Structural Changes in *Escherichia coli* Membranes Induced by Bacteriophage T4 at Different Temperatures

Y. S. Tarahovsky,* A. A. Khusainov,* R. Daugelavichus,‡ and E. Bakene‡
*Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino 142292 Russia, and ‡Vilnius University, Vilnius, Lithuania

ABSTRACT This paper presents some further evidence for our model of DNA translocation into *Escherichia coli* cells by bacteriophage T4 (see Tarahovsky, Y. S., Khusainov, A. A., Deev, A. A., Kim, Y. V. 1991. *FEBS Lett.* 289:18–22). When lowering the temperature, we succeeded in slowing down the infection process and in observing a few separate stages by electron microscopy. Also, potassium leakage at different temperatures was measured. At 0–6°C the phage was found to be irreversibly adsorbed on the cell surface, its tail to be contracted, and the outer membrane to be invaginated. Membrane fusion and formation of broad intermembrane bridges with a hole for potassium leakage were shown to start above 7°C. At about 17–20°C the diameter of the bridge decreased considerably, which could correspond to the sealing of the membrane.

INTRODUCTION

Early events of phage T4 interaction with *Escherichia coli* cells are well known (Simon and Anderson, 1967). The process begins with adsorption of virus particles onto the bacterial surface. After a primary contact, the phage particles are supposed to bind to certain parts on the cell surface, so-called adhesion zones, as a result of lateral diffusion (Bayer, 1968). It was shown that adhesion zones between the inner and the outer membranes of gram-negative bacterial cells were preferential sites for adsorption of T-even phage particles and subsequent DNA injection (Bayer, 1968; Bayer and Bayer, 1981).

The injection of DNA of T-even bacteriophages into the cell correlates with drastic structural changes in both phage particles and cell membranes. According to Simon and Anderson (1967) and Furukawa et al. (1979), the tip of the phage core penetrates both the outer membrane and the peptidoglycan layer of periplasmic space and intimately interacts with the cytoplasmic membrane or, possibly, penetrates it. The infection of *E. coli* by T-even phages is accompanied by a transient increase of permeability of the cytoplasmic membrane for ions and small solutes, the leakage reaction (Puck and Howard, 1954, 1955). The increase of permeability has been suggested to correspond to formation of localized lesions (Goldberg, 1980). A few minutes after the infection has begun, the leakage is sealed (Puck and Howard, 1955). As a matter of fact, these findings lay a foundation of the "syringe" model and "hole-theory" of infection by T-even bacteriophages (Simon and Anderson, 1967; Goldberg, 1980).

We have suggested another mechanism of phage DNA injection (Tarahovsky et al., 1991). The electron microscopy permitted us to assume the possibility of a direct intermembrane interaction with a subsequent fusion between outer and inner membranes during infection. Relying on numerous micrographs, we supposed that the phage core induced the outer membrane invagination and its approach to the cytoplasmic membrane with subsequent intermembrane interaction and fusion. Fusion should result in formation of a large hole and should be accompanied by leakage. Then the leakage should decrease as a result of a decrease of the bridge diameter and interaction of phage core with hydrophobic region of phospholipid bilayer. However, the above process was only suggested hypothetically. We had no direct evidence that the outer membrane invagination and fusion with the inner membrane precede the appearance of intermembrane bridges. In this work, we present the results of slowdown of infection of *E. coli* cells by bacteriophage T4 and describe a direct observation of different stages of the formation of the intermembrane bridges between the outer and inner cell membranes.

MATERIALS AND METHODS

Bacteria

E. coli B and K-12 strain AN180 were used. *E. coli* K-12 strain AN180 (F^-, argE3, thi, mtl, xyl, sir704) was kindly provided by Prof. F. Gibson (National University of Australia). The cells were grown to 5×10^8 cells per ml either in nutrient broth or in liquid salt medium M9 (pH 7.0) containing 38 mM Na_2HPO_4, 22 mM KH_2PO_4, 19 mM NH_4Cl, 8.5 mM NaCl, 1% kasein hydrolysate, 0.4% succinate, and thiamine hydrobromide (5 mg/ml). Night cultures of cells were diluted 20 times with fresh medium, and cell suspension was incubated for 14–16 h on a gyratory shaker at 37°C. After the growth had reached its stationary phase, the cells were pelleted at 4°C (Grinius and Daugelavichus, 1988).

