Characterization of Ion Channels Involved in the Penetration of Phage T4 DNA into Escherichia coli Cells*

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The hypothesis of a channel-mediated transport of phage DNA into Escherichia coli cytoplasmic membrane has been formulated for a long time. In this paper, we present experimental evidence in favor of this proposal. We have analyzed the kinetics of the K⁺ efflux induced by T4 phage and ghosts (phage depleted of DNA) using a potassium selective electrode. We show that the K⁺ efflux is not catalyzed by the K⁺ transport systems. The K⁺ of K⁺ efflux is the same for phage and ghosts. The rate of K⁺ efflux is linearly related to the multiplicity of infection. This suggests that phage and ghosts induce the formation of similar channels and that one channel is induced by one virion. The K⁺ efflux is associated with an influx of H⁺ and Na⁺ or Li⁺ which compete for entry through the channel. These ion fluxes may be responsible for the cell depolarization. The phage-induced channels allow the passage of DNA. They are only transiently opened, and their closing leads to cellular repolarization. The ghost-induced channels remain open. The insertion and conformation of the channels in the membrane depend on the temperature and their confirmation is voltage-dependent. We give an estimate of their size.

Despite an abundant literature, the mechanism of penetration of phage DNA into bacterial cells remains only partially understood. The first events which lead to the injection of phage T4 DNA into Escherichia coli cells are well characterized; they involve the binding of the phage tail fibers to the outer membrane receptor (the lipopolysaccharide in the case of E. coli B cells), the contraction of the tail, the penetration of the internal tube through the outer membrane, and the ejection of phage DNA from the capsid (1). On the other hand, very little is known about the mechanism by which the large hydrophilic DNA molecule crosses the cytoplasmic membrane. Several models have been proposed. In the case of phage T4 and T1, the requirement of the electrochemical gradient of protons ΔpH for injection of phage DNA (2-5) has led to the proposal that phage DNA would enter the cytoplasmic membrane in symport with protons (3,5). Yet, this hypothesis did not fit in with the experiments described by Filali Maltouf and Labedan (6, 7) who showed that phage T5 and λ inject their DNA into bacteria deprived of ATP and in the absence of ΔpH. These authors thus proposed a different but general scheme of DNA traversal: phage DNA would cross the cytoplasmic membrane by facilitated diffusion through specific pores or channels; the configuration or opening of these channels would depend on ΔpH in the case of phage T4 (6, 9).

The hypothesis of a channel-mediated transport of phage DNA has also been proposed by several authors who observed that different phage (T1, T4, T5, T7) caused changes in the permeability of the E. coli cytoplasmic membrane towards potassium (5, 10-15).

In this paper, we present experimental evidence in favor of the existence of these channels. For this purpose, we have analyzed and compared the kinetics of the early potassium fluxes induced by T4 phage and ghosts (i.e. phage depleted of DNA and internal proteins) under various experimental conditions. We have used a valinomycin-potassium-selective electrode which allows a direct measurement of the cation fluxes and avoids the drawbacks associated with the use of filtration and centrifugation techniques. The study further focuses on the ionic selectivity, voltage dependence, and configuration of these channels. Finally we give a rough estimate of their size.

EXPERIMENTAL PROCEDURES

Phage and Ghosts Preparation—Phage T4 was prepared and titrated as described in Ref. 16. T4 ghosts were prepared from the phage and tittered according to Ref. 17.

Growth of Bacteria and EDTA Treatment—E. coli B and B 525 were grown at 37 °C in NT broth (16) and E. coli TK 2240 in minimal medium supplemented with glucose as described (18). The bacteria were grown to an OD₆₀₀ of 0.5 (5 x 10⁷ cells/ml), harvested, washed once, and resuspended in 100 mM sodium phosphate buffer (pH 7.2).

Cells were permeabilized towards the ΔpH probe (TPP⁺) by a Tris-EDTA treatment (19). To prevent extensive loss of potassium during this treatment, the cells (2 x 10¹⁰/ml) were incubated, at 37 °C, in 100 mM Tris-HCl (pH 7.8) in the presence of 20 mM KC1 and exposed to only 0.2 mm EDTA for 3 min; they were then diluted 10-fold in 100 mM sodium phosphate buffer, centrifuged, and resuspended in the same buffer.

Intact and EDTA-treated cells were kept on ice at a density of 5 x 10¹⁰ cells/ml and used within the 2 h following the preparation.

Modification of the Intracellular Concentration of Potassium—For a partial depletion of internal potassium, the cells (1 x 10¹⁰/ml) were diluted 10-fold in ice-cold distilled water (20), maintained in water for 5 min, and then centrifuged 2 min at 5000 x g. They were resuspended in the experimental buffer at a concentration of 5 x 10¹⁰ cells/ml and used immediately. For a complete depletion of potassium, the shocked bacteria were maintained in ice-cold water for 1 h.

To increase the intracellular potassium concentration above 400 nmol/mg dry weight, the cells (2 x 10¹⁰/ml) were incubated in sodium phosphate buffer (pH 7.2) containing glycerol, 0.5 mM KCl, and variable concentrations of NaCl to change the osmolarity of the medium (21).

