DNA Injection During Bacteriophage T4 Infection of *Escherichia coli*

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The process of phage T4 DNA injection into the host cell was studied under a fluorescent microscope, using 4',6-diamidino-2-phenylindole as a DNA-specific fluorochrome. The phage DNA injection was observed when spheroplasts were infected with the artificially contracted phage particles having a protruding core. The DNA injection was mediated by the interaction of the core tip with the cytoplasmic membrane of the spheroplast. A membrane potential was not required for the process of DNA injection. On the other hand, DNA injection upon infection by intact noncontracted phage of the intact host cell was inhibited by an energy poison. Based on these observations, together with results from previous work, a model for the T4 infection process is presented, and the role of the membrane potential in the infection process is discussed.

By means of a reconstitution system, the roles of the cell surface components of *Escherichia coli* K-12 in bacteriophage T4 infection have been elucidated up to the stage of DNA ejection. Both lipopolysaccharide and the OmpC protein, major constituents of the outer membrane, function as the receptor (9, 10, 18, 24). The lipoprotein-bearing peptidoglycan layer that underlies the outer membrane allows the OmpC protein and lipopolysaccharide to assemble widely on its surface so that the receptor complex can interact with the individual distal ends of the long tail fibers of a single phage particle (9). It also serves as a rigid support for the outer membrane to help the penetration of the phage core through the flexible outer membrane (8). The core tip that has penetrated the cell surface interacts with a phospholipid bilayer, representative of the cytoplasmic membrane, and the interaction induces the ejection of phage DNA through the core (8). However, the DNA molecule thus ejected is not incorporated into the liposomes. This suggests that an additional factor(s) is required for DNA uptake into the host cytoplasm. It has been reported that a membrane potential across the cytoplasmic membrane is required for the injection of T4 DNA into the host cell (14, 16). However, it is unclear whether the membrane potential is directly involved in the transport of DNA across the cytoplasmic membrane or not.

In the present work, we studied the process of DNA injection across the cytoplasmic membrane by chasing the phage DNA labeled with 4',6-diamidino-2-phenylindole (DAPI) under a fluorescent microscope. The artificially contracted mutant phage particles having a protruding core interacted by means of the core tip with the spheroplasts of *E. coli* and injected the DNA into the cytoplasm. The DNA injection was independent of a membrane potential. On the other hand, the injection was inhibited by an energy poison when intact cells were infected with noncontracted phage particles. Based on these results, we propose a model for T4 infection in which a membrane potential is not required for the DNA injection itself but for the rapprochement of the cytoplasmic membrane to the outer membrane-peptidoglycan layer to facilitate the interaction of the cytoplasmic membrane with the core tip.

MATERIALS AND METHODS

Bacteria and bacteriophages. The *E. coli* strains used were wild-type B, ML308-225 (K-12; *lacI lacZ*) (13), and AN120 (K-12; F⁻, *uncA401 arg thr mtl xyl rpsL*) (5). The bacteriophage T4 strain used was T4Y122, a heat-sensitive T4 mutant having a mutation in baseplate gene 6 (23).

Preparation of phage T4 containing DAPI-labeled DNA. *E. coli* B was cultured to 0.5×10^8 cells per ml at 37°C in 100 ml of 3XD medium which contained (per liter) 4.5 g of KH_2PO_4, 10.5 g of Na_2HPO_4, 1.0 g of NH_4Cl, 0.3 g of $MgSO_4 \cdot 7H_2O$, 16 mg of $CaCl_2$, 15 g of Casamino Acids, and 25 g of glycerol and infected with T4Y122 (about five particles per cell). Simultaneously, 500 μg of DAPI was added to the culture medium. After 2 h, a few drops of chloroform were added to lyse the cells. The cell debris was removed by centrifugation at $3,000 \times g$ for 10 min. Phage particles in the

supernatant fluid were sedimented by centrifugation at 20,000 × *g* for 30 min and suspended in a small volume of T2 buffer. Phage particles were further purified on a sucrose gradient by centrifugation and stored in T2 buffer as described previously (18). The DAPI-stained phage particle was as active and stable as the unstained one in terms of infectivity and osmotic sensitivity provided that it was stored in the dark.