Phage

The wild-type T4D was grown and purified by the standard procedures described earlier (Khusainov et al., 1992).

Received for publication 10 February 1994 and in final form 17 October 1994.

Address reprint requests to Dr. Yury S. Tarahovsky, Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino Moscow Region, 142292, Russia. E-mail: tarahovsky@venus.iteb.serpukhov.su.

0006-3495/95/01/157/07 $2.00

Bacteriophage adsorption

After being grown, the cells were harvested, washed twice, and resuspended in a fresh nutrient broth that served as an adsorption medium. The cell concentration was determined by measuring the optical density of cell suspension. The calibrating curve of the optical density was determined by microbiological titration of cell suspensions. For measurements of the phage adsorption, a cell concentration range of 1×10^9–5×10^9 cells per ml was used. The procedure of measurements of irreversible adsorbed phage particles has been described in detail earlier in our work (Khusainov et al., 1992).

Assay of K^+ fluxes

The harvested cells were washed twice at 4°C with a medium containing 5 mM sodium salt of MOPS and 0.1 M NaCl (pH7.0). About 2–3 $\times 10^{11}$ viable cells were resuspended in 1 ml of this medium. The suspension was kept on ice and used within 3 h just after preparation. K^+ activity in incubation medium was registered with K^+-selective electrode EM KO1. The electrode was connected with J-115 ionometer (EV-1 M3 Ag/AgCl was used as a reference electrode). The electrode was connected with the incubation medium by 3% agar bridge, containing 20% choline chloride. About 50 ml of bacterial cell suspension was added into a 5 cm^3 glass vessel with a water jacket connected with ultrathermostate. The suspension was continuously stirred by magnetic stirrer. In our equipment after addition of definite quantities of 0.1 M KCl (calibration) the response lasted for 2–3 s. Apparent values of cytoplasmic potassium were calculated with the assumption that 1 g of cell dry weight was equal to 1.5 cm^3 of intracellular water (Grinius and Daugelavichius, 1988). To estimate the total cytoplasmic potassium content, the cation was released from the cells by addition of polymyxin B (320 units per ml) at 37°C.

Electron microscopy

For electron microscopy, we used *E. coli* B and K-12 strain AN180 cells at a concentration of 5×10^9 cells per ml in nutrient broth. Phages and cells were equilibrated at corresponding temperatures for 20 min before mixing. The phage was added to cell suspension with different multiplicity of infection (50–200) and incubated for some temperature-dependent time. At 0–10°C the time was about 1.5–3 h, at 10–15°C it was 30 min, and above 15°C it was 10 min. Then the suspension was fixed by 2.5% glutar aldehide in 100 mM phosphate buffer (pH 7.4) during 10 min and postfixed overnight in 2.5% glutar aldehide. Then the cells were fixed by 1% osmium tetroxide for 1 h, by 1% tannic acid for 1 h, and again by 1% osmium tetroxide for 1 h (GOTO-fixation) as described earlier (Tarahovsky et al., 1991). After every stage of fixation, the cells were washed 5 times with 100 mM phosphate buffer (pH 7.4). All procedures of fixation and washing were performed upon a discontinues mixing of the solution by a slow rotation of flakes. The fixed cells were dehydrated by alcohol and acetone and embedded in Epon 812 resin. Ultrathin sections about 500 Å thick were stained with uranil acetate and lead citrate. The ultrastructural analysis was performed by JEM 100B electron microscope.

RESULTS

The irreversible adsorption of phage T4 on *E. coli* cells strongly depends upon temperature (Gamov, 1969). As it is seen in Fig. 1 *A*, in the temperature range of 4–8°C a small number of phage T4 particles (5–15%) can be irreversibly adsorbed on the surface of cells. The number of irreversibly adsorbed particles starts markedly increasing upon an increase of temperatures higher than 8–10°C. It should be noted that the temperature dependence shown in Fig. 1 *A* is typical for the entire time interval, in which the experiments of this work were carried out.