Determination of the Potassium Content of the Cells—The variations in the potassium content of the cells (K₉) were determined by

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The abbreviations used are: ΔpH, electrochemical ion gradient for H⁺; m.o.i., multiplicity of infection; m.o.k., multiplicity of killing; TCS, 3,3',4',5-tetrachlorosalicylanilide; TPP⁺, tetraphenylphosphonium ion.
measuring the changes of the potassium concentration in the external medium (K\textsuperscript{ext}) with a potassium-valinomycin-selective electrode (radiometer) associated with a calomel reference electrode containing a secondary salt bridge filled with a solution of 100 mM NaCl. The cation electrode potential was calibrated with KCl solutions of known concentrations, which were prepared in the experimental buffer.

To estimate the total potassium content of the bacteria, the cation was released from the cells either by osmotic shock as described above or by addition of colicin E1 (1 \( \mu \)g/ml) (22). The amount of released cation was then estimated with the potassium electrode. Both techniques of potassium release led to similar values of internal potassium. This was taken as an indication that the release of potassium was complete. K\textsubscript{c} was calculated from the value of K\textsubscript{t} and expressed in nanomoles/mg cell dry weight (nmol/mg\textsuperscript{-1}) assuming that the vessel containing cells correspond to 1 mg cell dry weight/ml.

Measurement of the Cytoplasmic Volume of \( \Delta \phi \) and \( \Delta \psi \) was determined from the accumulation of \(^{38}\text{Cl}\text{TPP}^+\) (10 \( \mu \)M, final concentration, 2.17 GBq/mmol) (23). The EDTA-treated cells were filtered on glass fibers filters. \(^{38}\text{Cl}\text{TPP}^+\) uptake was corrected for unspecific binding by subtracting a blank obtained under identical conditions except that the cells were pretreated either with the protonophore TCS (10 \( \mu \)M, final concentration) or with colicin E1.

\( \Delta \phi \) was estimated from the distribution of \(^{14}\text{C}\) benzole (10 \( \mu \)M, 19.7 MBq/mmol) in the presence of \(^{38}\text{H}2\text{O}\) (0.11 MBq/ml) and using centrifugation (23).

The cytoplasmic volume was determined with \(^{38}\text{H}2\text{O}\) (0.11 MBq/ml) and \(^{14}\text{C}\) sucrose (0.3 \( \mu \)M, 20 GBq/mmol) according to Ref. 24 and using centrifugation.

Lysis of the infected cells occasionally occurred during the centrifugation leading to an incorrect estimation of the cytoplasmic volume and of \( \Delta \phi \). We thus checked for released material by comparing the OD of a similar bacterial suspension which was lysed by adding 1 M NaOH prior to the centrifugation. The values of the cytoplasmic volume were obtained under conditions where no more than 5% of the bacteria were lysed.

Monitoring of the External pH Changes—The experiments were performed according to the principle described by West and Mitchell (25). Bacteria (2 \( \times \) 10\textsuperscript{9} cells/ml) were added to a closed and thermostated vessel containing 5 ml of 1 mM sodium phosphate buffer, 100 mM NaCl, carbonic anhydrase (25 \( \mu \)g/ml), and sodium iodoacetate (1 mM). The pH was adjusted to the desired value with 100 mM NaOH. The suspension was stirred and bubbled with a stream of \( \text{N}_2 \), and the experiment was started when the suspension had become completely anaerobic. pH titration curves were obtained by adding standard solutions of NaOH or HCl to the experimental buffer.

Measurement of Oxygen Consumption—Oxygen consumption was measured simultaneously with potassium efflux by using a Clark electrode connected to a Gilson oxygraph.

Measurement of the Lysosome Activity of Infected Cells—The cells (5 \( \times \) 10\textsuperscript{9} cells/ml) were suspended in the appropriate buffer, infected at 37 °C, and at m.o.i. = 4. Five min later, they were centrifuged, concentrated in 50 mM Tris (pH 7.8), and sonicated. After centrifugation, the lysosome activity of the supernatant was measured using chloroformed E. coli B cells as substrate (26).

RESULTS

Experimental Evidences in Favor of the Existence of Channels

Kinetics of Potassium Efflux Induced by Phage and Ghosts—Freshly prepared E. coli B cells retained 420 ± 20 nmol of K\textsuperscript{+} cell dry weight. When these cells were kept concentrated on ice they lost potassium, but at 37 °C, in the presence of an energy source and 0.5 mM KCl, they reaccumulated potassium at an initial rate (70 nmol·mg\textsuperscript{-1}·min\textsuperscript{-1}) compatible with that of the K\textsuperscript{+} constitutive transport system TrkA (18), which was the only one present in these cells (Fig. 1). When the steady state was attained, phage T4 was added (m.o.i. = 3), a multiplicity at which 95% of the cell population was infected. Fig. 1 shows that the efflux of potassium occurred immediately after phage addition at an initial rate of 340 ± 20 nmol·mg\textsuperscript{-1}·min\textsuperscript{-1}. After 2 min, a new steady state was attained (K\textsubscript{c} = 150 nmol·mg\textsuperscript{-1}); this steady state was maintained for at least 6 min.