Preparation of contracted phage T4. Phages with a contracted tail sheath were prepared from T4Y122 by heat treatment at 55°C for 5 min as described previously (8).

Preparation of spheroplasts. Spheroplasts were prepared from ML308-225 grown in modified Fraser and Jerrel medium as described previously (17). The spheroplast suspension was diluted with 9 volumes of 0.5 M sucrose–0.1 M sodium phosphate buffer (pH 7.1) and supplemented with magnesium chloride and glucose to final concentrations of 5 mM and 1%, respectively.

Microscopic observation of phage DNA injection. A suspension of spheroplasts (100 μl, containing about 1 × 10⁷ spheroplasts) was mixed with 10 μl of DNase I (1 mg/ml; Worthington Diagnostics) and a phage suspension (3 μl, containing about 1 × 10⁸ phage particles) at 37°C. When intact cells were to be used, AN120 was grown in antibiotic medium 3 and suspended in the same volume of fresh antibiotic medium 3. A portion (100 μl, 5 × 10⁷ cells) of the suspension was supplemented with 5 μl of 0.1 M MgCl₂ and 1 μl of DNase I (1 mg/ml) and mixed with 10 μl of a phage suspension (5.6 × 10⁸ phage particles). Portions (2 μl) of these mixtures were taken out and observed under an optical microscope. Fluorescent microscopy was carried out with an Olympus BHS-RFK epifluorescence microscope, which was equipped with a high-pressure mercury vapor lamp (HBO, 100 W), a 334-nm excitation filter, a 420-nm suppression filter, and a UVFL 100/1.30 objective. Photographs were taken 10 to 20 min after the mixing. The same preparations were also observed by phase-contrast microscopy. For electron microscopy, original spheroplast preparations were centrifuged at 3,000 rpm for 5 min, and the spheroplast pellets were suspended in the same volume of 0.5 M sucrose–0.1 M sodium phosphate buffer (pH 7.1)–5 mM MgCl₂–1% glucose. The suspension was mixed with the same volume of a T4 suspension (2.8 × 10¹¹/ml), incubated at 37°C for 10 min, and negatively stained with 1% sodium phosphotungstate (pH 6.2). A Hitachi HS-9 electron microscope was used.

RESULTS AND DISCUSSION

Phage DNA injection into host cell cytoplasm. T4Y122 is a heat-sensitive T4 mutant having a mutation in baseplate gene 6 such that heat treatment induces the contraction of the tail sheath and extrusion of the core (23). The contracted phages thus prepared are adsorbed with the core tip to liposomes containing phosphatidylglycerol or cardiolipin, and the DNA is ejected (8). However, DNA injection into the liposomes was not observed. Since the liposomes that we used in a previous study were relatively small, they might not have been large enough to take up an entire phage DNA molecule. We

prepared, therefore, larger liposomes by the method of Francis and Papahadjopoulos (7). We also prepared membrane vesicles by the method of Kaback (13). However, the adsorption of the contracted phages to these membranous vesicles was not significant; hence, DNA injection could not be studied critically.

The intensive adsorption of contracted phages and subsequent DNA injection were observed only when spheroplasts were used. The adsorption was rather stable (Fig. 1A). Since adsorption was observed neither with the combination of noncontracted phages and spheroplasts (Fig. 1B) nor with that of contracted phages and intact cells (data not shown), it was concluded to be due to the interaction of the core tip with the cytoplasmic membrane of the spheroplasts.