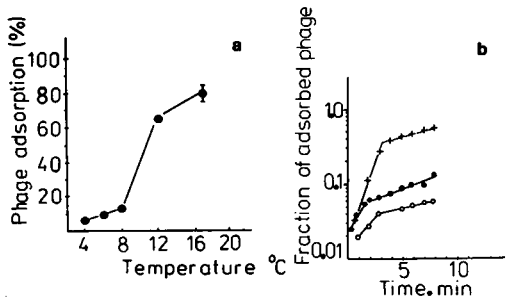

FIGURE 1 (*A*) Temperature dependence of the irreversibly adsorbed hage T4D on the surface of host cells 8 min after mixing. (*B*) The time course of irreversible adsorption of bacteriophage T4 at different temperatures: (□) −4°C; (■) −8°C; (+) −12°C. The cells (*Escherichia coli* AN180) were infected by phage T4 at an infection multiplicity of 2.

Earlier, some works were dealing with the study of the dependence of irreversible adsorption on the infection time at different temperatures (Gamov, 1969). It was shown that for phage T4 there were two inclinations of the time-dependent adsorption curve within 0–18°C. Fig. 1 *B* depicts typical curves of the irreversible adsorption kinetics for this temperature range, which have been obtained in our experiment. To explain this dependence of adsorption kinetics, Gamov (1969) has advanced the idea about the presence of active (quickly adsorbed) and inactive (slowly adsorbed) phage T4 particles. It was suggested that there could be some dynamic equilibrium between quickly and slowly adsorbed phage T4 particles, which is shifted upon a temperature change. Later on, the phage activity was assumed to be exactly related to unfolding of long fibers. Such an assumption was based on the results obtained from the study of the temperature dependence of unfolding of phage T4 long fibers (Greve and Block, 1975) and irreversible adsorption efficiency on the number of long fibers simultaneously bound with the receptors (Goldberg, 1983). In our opinion, the inclination in the initial part of the curves represented in Fig. 1 *B* reflects the exit of the system into a quasi-stationary state after phage has been mixed with the cells. The temperature-dependent adsorption value indicates that at temperatures close to 0°C a relatively small part of the phage population is capable of being irreversibly adsorbed on the cellular surface within the time used in our experiments. The presented results suggest that even at physiologically low temperatures there are particles of phage T4 capable of right irreversible binding to the "host" cells and, possibly, of infecting the cells. This allows us to suppose the interaction between phage and cell to occur at low temperatures just in the way as it does at normal physiological ones. But in this case all the processes are essentially slowed down.

The processes of irreversible adsorption of phage T4 onto *E. coli* cells and those of translocation of its DNA into cytoplasm are related to changes in permeability of the cellular membranes. In connection with that, we studied the temperature dependence of the exit of potassium ions out of the cell after their interaction with phage T4. Fig. 2 represents a change of the potassium content in the measured cell in a

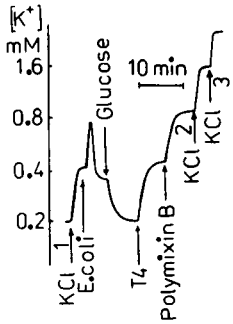

FIGURE 2 The diagram of a typical registration course of potassium fluxes induced by glucose and phage T4 as it was measured by potassium selective electrode. 5 ml of incubation medium (pH 7.0, 37°C) contained 5 mM Mops, 100 mM NaCl, and 0.2 mM KCl. The additions marked with arrows were as follows: 1 mM (1) or 3 mM (2 and 3) KCl; 2 mg of glucose; 1×10^{10} per ml *Escherichia coli* AN 180 cells; phage T4D with multiplicity of 2 infective phage per cell and 1600 units (0.2 mg) of polymyxin B sulfate.

typical experiment. The curve demonstrates the sequence of different additions into the measured cell and the subsequent typical changes of potassium content in the medium. The measurements allowed us to estimate the temperature dependence of such parameters of the potassium exit out of cytoplasm as the initial rate and the lag-period. The lag-period before the potassium release from cytoplasm is usually observed after addition of phages to cell suspension, Fig. 3. It strongly depends on the temperature and sharply increases below 14°C. Below 6–7°C it tends to infinity. This is the lowest temperature, at which the potassium release from cytoplasm of bacterial cells is registered even after infection within several hours. This is in agreement with the results of Grinius and Daugelavichus (1988). A strong increase of the lag-period below 14°C likely explains the absence of the potassium release in the studies performed by some authors (Boulanger and Letellier, 1989).