Phage depleted of DNA and internal proteins were prepared by submitting T4 phage to an osmotic shock (17). Although these ghosts have lost infectivity, they inhibit several bacterial functions and are able to kill the bacteria (17, 27). Fig. 1 shows that at equivalent multiplicity (m.o.k. = 3) the initial rate of potassium efflux was six times larger for ghosts than for phage (V\textsubscript{c} = 2 µmol·mg\textsuperscript{-1}·min\textsuperscript{-1}). Furthermore, the ghost-induced potassium efflux did not stop after 2 min, and the cells were almost irreversibly depleted of potassium after 4 min.

The kinetics of potassium efflux induced by T4 phage or ghosts was not modified upon treatment of E. coli cells with EDTA (data not shown).

The Initial Rate of Potassium Efflux Is Linearly Related to the Phage or Ghost Multiplicity—Since the initial rate of potassium efflux was too high to be estimated with accuracy when the multiplicity was higher than 5, we performed part of the experiments at 25 °C, a temperature at which T4 phage still injected its DNA (4). Under these conditions, the rate of efflux was constant for at least 20 s. Fig. 2 shows that the initial rate of potassium efflux increased linearly with the number of phage or ghosts and that the value of K\textsubscript{c} at steady state was also dependent on the number of virions (Fig. 2, inset).

Phage-induced Potassium Efflux Is Not Mediated by the Different K\textsuperscript{+} Transport Systems—E. coli possesses a constitutive (TrkA) and an inducible (Kdp) potassium transport system which catalyzes the exchange of cellular K\textsuperscript{+} with external K\textsuperscript{+} (20, 28). At steady state, the rate of K\textsuperscript{+} exchange and thus the rate of K\textsuperscript{+} efflux is maximum (40 and 15 nmol·mg\textsuperscript{-1}·min\textsuperscript{-1} for the TrkA and Kdp systems, respectively) (28, 29). Potassium retention mutants have also been isolated from E. coli cells; one of these strains (B 525) loses potassium spontaneously at an initial rate of 70 nmol·mg\textsuperscript{-1}·min\textsuperscript{-1} when transferred in a medium free of potassium (30). Table I shows that the rate of K\textsuperscript{+} efflux induced upon infection of these different strains by phage T4 was larger than the rate of efflux catalyzed by the K\textsuperscript{+} transport systems. These results indicate that a new route for potassium efflux has been opened upon T4 infection.
Characterization of DNA Channels in E. coli Cells

The Initial Rate of Phage and Ghost-induced K+ Efflux Depends on the Value of $K_w$ before Infection—Submitting E. coli cells to a gentle osmotic shock in cold water decreased $K_w$ from 420 to $150 \pm 30$ nmol·mg$^{-1}$. Upon addition of glycerol and limiting concentrations of potassium (0.1, 0.2, 0.35, and 0.5 mM KCl), the cells reaccumulated potassium by the TrkA system, and at steady state, the respective $K_w$ values were 225, 270, 280, and 360 nmol·mg$^{-1}$. $K_w$ was increased above 420 nmol·mg$^{-1}$ by incubating the cells in the presence of glycerol, 0.5 mM KCl, and various concentrations of NaCl (50, 100, and 200 mM) (21). Under these conditions, the respective values of $K_w$ were 435, 460, and 560 nmol·mg$^{-1}$, and the efficiency of phage adsorption and infection were not modified (data not shown). Attempts to increase $K_w$ above 560 nmol·mg$^{-1}$ severely impaired the infectious process. When the new steady state of internal potassium was attained, T4 phage or ghosts were added. Fig. 3 represents the double-reciprocal plots of the initial rate of potassium efflux as a function of the internal potassium concentration for various multiplicities. These plots were well fitted with a series of straight lines suggesting that the kinetics of potassium efflux can be described by an equation of the Michaelis-Menten form. The $V_{\text{max}}$ values were 1.7, 2.9, 4, and 8 µmol·mg$^{-1}$·min$^{-1}$ for m.o.i. = 2, 4, 5, and m.o.k. = 0.5, respectively. The $K_m$ was identical for phage and ghost ($K_m = 2 \pm 0.5$ M if one considers that uninfected cells have a cytoplasmic volume of $1 \pm 0.1$ µl·mg$^{-1}$); this suggests that they induce the formation of similar channels. Furthermore, since the rate of potassium efflux was linearly related to the multiplicity (see Fig. 2), it is reasonable to assume that each phage or ghost induces the formation of one channel. Fig. 1 shows that the phage-induced potassium efflux always ceased 2 min after the addition of the phage whatever the multiplicity. Furthermore, this steady state was defined independently of the functioning of the different influx systems (data not shown). On the other hand, the ghost-induced potassium efflux continued until complete depletion. This suggests that the phage-induced channel would

**Table 1**

Initiation of K⁺ efflux catalyzed by the different transport systems and induced by phage T4

<table>
<thead>
<tr>
<th>Strain</th>
<th>BTK2240</th>
<th>B635</th>
</tr>
</thead>
<tbody>
<tr>
<td>K⁺ transport system</td>
<td>TrkA</td>
<td>Kdp</td>
</tr>
<tr>
<td>Steady-state K⁺ exchange*</td>
<td>40</td>
<td>15</td>
</tr>
<tr>
<td>Phage-induced K⁺ efflux*</td>
<td>340</td>
<td>300</td>
</tr>
</tbody>
</table>

*The rates are expressed in nanomoles·mg$^{-1}$·cell dry weight·min$^{-1}$. *Infection at a phage multiplicity of 3.

