MOI). Such a response has been reported for the leakage of ^{42}K and ^{28}Mg ions (26) and ^{15}N-labeled material (2, 5) from phage-infected cells. However, we find enlargement of the host cell to be a permanent and irreversible alteration which occurs after the adsorption and successful infection by at least one viable or killed phage or phage ghost. Additionally, experiments in which *E. coli* B/r cultures in balanced growth were exposed to T4 lysozyme did not result in any increases in MCV (*unpublished data*). It is now thought unlikely that this phage enzyme is responsible for cell disruption at the time of infection, but rather that it functions in the liberation of mature progeny phage (10).

Over the range of 22 to 37 C, a strong temperature dependence exists for the enlargement process. This suggests that a biochemical mechanism, rather than osmosis, governs all or part of the enlargement of infected cells. Also, the fact that the rate of enlargement of infected cells shows the same Q_{10} as the rate of increase of uninfected cell numbers over this temperature range is consistent with the concept that the mechanism of enlargement of infected cells is closely related to the mechanism of growth of uninfected cells. It does not, of course, prove such a relationship.

In concentrations of sucrose that cause abrupt plasmolysis in *E. coli* (3) cell enlargement is still evident, further indicating that a rapid influx of extracellular water and efflux of intracellular ions produce only a portion of the increase in volume of the infected cell. Sucrose, which is not transported into the cells, has been used to stabilize protoplasts against osmotic rupture (15).

Cyanide concentrations that block energy metabolism and, hence, cell division also block most cell enlargement, although there is evidence that neither phage adsorption nor penetration is inhibited (9). Perhaps only the cyanide-insensitive fraction of bacterial enlargement (20%) is osmotic in nature. The energy dependence of host cell enlargement is also suggested by the fact that such enlargement usually ceases after about 30 min, as demonstrated in several figures in this paper and in reference 11. Oxygen uptake by T4-infected *E. coli* continues normally for about 30 min and then rapidly diminishes to zero (18). Progeny phage production alone probably does not account for infected cell enlargement, because we have described MCV increases after infection with UV-killed phage and with phage ghosts (11).

Thus, the data obtained indicate that the well documented cell permeability changes, involving the loss of cellular ^{35}S, ^{32}P, ^{28}Mg, ^{42}K, putrescine, and NAD after T-even phage infection, can explain only part of host cell enlargement Although infection by these phages terminates host cell macromolecular synthesis, cell surface components including phospholipids are synthesized (6, 12), perhaps under phage direction (25), and damage to the cell surface from phage infection presumably is repaired, as evidenced by the cessation of leakage (22, 26). Our results for DAP uptake and incorporation into cold acid-insoluble cell components suggest that the rate at which cell wall is synthesized is not significantly reduced by phage infection. Continuing growth of the bacterial cell wall or membrane (possibly in an altered form), or both, combined with blockage of cell division as well as other host cell macromolecular synthesis, could account for the observed increase in MCV and decrease in MCD.

ACKNOWLEDGMENTS

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