The process of DNA injection was examined by fluorescent microscopy, using DAPI as a DNA-specific fluorochrome. Under the microscope, the DAPI-containing contracted phage particles appeared as light-diffusion spots showing vigorous Brownian movement (Fig. 2). When the fluorescent phages were osmotically shocked in the presence of DNase I, only 2% of the fluorochrome was recovered with the ghost phages, indicating that DAPI stained DNA specifically (data not shown). In addition, the binding of DAPI to DNA molecules has been shown to be very stable (22). Upon the addition of spheroplasts, the fluorochrome in the contracted phage particles was transferred into the spheroplasts (Fig. 2A), indicating that the phage DNA was injected into the spheroplasts. Since the medium was supplemented with DNase, the DNA transfer must have taken place directly across the cytoplasmic membrane. Consistent with the electron microscopic observations described above, contracted phages and spheroplasts were the only combination that resulted in the transmembrane transfer of the fluorochrome into the host cell. Rod-shaped cells, most likely representing intact cells in the spheroplast preparation (indicated by arrows in Fig. 2A), were not stained with the fluorochrome. Noncontracted phage particles also failed to transfer the fluorochrome into the spheroplasts (Fig. 2B). These results excluded the possibility of the indirect fluorochrome transfer from phage particles to host cells via the medium. This possibility was further excluded by the facts that DAPI (5 μg/ml) added to the culture medium was hardly taken up by intact cells or spheroplasts within experimental periods (10 to 20 min), whereas it was rapidly taken up when the cells were treated wih glutaraldehyde (data not shown).

Effect of a membrane potential on the DNA injection. A membrane potential is reported to be required for the DNA injection in the process
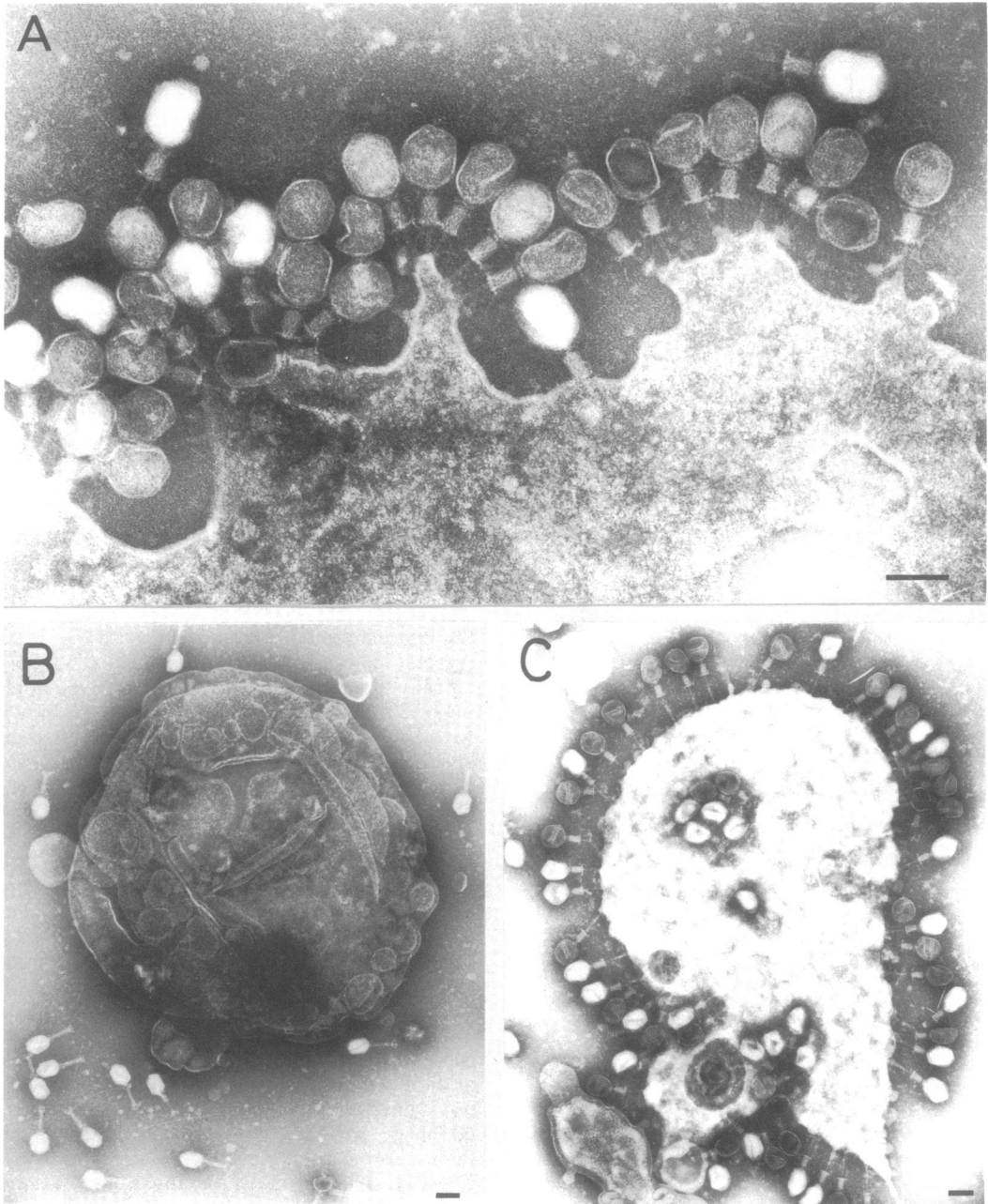
FIG. 1. Phage T4 adsorption to and DNA ejection into *E. coli* spheroplasts. Phage particles were incubated with spheroplasts of ML308-225 at 37°C for 10 min, directly negatively stained, and examined under an electron microscope. The phages used were contracted T4Y122 (A and C) and noncontracted T4Y122 (B). In (C) CCCP was added to the spheroplast suspension at a final concentration of 10 μM before the addition of phages. The bars represent 100 nm.