From the experiments described in Fig. 2, we have calculated that at 25 °C, one bacterium infected by one phage or one ghost lost potassium at respective rates of $2.6 \times 10^4$ and $1.4 \times 10^5$ K⁺ s$^{-1}$. These rates are many orders of magnitude higher than one expects for passive efflux through the lipid bilayer (20 ions·s$^{-1}$)/cell if one assumes a permeability coefficient of $10^{-14}$ m·s$^{-1}$ (31)). These fluxes are also too high to be mediated by a mobile carrier-like valinomycin whose maximum turnover is $3 \times 10^6$ ions·s$^{-1}$ (32). On the other hand, these rates are close to the rate of potassium efflux through the gramicidin or colicin channels: $10^7$ and $10^8$ ions·s$^{-1}$, respectively (33, 34). This suggests that phage- and ghost-induced potassium efflux occur through channels.
be open only transiently, whereas the ghost-induced channel would remain open.

Ion Selectivity of the Channel: Relationship between Ion Movements and Membrane Potential Changes

The large efflux of potassium must be electrically compensated by a concomitant influx of cations or efflux of anions. At least two cations may fulfill this requirement, sodium and protons. Respiring E. coli cells generate an electrochemical gradient of protons:

\[
\frac{(\Delta \psi)n}{F} = \Delta \psi - \frac{(2,3RT/F)}{\Delta \text{pH}}
\]

where \(\Delta \psi\) represents the membrane potential difference (negative inside the cell) and \(\Delta \text{pH}\) the pH difference across the membrane (alkaline inside the cell). Thus, if the phage-induced channel is permeable to protons, one expects that its opening will tend to pull the protons back into the cytoplasm.

The same situation may prevail for sodium and lithium. Indeed, in energized cells, sodium is maintained at a very low internal concentration (a few millimolar) by a Na\(^+/\text{H}^+\) antiport (36, 37) which is also recognized by Li\(^+\) (38). Thus, if the external concentration of lithium or sodium is high, these ions should diffuse down their electrochemical concentration gradients upon channel opening.

Selectivity of the Channel towards Sodium and Lithium—If the efflux of potassium is accompanied by the influx of sodium, one expects that the amplitude of the sodium gradient will affect the rate of potassium efflux. The study of the influence of the external sodium concentration on the rate of potassium efflux is complicated by the fact that the adsorption of T4 phage or ghosts requires the presence, in the infecting buffer, of at least 50 mM NaCl (39). T4 phage and ghosts were thus preadsorbed during 15 min to a concentrated bacterial suspension maintained at 4 °C in a buffer containing 100 mM NaCl. Under these conditions the DNA was not injected, and the efflux of potassium was insignificant (data not shown).

The efflux of potassium was then initiated by rapid dilution of the cells into a buffer prewarmed at 37 °C and containing various concentrations of NaCl or LiCl and to which variable amounts of lactose (which is not transported in this strain) were added in order to maintain a constant osmolarity. Aliquots of these infected cells were taken and checked for irreversible adsorption which was higher than 90%.

Fig. 4 shows that the initial rate of phage-induced potassium efflux increased by a factor of 10 when the external sodium or lithium concentrations were raised from 10 to 100 mM. Above 100 mM NaCl or LiCl, the rate of potassium efflux remained constant. The same dependence of the initial rate of potassium efflux with the concentration of NaCl was found with ghosts.

Selectivity of the Channel toward Protons—We first checked for a change of \(pH_{\text{in}}\) by comparing the accumulation of \([^{14}C]\) benzoate in infected and uninfected cells. Results of six independent experiments indicated that the internal pH of E. coli cells incubated at pH 6.6 was irreversibly decreased from 7.60 ± 0.09 to 7.33 ± 0.09 upon infection by phage T4 at m.o.i. = 3. This decrease was not accompanied by a significant change of the cytoplasmic volume (1.10 ± 0.10 \(\mu\text{l} \cdot \text{mg}^{-1}\) before infection compared to 0.98 ± 0.10 \(\mu\text{l} \cdot \text{mg}^{-1}\) after infection). To correlate the change of \(pH_{\text{in}}\) with a proton influx, we have measured the kinetics of proton uptake from the changes of \(pH_{\text{out}}\) (25). Fig. 5A shows that the addition of phage T4 (m.o.i. = 6) resulted in a change in two steps of \(pH_{\text{out}}\) which first decreased by 0.008 pH unit during the first 15 s and then increased, indicating that protons had entered the cells. This proton influx ceased simultaneously with the potassium efflux, and the new steady state of \(pH_{\text{out}}\) was maintained at least for 6 min. Fig. 5A shows that the initial decrease of \(pH_{\text{out}}\) also occurred at 4 °C, i.e. when phage T4 was only adsorbed to the cells. However, this decrease was irreversible. Since the formation and/or opening of the channel does not take place at 4 °C (see below), we think that this decrease of \(pH_{\text{out}}\) is not related to the formation of the channel, but may be the consequence of a reorganization of the outer membrane due to phage adsorption.