The Arrhenius representation of the initial rate of potassium efflux from cell cytoplasm induced by phage infection has a complex character (Fig. 4). The weak potassium efflux only starts at the temperatures above 6–8°C. In the absence of glucose, the efflux rapidly increases at the temperatures

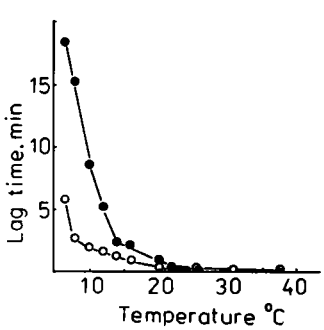

FIGURE 3 Temperature dependence of lag-period defined as a duration after addition of phage particles and before beginning of potassium leakage. Experimental conditions were identical to those described in the legend to Fig. 2. The measurements were performed in the absence (●) and in the presence of glucose (○).

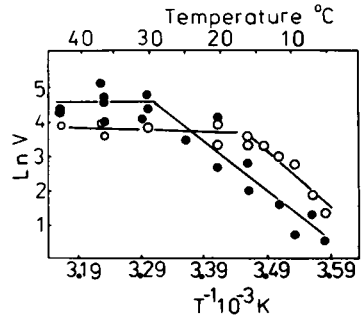

FIGURE 4 Arrhenius representation of the rate of T4-induced K^+ efflux in the absence (●) and in the presence (○) of glucose. Experimental conditions were identical to those described in the legend to Fig. 2. The rate of potassium efflux is expressed in nmol min^{-1} mg^{-1}.

above 10–12°C. At 30°C the discontinuity of the slope of the Arrhenius representation of potassium efflux is observed, which corresponds to the data obtained by other authors (Boulanger and Letellier, 1989). In the presence of glucose, the discontinuity of the slope is detected at a temperature interval of 15–20°C. This bears witness to an essential influence of the energy source on the temperature dependence of plasma membrane permeability value of infected cells.

The ultrastructural study reveals a valid adsorption of few phage particles onto the cell surface even at 0°C (Fig. 5, *A* and *B*). At this temperature, the adsorbed bacteriophages are able to contract the tail and invaginate the outer membrane, which causes its interaction with the inner membrane. Such an interaction of the cell membranes never initiates the membrane fusion at low temperatures even after a joint incubation of phages and cells for some hours. The structural changes typical for the fusion process could be firstly observed only above 6–7°C. An example of structural changes, like semifusion of two membranes, results in formation of one bilayer presented in Fig. 5, *C* and *D*. At a higher temperature, the complete membrane fusion and creation of intermembrane bridges are rather typical (Fig. 6). Formation of the first few bridges can be observed at 7–8°C, and above 10°C they are spread rather widely. At these temperatures, the diameter of the bridges considerably exceeds the phage core and the diameter of bridges observed by us earlier (Tarahovsky et al., 1991). The diameter, which is most often observed, is about 14–15 nm. The narrow intermembrane bridges with an inner diameter corresponding to that of the phage core could be only observed above 20°C. Probably, the formation of the narrow bridges results from an immediate interaction between the phage core and the membrane. This prevents the ion leakage as we have suggested earlier (Tarahovsky et al., 1991).

It is noteworthy that the thickness of sections was about 500 Å, which considerably exceeded the sizes of the observed intermembrane bridges and invaginations of the outer membrane. These structures could be involved, therefore, in a single epon section. This permitted us to see the side projection of structures on the whole. Indeed, if such small structures were cut, their contrast should have substantially decreased because a part of the material would have been lost.