of T4 infection. We confirmed the results by fluorescent microscopy with the combination of an *uncA* mutant and phages carrying a DNA-specific fluorochrome (Fig. 3). Because in *uncA* cells there is no F_1-ATPase activity, the ener-

gized membrane state is completely dependent on the electron transport chain (5). Potassium cyanide, a specific poison for the respiratory chain, inhibited the phage DNA injection when the intact *uncA* cells were infected with intact
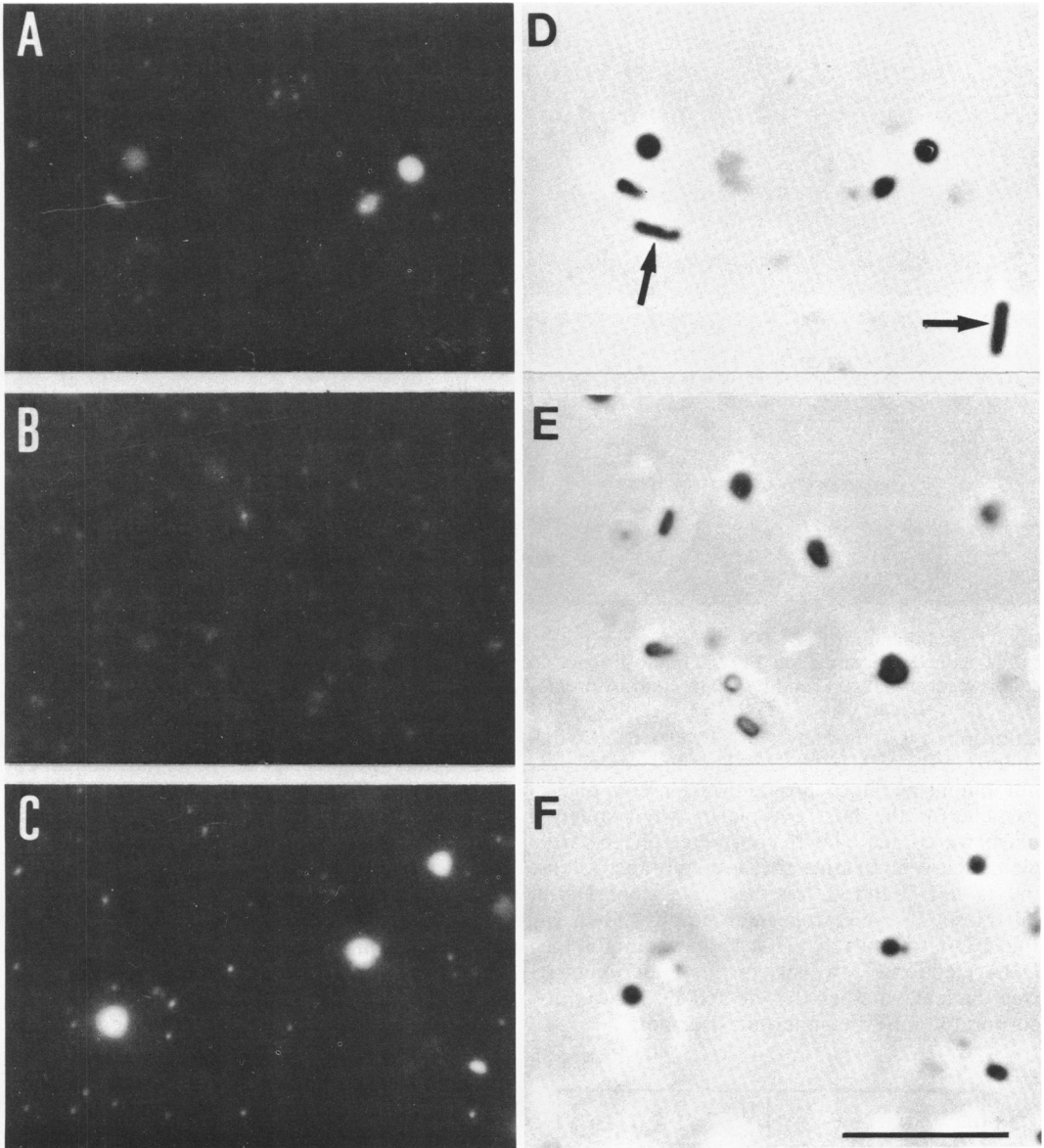
FIG. 2. Injection of phage DNA into *E. coli* spheroplasts. The injection of DAPI-stained DNA of T4Y122 into spheroplasts of ML308-225 was examined under the epifluorescence microscope with the following combinations: (A and D) spheroplasts and contracted phages; (B and E) spheroplasts and noncontracted phages; (C and F) CCCP-treated (10 μM) spheroplasts and contracted phages. (A to C) Fluorescent profiles; (D to F) phase-contrast profiles of the same field. The arrows in (D) indicate intact cells in the spheroplast suspension. The bar represents 10 μm.

noncontracted phages. Many small light-diffusion spots representing DNA-containing phage particles were observed attached to the host cells, whereas the host cells were almost free from DAPI (Fig. 3B): more than 60% of the host cells were unstained with the fluorochrome, and the others were weakly stained. In the experiments shown in Fig. 1 and 2, spheroplasts were