On Fig. 5B, we have compared the initial rate of proton influx with the rate of potassium efflux for different multiplicities. Both fluxes were measured under anaerobiosis. This may explain why the rate of potassium efflux was smaller than the rate determined above (see Figs. 1 and 2). The rate of proton influx showed the same linear dependence with the multiplicity as the rate of potassium efflux. Fig. 5C shows that the initial rate of proton influx and the total number of protons translocated increased when the external pH was increased from 6.9 to 7.8. Over this range of external pH, the rate of potassium efflux was not modified (130 nmol·mg\(^{-1}\)·min\(^{-1}\)) (data not shown). Furthermore, the rate of proton influx was always smaller than the rate of potassium efflux. Fig. 5D shows that the initial rate of proton influx increased by a factor of 3 when the NaCl concentration was decreased from 100 to 20 mM.

The experiments described above indicate that the efflux of potassium is associated with an influx of sodium and protons. The rate of proton influx was shown to depend on the external NaCl concentration; this suggests that both cations compete for entry in the cell. As it seems unlikely that such a high proton influx occurs through the lipid bilayer, we think that protons also move through the channel. It is thus reasonable to assume that the competition between sodium and protons takes place at a site located on the channel.
Characterization of DNA Channels in E. coli Cells

Fig. 5. Protons translocation in an anaerobic suspension of E. coli infected by phage T4. Experimental conditions were described under "Experimental Procedures." A, kinetics of the change of pHout upon infection at m.o.i. = 6. The infection was performed either at 37 °C (—) or at 4 °C (---). B, effect of phage multiplicity on the initial rates of proton influx and of potassium efflux. Both fluxes were measured under anaerobiosis in a slightly buffered medium containing 100 mM NaCl. The preinfectious value of pHout was 6.870 ± 0.002. The initial rates were expressed in nanomoles mg⁻¹ min⁻¹. C, initial rate of H⁺ influx and total H⁺ translocated for different values of pHout. The cells were washed and concentrated in the buffer at the desired pH before the infection. The buffer contained 100 mM NaCl. Phage multiplicity = 6. The rate of proton influx was expressed in nanomoles mg⁻¹ min⁻¹ and the H⁺ translocated in nanomoles mg⁻¹. D, effect of the external NaCl concentration on the initial rate of H⁺ influx. The principle tof the experiment is identical to those described in the legend in Fig. 4. The experiment was performed at pH 7.1 and at a phage multiplicity of 6.

The experiments described in Fig. 6, A and B, indicate that the efficiency of the phage-induced repolarization decreased with the potassium content of the phage-infected cells. This was shown either by increasing the phage multiplicity which is known to decrease Kᵣ (see Fig. 2) or, for a given multiplicity, by changing the potassium content of the cells before the infection.

We have recently shown that the decrease of the potassium content of uninfected and energized E. coli cells was accompanied by a parallel decrease of the respiratory activity (44). In order to see if in infected cells the value of Kᵣ was related to the respiratory activity, we measured both parameters at various times after the infection and for different multiplicities. The respiratory activity started to decrease 10–15 s after the addition of phage and 1 min after a new steady state was attained whose level was dependent on the multiplicity: the higher the multiplicity, the lower the respiration. Fig. 7 shows that there was a unique linear relationship between Kᵣ and the rate of respiration in infected and uninfected cells. Since the functioning of the respiratory chain plays a central role in the generation and the maintenance of A⁺, we think that it is the degree of functioning of the respiratory chain which defines the state of repolarization of the infected cells.

The high external NaCl concentration (NaCl = 100 mM), the H⁺/K⁺ stoichiometry varied from 0.1 (pH 6.9) to 0.5 (pH 7.8). This suggests that protons alone cannot insure the electrical compensation of the potassium efflux and that sodium may be required to compensate the cation efflux. Anion efflux may well accompany potassium efflux; nevertheless, the chloride content of the cells (40) and the amounts of organic anions (pyruvate and glucose 6-phosphate) which may be lost by the cells (41) appear to be too small to allow the electrical compensation of potassium efflux.

Relationship between the Membrane Potential Changes Induced by Phage and Ghosts, Kᵣ, and the Respiratory Activity—We have previously shown that immediately after adsorption of phage T4, the E. coli cytoplasmic membrane becomes partially and transiently depolarized (19). Fig. 6A shows that this depolarization occurred at the same time as the H⁺ and K⁺ fluxes and ceased simultaneously with the cation fluxes. We thus think that the membrane potential changes are the consequence of the different ion fluxes and are related to the formation of the channel. In the preceding paragraph, we proposed that the arrest of potassium efflux would correspond to the closing of the channel. This closing may well explain the sudden arrest of the depolarization and the repolarization.

Ghosts also triggered the depolarization of the membrane, but this was not followed by repolarization (Refs. 42 and 43 and Fig. 6A). Furthermore, the cells were completely depolarized at m.o.k. = 3; this sustains the hypothesis formulated above that the ghost-induced channel would remain permanently open.

Effect of Temperature on the Initial Rate of Phage and Ghost-induced Potassium Efflux—Decreasing the temperature
Characterization of DNA Channels in E. coli Cells

Fig. 6. Membrane potential changes induced by T4 phage and ghosts. Δψ was measured on EDTA-treated cells and using [3H]TPP. A, effect of phage and ghost multiplicities. B, T4-induced Δψ changes upon infection at m.o.i. = 3 and for variable concentrations of K+ (nanomoles·mg⁻¹) before the infection. The bacteria were depleted of potassium by osmotic shock as described under "Experimental Procedures" and then infected in the absence of added potassium.