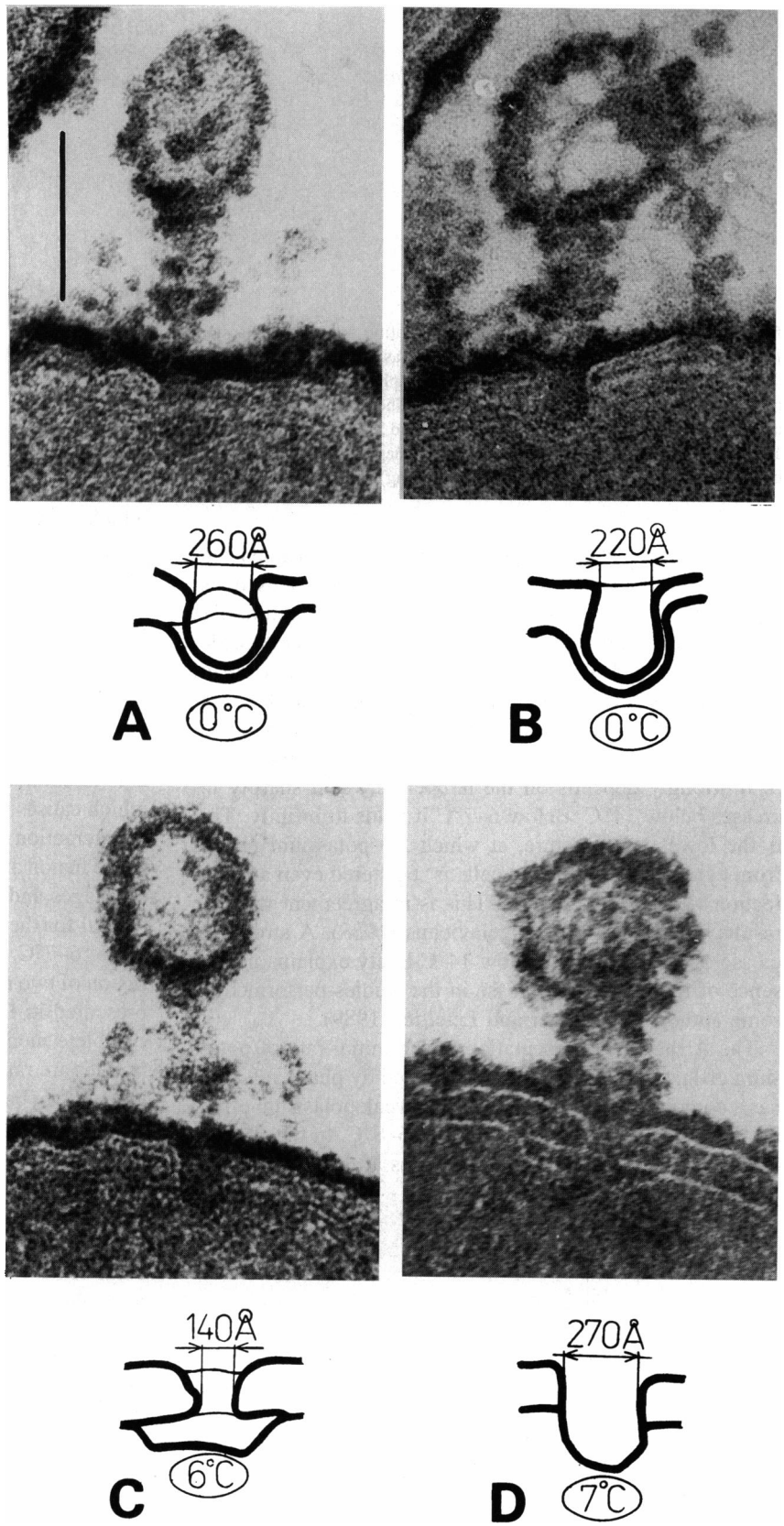

FIGURE 5 Ultrastructural changes in *Escherichia coli* membranes induced by phage T4D at temperature interval 0–7°C. Here and later *Escherichia coli* B and AN 180 cells were used in the experiments. Phage T4 was added at a multiplicity of 100. Under each of the micrographs, the schematic illustrations of membrane structures induced by phage are presented. The bar represents 1000 Å.

On the contrary, the presented micrographs demonstrate the examples of the most contrast and most appropriately oriented objects in the epon section plane. Therefore, a greater part of the material of structures observed should be involved inside the section. In this case, the errors of identification of membrane structures and, to some