incubated in the presence of glucose. Therefore, the cytoplasmic membrane was thought to be energized. The effect of energy poisons on the process of DNA injection was studied with spheroplasts (Fig. 2C). Since it was difficult to prepare stable spheroplasts from the *uncA* mutant, potassium cyanide was not used to block the energy supply. Instead, carbonyl cyanide-*m*-
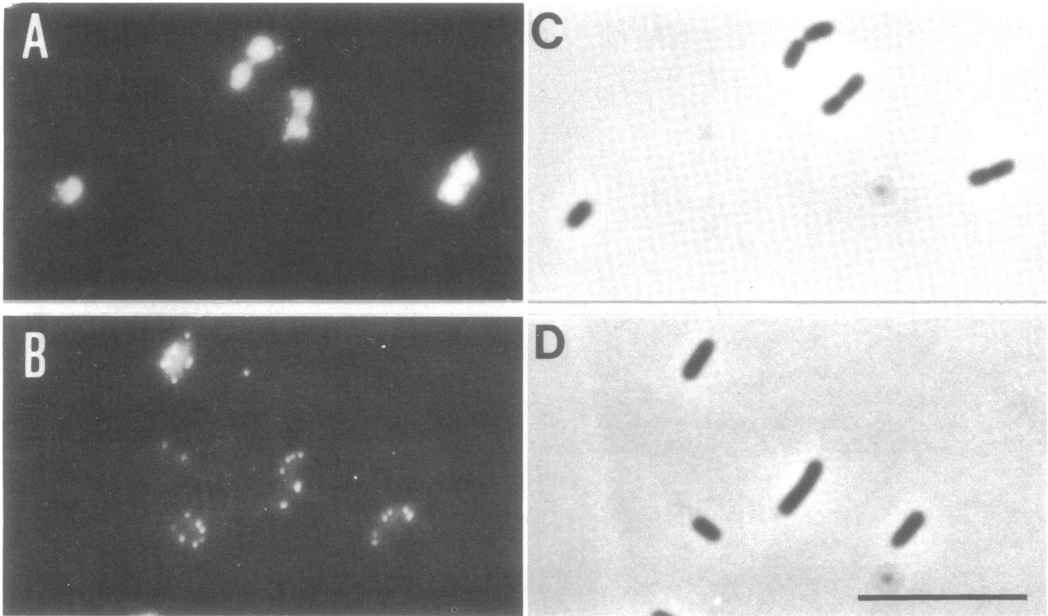
FIG. 3. Inhibition of T4 DNA injection by potassium cyanide. AN120 (*uncA*) cells were incubated with DAPI-stained noncontracted T4Y122 in the absence (A and C) or presence (B and D) of 5 mM KCN. (A and B) Fluorescent profiles; (C and D) phase-contrast profiles of the same fields. The bar represents 10 μm.

chlorophenyl hydrazone (CCCP) was used with spheroplasts of ML308-225. This uncoupler did not inhibit the fluorochrome transfer from phage particles to the host cytoplasm when sphero-plasts were infected with contracted phage parti-cles, clearly indicating that a membrane poten-tial is not required for the process of DNA injection. The electron micrograph shown in Fig. 1C also shows that CCCP did not inhibit the DNA ejection. However, in this experiment it was unclear whether the ejected DNA was in-corporated into the spheroplast or not.

Proline transport across the cytoplasmic membrane is membrane potential dependent (11). To confirm that both cyanide and CCCP had actually destroyed the membrane potential of intact cells and spheroplasts under the condi-tions described in the legends to Fig. 3 and 2, respectively, proline uptake activity was as-sayed (Fig. 4). Both poisons almost completely inhibited proline transport activity.

For what is the membrane potential required? It is clear that metabolic energy is required for the DNA injection into the host cell in the
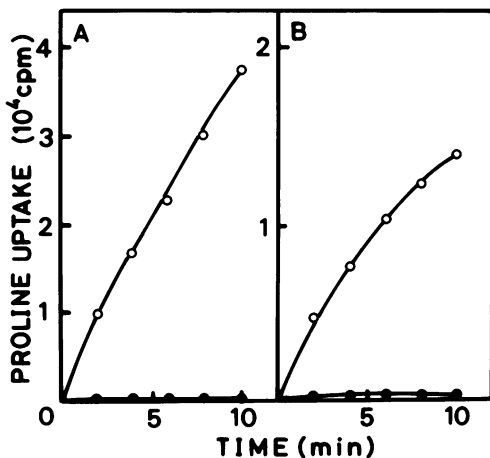

FIG. 4. Effect of energy poisons on proline uptake activity of intact AN120 cells and spheroplasts pre-pared from ML308-225. (A) For experiments with intact cells, a 1.4-ml culture of AN120 cells grown in M9–0.2% glucose (5×10^8 cells per ml) was mixed with 14 μl of 0.18 mM L-[U-^{14}C]proline (2.74 Ci/mol) in the presence (●) or absence (○) of 5 mM KCN, and incubation was carried out at 37°C. Portions (200 μl) were taken out at the indicated times, filtered through a cellulose-acetate membrane filter (0.5-μm pore size; Millipore Corp.), and washed with M9–0.2% glucose. (B) For experiments with spheroplasts, 1.4 ml of the spheroplast suspension of ML308-225 (700 μg of pro-tein) was mixed with 14 μl of 0.18 mM L-[U-^{14}C]pro-line (2.74 Ci/mol) in the presence (●) and absence (○) of 10 μM CCCP. Incubation and sampling were the same as described for (A) except that 0.5 M sucrose–0.1 M sodium phosphate buffer (pH 7.1) was used for washing. Membrane filters were dried, and the radio-activity was counted in 5 ml of toluene scintillation fluid with an Aloka scintillation spectrometer.