Fig. 7. Relationship between respiratory activity and K⁺ in infected and uninfected cells. The O₂ consumption and K⁺ were measured simultaneously at different times after the infection. Other experimental conditions were as described in the legend to Fig. 1. For uninfected cells, the O₂ consumption was measured on cells in which K⁺ content was varied as described under "Experimental Procedures." Δ, ○; ○ correspond to respective m.o.i. of 2, 3, 5, 8; △, uninfected cells.

decreased the rate of the potassium efflux induced by the phage and ghosts and increased the time required to initiate efflux: between 39 and 29 °C the efflux started immediately after the addition of the virion, below 29 °C the delay increased with decreasing temperature, and at 16 °C the efflux started only 2 min after addition of the virion. Below 14 °C, there was no significant efflux. Fig. 8 shows that the Arrhenius plot of the initial rate of potassium efflux induced by the phage and ghosts was well fitted by two straight lines: below 29–31 °C the rate of efflux was strongly dependent on temperature with a Q₁₀ higher than 10; above 29 °C, the rate of efflux was little dependent on temperature and the Q₁₀ was smaller than 1.5. The same dependence of potassium efflux with temperature was observed whatever the m.o.i. and m.o.k. (data not shown).

Labedan and Goldberg (4) previously determined the kinetics of penetration of the DNA of a T4 mutant as a function of the temperature by measuring the extent of the degradation of the injected DNA by a cytoplasmic exonuclease. Below 15 °C they observed little degradation of the DNA, above 15 °C the rate of DNA degradation increased with temperature. Fig. 8 shows that the Arrhenius plot of the initial rate of DNA breakdown presented the same discontinuities and had the same slopes as the Arrhenius plot of potassium efflux. This result strongly suggests that the kinetics of phage DNA penetration is related to the kinetics of potassium efflux through the channel.

Potassium Efflux Induced by Superinfecting Phage—Within 1 min after the adsorption of the first virions, phage T4 develops a mechanism which prevents an efficient infection by secondary phage. These "superinfecting phage" adsorb normally, about half of them eject their DNA which is degraded into acid-soluble material, but 80–85% of these phage are not able to inject their DNA into the cytoplasm (45). This exclusion of superinfecting phage can be overcome by addition of chloramphenicol prior to the first infection, and under these conditions more than 80% of the DNA of the secondary phage is injected (45). We have measured the extent of potassium efflux induced by the primary and secondary phage. Cells were first infected at m.o.i. = 3 in the presence or in the absence of chloramphenicol. Table II shows that the initial rate of potassium efflux was not significantly modified by the addition of chloramphenicol. When the new steady state of internal potassium was attained, a second infection was per-
Experimental conditions were identical to those described in the legend to Fig. 1. The secondary infection was performed 3 min after the first infection. The value $K_+$ before the secondary infection was 200 nmol·mg$^{-1}$·min$^{-1}$. CAM, chloramphenicol.

| First infection | +CAM | 420 | 285 | 15 |
| CAM | 420 | 270 | 15 |
| Superinfection | +CAM | 200 | 160 |
| CAM | 200 | 96 | 70–80 |

**TABLE III**

**Effect of the addition of the protonophore TCS on the $\Delta\psi$ changes induced by phage T4**

EDTA-treated $E$. coli cells were preincubated in sodium phosphate buffer 100 mM (pH 7.8), containing glycerol and various concentrations of TCS. They were infected at m.o.i. = 3 and $\Delta\psi$ was measured at different times after infection. The values of $\Delta\psi$ corresponding to the depolarization maximum and to the steady state were obtained, respectively, 1 and 6 min after the infection.

<table>
<thead>
<tr>
<th>TCS</th>
<th>Membrane potential before infection</th>
<th>Depolarization maximum</th>
<th>Repolarization steady state</th>
</tr>
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<tbody>
<tr>
<td>0.25</td>
<td>180</td>
<td>135</td>
<td>160</td>
</tr>
<tr>
<td>0.5</td>
<td>145</td>
<td>90</td>
<td>125</td>
</tr>
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<td>2</td>
<td>110</td>
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<tr>
<td>5</td>
<td>80</td>
<td>50</td>
<td>50</td>
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</table>

**Fig. 9.** Effect of the preinfectious level of $\Delta\phi$ on the phage and ghost-induced initial rate of potassium efflux. The EDTA-treated cells were preincubated at pH 7.8 in the presence of various concentrations of TCS. They were then infected at m.o.i. = 3 (○) or m.o.k. = 0.5 (△). The rate of K$^+$ efflux was calculated after calibration of the potassium electrode in the presence of various concentrations of TCS. Control experiments showed that in uninfected cells the efflux of K$^+$ induced by the addition of TCS never rose above 20–40 nmol·mg$^{-1}$·min$^{-1}$.

Effect of superinfecting phage on the initial rate of K$^+$ efflux

Table II shows that in the presence of chloramphenicol, the initial rate of potassium efflux induced by the secondary phage was comparable to the rate measured for a primary infection performed in cells containing 200 nmol of K$^+$·mg$^{-1}$. On the other hand, when chloramphenicol was omitted, the initial rate of potassium efflux induced by the secondary phage was decreased by a factor of 4. These results indicate that there is a relationship between the extent of phage DNA penetration and the extent of potassium efflux.