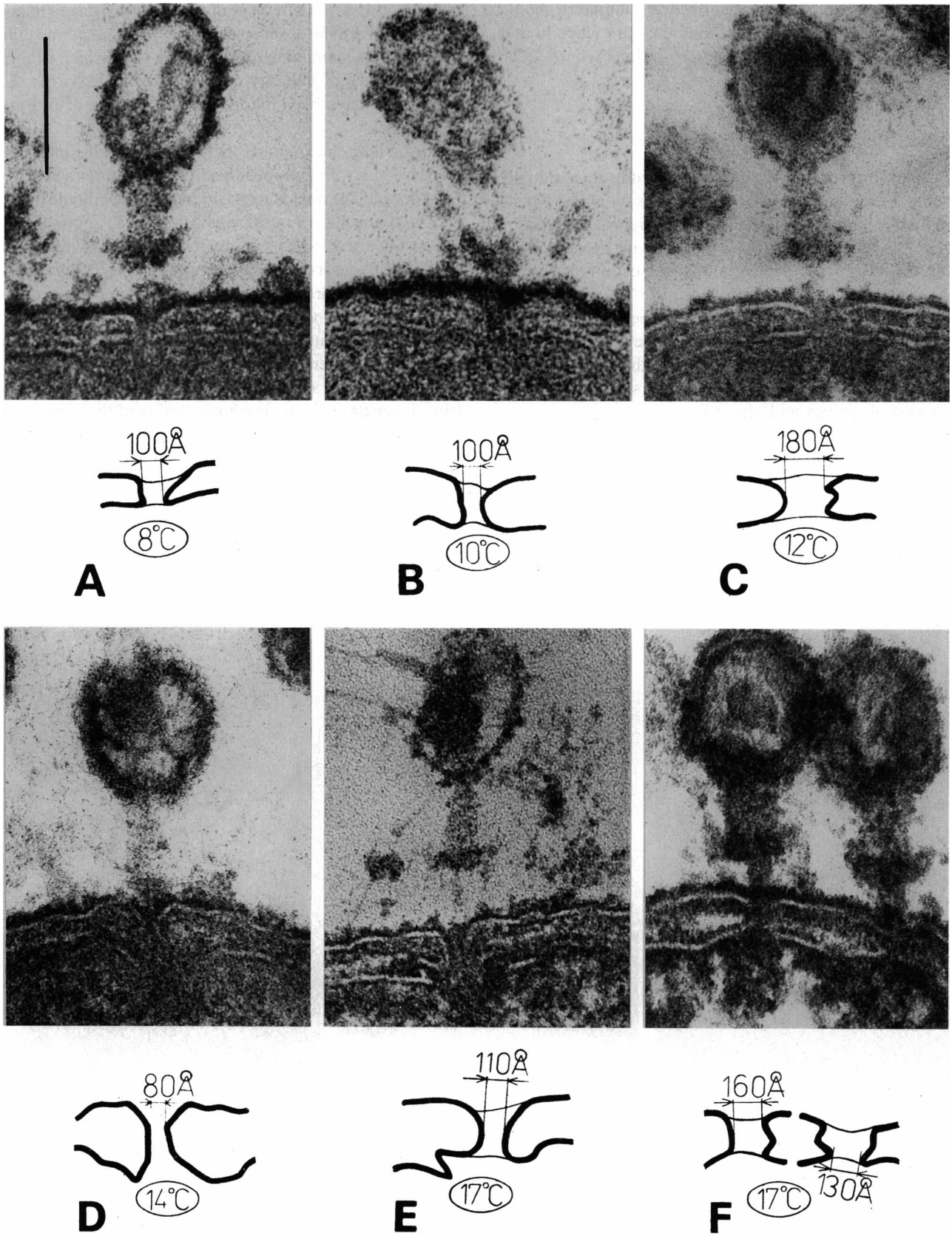

FIGURE 6 Wide pores in *Escherichia coli* envelope induced by phage T4 at temperature interval 8–17°C. The details are in the description to Fig. 5.

degree, those of measurements of their diameters should be considerably less sufficient than they are when bigger objects are analyzed in other investigations where serial sections are mainly used.

DISCUSSION

The use of lowered temperatures permitted us to essentially slow the infection of *Escherichia coli* cells by phage T4 and to apply this phenomenon for observing individual infection stages. The temperature was revealed to influence considerably the adsorption of phages onto the cellular surface.

When the temperatures were close to 0°C, the adsorbed phages were still capable of contracting the tail and ejecting the DNA molecule. This process was, however, substantially slower at lowered temperatures, and the ultrastructural changes presented by us could only be seen after a long incubation of phages with the cells.

When the temperatures ranged from 0 to 6°C, there were few phages able to be irreversibly adsorbed on the cellular surface. In most cases, adsorption led to the tail contraction and to the outer membrane invagination. Despite an approach of inner and outer membranes, and despite formation of a direct contact between them, we have never seen the pictures suggesting a fusion of the membranes. In this case, we often saw phages with empty heads. This is testimony to the possibility of injecting DNA into the pocket formed by the outer membrane as a result of invagination. These data are in good agreement with the absence of phage effect on permeability of the plasma membrane for potassium ions, which suggests a maintenance of integrity of the hydrophobic barrier. The latter could have been hardly possible in case of fusion of membranes.