Stage: I, II, III

Events:
- I: Long tail fiber adsorption
- II: Tail pin adsorption
- III: Tail sheath contraction / Needle insertion

Host cell factors required:
- I: OmpC protein / Lipopolysaccharide
- II: Lipopolysaccharide
- III: Peptidoglycan layer

Stage: IV, V, VI

Events:
- IV: Approach of cytoplasmic membrane to outer membrane
- V: Interaction of core tip with cytoplasmic membrane
- VI: DNA injection into cytoplasm

Host cell factors required:
- IV: Membrane potential
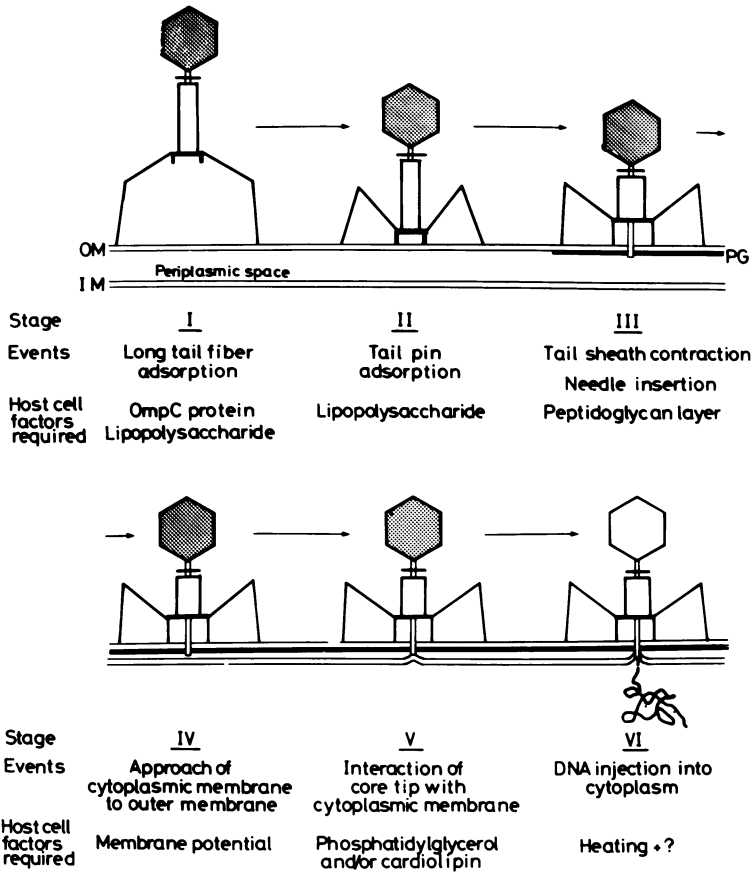- V: Phosphatidylglycerol and/or cardiolipin
- VI: Heating + ?

FIG. 5. Summary of process of phage T4 infection. OM, Outer membrane; CM, cytoplasmic membrane; PG, peptidoglycan layer.

process of phage T4 infection (14, 16; Fig. 3). Moreover, a membrane potential across the cytoplasmic membrane has been shown to be responsible for it (16). In the presence of uncouplers, such as cyanide, azide, dinitrophenol, and CCCP, the DNA injection is inhibited, although phage adsorption and contraction of the tail sheath are not inhibited by these uncouplers (16). Using reconstituted cell surfaces, we have studied the roles of cell surface components in the process of phage T4 infection (8, 9, 18, 24). Together with the results obtained in the present work, these studies led us to propose the process of T4 infection summarized in Fig. 5. We are not going to discuss the details of the individual stages from a general point of view, for the process up to stage V (except stage IV) has already been discussed in a previous paper (8). Here we discuss them in terms of the role of a membrane potential.