Voltage Dependence of the Channel Formation and/or Opening—The membrane potential changes and initial rate of potassium efflux were measured in cells which were gradually depolarized before the infection by addition of the protonophore TCS. Table III shows that the phage-induced transient depolarization occurred normally when $\Delta\psi$ was decreased from 180 to 75 mV but was not observed any more below 75 mV.

**Fig. 9** shows that the potassium efflux strongly depended on $\Delta\psi$ both for phage and ghosts: the efflux was constant when $\Delta\psi$ was varied between 170 and 140 mV; it then increased to a maximum obtained at $\Delta\psi$ = 85 mV and decreased gradually below 85 mV. For $\Delta\psi$ = 0, the efflux of potassium was negligible in the case of phage and strongly reduced in the case of ghosts.

Labeledan and Goldberg (8) previously demonstrated that phage T4 DNA was not injected into the cells when $\Delta\phi$ was decreased below 80 mV. Since the efflux of potassium was strongly decreased below 80 mV, we controlled the penetration of phage DNA under our experimental conditions. As a criterion for phage DNA penetration, we measured the activity of the lytic enzyme lysozyme which is produced very early after the infection by T4 phage (26). In the absence of TCS ($\Delta\psi$ = 170 mV), the lysozyme activity corresponded to an OD of 0.85 unit·min$^{-1}$, whereas in the presence of 5 μM of TCS ($\Delta\psi$ = 80 mV), the lysozyme activity was 0.01 unit·min$^{-1}$. These experiments confirm that the penetration of phage DNA was impaired at low $\Delta\psi$.

Thus, decreasing $\Delta\psi$ below 85 mV has two consequences: it greatly inhibits the potassium efflux and prevents the penetration of phage T4 DNA. Since the potassium efflux induced by ghosts was still observed at low $\Delta\psi$, this suggests that the channel is still inserted in the cytoplasmic membrane, but that its conformation is modified in such a way that it does not allow the penetration of the DNA molecule.

Estimation of the Minimum Size of the Channel—The size of the channel can be estimated by determining the rate of potassium efflux for $\Delta\psi$ = 0. Under these conditions, and for values of $\Delta C$ well below the $K_m$, the radius $r$ of the channel can be deduced from the value of the potassium flux $J$ through the Fick equation:

$$r^2 = \frac{J \cdot h}{\Pi \cdot \Delta \cdot \Delta C \cdot D}$$

where $h$ is the length of the channel, $\Delta C$ the potassium concentration difference, $D$ the potassium diffusion coefficient, and $\Delta t$ the time interval(s).

Since the conformation of the channel was modified below 85 mV, the experimental determination of the fluxes for $\Delta\psi = 0$ was not possible. Nevertheless, since the rate of potassium efflux increased when $\Delta\psi$ was decreased, estimation of this...
rate for $\Delta \psi = 85$ mV should permit calculation of a minimum size for the channel. The calculation was based on several assumptions: (i) the channel is freely permeable to potassium: this implies that the movement of $K^+$ is not hindered by the presence of DNA in the channel. The rate of potassium efflux was thus determined from the experiments performed with ghosts, in a medium containing 100 mM NaCl (see Fig. 4) and at 37°C (i.e. under conditions where the $Q_{10}$ of potassium efflux approached that of the ion in solution). (ii) The length of the channel is equal to the mean thickness of the cytoplasmic membrane. Since the main function of the channel is to facilitate the passage of DNA through the cytoplasmic membrane, its minimum length should be at least the thickness of the cytoplasmic membrane. (iii) The diffusion coefficient $D$ of potassium is similar in the membrane and in solution. Since $D$ can only be smaller in the membrane than in solution, we can only underestimate the size of the channel (see Equation).

For m.p.k. = 1 and $\Delta \psi = 85$ mV, the initial rate of potassium efflux was 2.8 $\mu$mol-mg$^{-1}$-min$^{-1}$. This corresponds to a value of $0.2 \times 10^{-16}$ mol-s$^{-1}$/bacteria and thus per channel. From the values of $D = 1.96 \times 10^{-20}$ m$^2$.s$^{-1}$ (46), $h = 8 \times 10^{-7}$ m, and $C = 400$ M·m$^{-2}$, we obtained a minimum diameter of the channel of 0.6 nm.

**DISCUSSION**

The increased permeability of the bacterial cell towards potassium which occurs upon infection by different phage was first qualitatively described by Puck and Lee (11, 12) and then by Silver et al. (13). It was proposed that this leakage would correspond to the formation of "localized lesions or holes" in the membrane accompanying the insertion of the phage tail tube through the cell wall. The hypothesis of a pore was also formulated by Gleen and Duckworth (14) in the case of phage T5. On the other hand, Ponta et al. (15) proposed that the drop in the intracellular ionic strength of T7- and T3-infected cells would be a regulatory step providing an optimal environment for the function of the phage-induced enzymes. Keweloh and Bakker (10) have gone into the details of the T1-induced permeability changes. Although they did not exclude that the permeability changes were due to the formation of channels, they favored a mechanism by which the function of the permeability changes would be to depolarize the cells completely so that upon entry, the DNA polyanion should not have to overcome the membrane potential existing in noninfected cells. More recently, Kuhn and Kellenberger (47) claimed that the ion leakage was accidental and had no function in the translocation of DNA.