Fusion of the membranes and formation of the intermembrane bridges were only observed at temperatures above 7°C. Here it should be noted that the broad intermembrane bridges we saw in the temperature range from 8°C to approximately 17–20°C had a considerably greater diameter than the narrow ones we had observed at temperatures above 20°C. (Fig. 7). The diameter of broad bridges seen at lowered temperatures could essentially vary, as shown in Fig. 6. Yet the chief distinguishing feature of narrow bridges is that their inner diameter is smaller than the diameter of phage core. Earlier, this allowed us to assume the possibility for phage core proteins to penetrate the hydrophobic region of the surrounding membrane (Tarahovsky et al., 1991). This could result in formation of a specific intermembrane structure necessary for sealing the plasma membrane. Unfortunately, the measurements of only initial rates of potassium release from the cytoplasm carried out in our work did not permit an identification of sealing. However, the Arrhenius curve (Fig. 4) showed a discontinuity within 15–20°C for cells in the presence of glucose and within about 30°C in the absence of energy sources. This suggested the possibility of a structural

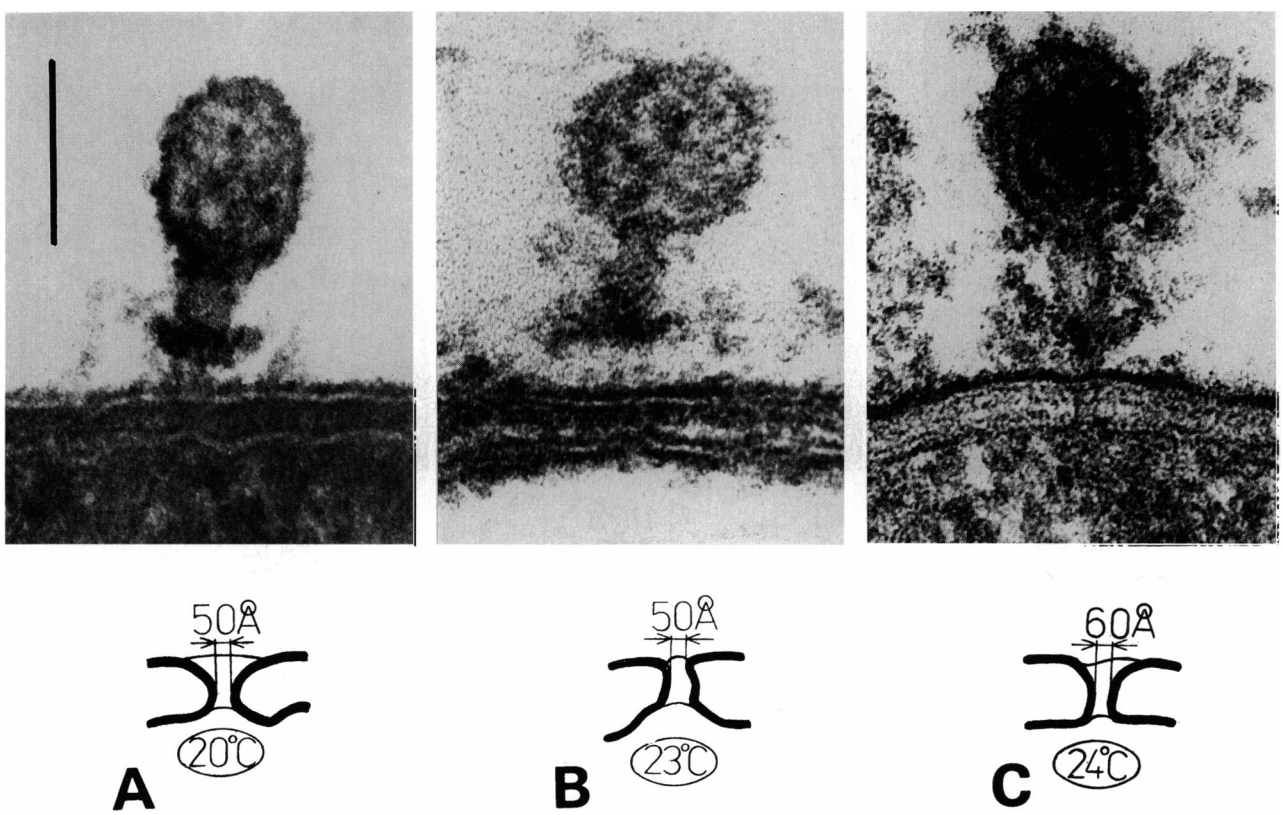

FIGURE 7 Narrow pores in the envelope of *Escherichia coli* cells treated by phage T4 at temperatures above 20°C. The details are in the description to Fig. 6.