The cell surface reconstituted from outer membrane protein OmpC, lipopolysaccharide, and the lipoprotein-bearing peptidoglycan layer

functions in the first three stages without any additional energy supply, indicating that the energization of the cytoplasmic membrane is not required for the phage adsorption and the core insertion. This is consistent with observations that membrane energization is not required for the phage adsorption to intact cells and the subsequent tail sheath contraction (9). By introducing lipsomes as representative of the cytoplasmic membrane, we have further shown that a membrane potential, as well as a pH gradient (ΔpH), is not required for the DNA ejection from the contracted phage (8). Furthermore, we showed here that the transfer of phage DNA into the host cell cytoplasm upon interaction of the core tip with the cytoplasmic membrane does not require a membrane potential. As far as individual reactions in the T4 infection process so far studied are concerned, taken together, none of them requires a membrane potential. Then, for what stage is the membrane potential required?

In gram-negative bacteria, the cell surface

layer (the outer membrane-peptidoglycan layer) is separated from the cytoplasmic membrane by the periplasmic space. Previous experiments with a reconstituted cell surface show that only a very short distal end of the core of an infected phage particle can be protruded out from the inner surface of the outer membrane-peptidoglycan layer (9), and this protrusion most probably is not long enough to interact by its tip with the cytoplasmic membrane across the periplasmic space. Provided that this is the case, a stage should exist in which the cytoplasmic membrane comes close to the outer layer, thus enabling the interaction of the core tip with the cytoplasmic membrane. In this respect, it should be noted that the membrane potential is not required for the phage adsorption and DNA ejection on the reconstituted cell surface where liposomes, representative of the cytoplasmic membrane, can easily reach the inner surface of the outer membrane-peptidoglycan layer (8). As a working hypothesis, therefore, we propose that the membrane potential is required for this process. Some circumstantial evidence supports this view. For example, the energization of the cytoplasmic membrane is required for the translocation of phospholipids from the cytoplasmic membrane to the outer membrane (6) and also for the transfer of externally added colicins Ia, K, and E1 to the target organelle, the cytoplasmic membrane (12, 19, 20), and an idea similar to that proposed here has been presented elsewhere (15). Similar phenomena have been reported for mitochondria, which also have outer and inner membranes. The incorporation of externally synthesized mitochondrial proteins across the outer membrane into the inner membrane requires the energization of the mitochondria (21).

In gram-negative bacteria, such as *E. coli*, the outer and cytoplasmic membranes adhere to each other at 200 to 400 localized sites (1). This adhesion zone is reported to be involved in infection by phages, including T4 (2, 4). Obviously, even in the light of our model (Fig. 5), an adhesion zone is a preferred site for the core insertion and DNA injection to occur sequentially, provided that a phage particle that lands on the cell surface can move to this zone, as suggested by Bayer and Bayer (3). However, we have shown in previous papers that every part of the cell surface has the ability to induce irreversible T4 infection to the stage of core insertion (stage III) (9) and the core tip that has penetrated the cell surface interacts with a phospholipid bilayer as if the tip was sucked up the bilayer, thus forming a junction which is similar to the adhesion zone (8). If these facts are taken into consideration, it would be rather difficult for a phage that had already inserted the core through

the cell surface to move around to reach a preexisting adhesion zone. Fluorescent microscopic observation showed that the phage DNA injection takes place right after the stable landing of a phage particle onto the host cell surface (data not shown). Therefore, we would prefer the model shown in Fig. 5, in which T4 infection results in the formation of a "pseudoadhesion" zone between the two membranes in the presence of a membrane potential. However, it should be noted that at present there is no direct evidence for the energy-induced interaction of the outer and cytoplasmic membranes.

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