In this study, we have quantitatively analyzed the potassium fluxes induced by T4 and have shown that phage and ghosts induce the formation of channels whose number corresponded to the number of infected particles. Several experiments indicated that the phage and ghost-induced channels were similar: the $K_m$ of potassium efflux was identical for both types of particles; both channels showed the same ionic selectivity and structural properties. The characteristics of the channel are therefore defined independently of the presence of phage DNA. Nevertheless, three independent experiments indicate that the presence of the channel is required for the passage of the DNA: first the amplitude of the potassium efflux was correlated to the extent of DNA penetration; second, the rate of potassium efflux and of DNA penetration showed the same temperature dependence (i.e. same slope and break in the Arrhenius plot). Finally, the inhibition of the potassium efflux which occurred when $\Delta \psi$ was decreased below 85 mV was correlated with the inhibition of phage DNA penetration.

The complex dependence of the rate of potassium efflux and of DNA penetration (4) on temperature indicates that the channel may exist at least in three different conformation states: above 29°C, the small $Q_{10}$ suggests that both potassium and DNA could freely diffuse through the channel. Between 29 and 15°C, two important changes were observed. First, the $Q_{10}$ was higher than 10 indicating that the channel conformation had changed thus reducing the efficiency of the penetration of DNA. Second, the efflux of potassium was delayed. Since the irreversible adsorption of the phage occurs in less than 1 min, even at 4°C (4), we think that the significant delay in the potassium efflux (2 min at 16°C) may reflect the increasing difficulty of the channel to be inserted in the cytoplasmic membrane. Below 14°C, the potassium efflux was stopped both for the phage and ghosts. This is also the temperature at which the penetration of DNA was severely impaired. Since the disorder-order transition of the hydrocarbon chains of E. coli lipids takes place at this temperature (48), we think that the ordering of the lipids may prevent either the insertion of the channel in the membrane or its opening, thus hindering the DNA penetration. What causes the change of conformation which occurs at 29°C, a temperature at which the lipid hydrocarbon chains are in a disordered state (48)? Van Alphen et al. (49) have shown that the midpoint of the order-disorder transition of the isolated lipopolysaccharide (the receptor of phage T4) takes place around 29°C. Thus, if one speculates that the components of the channel are either carried by the phage or are even present in the external membrane, then the discontinuity in the Arrhenius representation observed at 29°C could reflect the influence of the conformation of the lipopolysaccharide on the conformation of the channel. The possibility that the conformation of the channel itself changed at 29°C cannot of course be ruled out.

The properties of the channel were also dependent on the value of the membrane potential. Indeed, we have shown that the phage and ghost potassium efflux decreased below 85 mV and we have confirmed the results of Labedan and Goldberg (4) who showed that DNA penetration was impaired below this threshold of $\Delta \psi$. Since the ghost-induced potassium efflux was still observable when $\Delta \psi$ was decreased to zero, we think that the insertion of the channel in the cytoplasmic membrane occurs whatever the value of $\Delta \psi$, but that its conformation will depend on $\Delta \psi$.

The high specificity and efficiency with which phage DNA is recognized and injected into the host cell membrane has led Kornberg (50) to propose that a "pilot protein" attached to the DNA tip would be involved in recognition and formation of a postulated DNA pore. The similarity between the channels induced by T4 ghosts and phage strongly suggests that the formation and opening of the pore does not require the presence of DNA. Thus, if a pilot protein is required to recognize and form the channel, it could only be associated with the phage capsid and not with the DNA tip. We have shown that the potassium efflux always ceased 1–2 min after phage addition, whereas it continued until complete depletion upon ghost infection. This leads us to conclude that the phage-induced channel in contrast with the ghost-induced channel would be only transiently open. We thus propose that the pilot protein carried by the DNA would not be required to open the channel but to close it.

Although the mechanism of DNA entry into the bacterial cells may differ in several details, we think that the data available for most phage are compatible with the hypothesis
Characterization of DNA Channels in E. coli Cells

of a channel-mediated transport of DNA (51). From the point of view of energetics, the following unique general sequence of events emerges: upon attachment of the phage, a channel is opened in the cytoplasmic membrane. This opening induces an efflux and an influx of the actively transported ions down their electrochemical concentration gradient; as a consequence, the membrane becomes partially or totally depolarized depending on the number of phage added, on the size of the channel, and on the time during which the channel remains open. The depolarization results in an inhibition of the transport of metabolites which are coupled to the electrochemical proton gradient.

These channels appear to share common properties: indeed, it seems a general statement that the injection of phage DNA is prevented below the disorder-order transition of E. coli lipids. This was shown not only in the case of phage T4 (4) but in the case of phage T5 (52-54) and λ (55). On the other hand, some properties of the channels appear to be different: for instance, the DNA of phage T5 and λ can be injected into de-energized cells (6,7). Thus, it seems unlikely that the formation and opening of the corresponding channel should be voltage-dependent as it is for T4 (10,34).

The characterization and the isolation of these channels should lead to a better understanding of this process.

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