reorganization of the channel responsible for ionic leakage. At these temperatures, a structural reorganization of lipids of the plasma membrane is known as well (Lugtenberg and Van Alphen, 1983). Some considerable differences in diameter of intermembrane bridges observed in the media rich in energy sources at above and below 15–20°C can be supposed to correlate with the corresponding discontinuity of the Arrhenius curve at these temperatures.

We thank Dr. K. A. Dawson, University college of Dublin, Ireland for his helpful suggestions and valuable discussions. We also thank Dr. V. Alekseenko for assistance in K^+ efflux measurements. The technical and secretarial help of A. S. Kharitonov is also acknowledged.

This work was supported by Russian Foundation of Fundamental Investigations grant 93–04–22040 and partially supported by grant INTAS-93-2084.

REFERENCES

Bayer, M. F. J. 1968. Adsorption of bacteriophages to adhesions between wall and membrane of *Escherichia coli*. *J. Virol.* 2:346–356.

Bayer, M. F. J., and M. H. Bayer. 1981. Fast responses of bacterial membranes to virus adsorption: a fluorescence study. *Proc. Natl. Acad. Sci. USA.* 78:5618–5622.

Boulanger, P., and L. Letellier. 1989. Characterization of ion channels involved in the penetration of T4 DNA into *Escherichia coli* cells. *J. Biol. Chem.* 263:976–9775.

Furukawa, H., H. Yamada, and S. Mizushima. 1979. Interaction of bacteriophage T4 with reconstituted cell envelopes of *Escherichia coli* K12. *J. Bacteriol.* 140:1071–1080.

Gamov, R. I. 1969. Thermodynamic treatment of bacteriophage T4B adsorption kinetics. *J. Virol.* 4:113–115.

Goldberg, E. B. 1980. Bacteriophage nucleic acid penetration. *In* Receptors and Recognition, Series B, Vol. 7. L. L. Randall and L. Phillipson, editors. Chapman and Hall, London. 115–141.

Goldberg, E. 1983. Recognition, attachment and injection. *In* Bacteriophage T4. C. K. Mathews, E. Kutter, G. Mosig, and P. B. Berget, editors. American Society for Microbiology, Washington, D.C. 32–39.

Greve, J., and J. Block. 1975. Transient electric birefringence of T-even bacteriophages. II. T4B in the presence of tryptophan and T4D. *Biopolymers.* 14:139–154.

Grinius, L., and R. Daugelavichus. 1988. Depolarization of *Escherichia coli* cytoplasmic membrane by bacteriophage T4 and lambda: evidence for induction of ionpermeable channels. *Bioelectrochem. Bioenerg.* 19:235–245.

Khusainov, A. A., G. V. Shilnikov, V. E. Emelyanenko, and G. R. Ivanitsky. 1992. Effect of thermoinduced changes in T4 bacteriophage structure on the process of molecular recognition of host cells. *Biochim. Biophys. Acta.* 1118:211–217.

Lugtenberg, B., and L. Van Alphen. 1983. Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other Gram-negative bacterium. *Biochim. Biophys. Acta.* 737:51–115.

Puck, T., and H. L. Howard. 1954. Mechanism of cell wall penetration by viruses. I. An increase in host cell permeability induced by bacteriophage infection. *J. Exp. Med.* 99:481–494.

Puck, T., and H. L. Howard. 1955. Mechanism of cell wall penetration by viruses. II. Demonstration of cyclic permeability change accompanying virus infection of *Escherichia coli* B cells. *J. Exp. Med.* 101:151–175.

Simon, L. D., and T. F. Anderson. 1967. The infection of *Escherichia coli* by T2 and T4 bacteriophage as seen in the electron microscope. I. Attachment and penetration. *Virology.* 32:279–297.

Tarahovsky, Y. S., A. A. Khusainov, A. A. Deev, and Y. V. Kim. 1991. Membrane fusion during infection of *Escherichia coli* cells by phage T4. *FEBS Lett.* 289:18–22.