Involvement of the Bacterial *groM* Gene Product in Bacteriophage T7 Reproduction

II. A Reduced Level of Ion Concentrations Causes the Blockage of T7 Maturation in K-12-M Cells

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Cellular leakage observed in *Escherichia coli* K-12-M shortly after T7 infection might be the cause of arrested phage morphogenesis. We observed in this strain, but not in the normal host, a drastic reduction of the intracellular concentration of potassium (60%), magnesium (40%), putrescine (90%), and spermidine (40%), whereas ATP was not significantly reduced. Leakage started about 1 min after the addition of phage and was arrested 3 to 5 min postinfection. Larger molecules such as *o*-nitrophenyl- β -D-galactopyranoside could not enter the cells, showing that the permeability of the membrane was not generally affected. To prevent their leakage, we increased the outside concentrations of several small molecules and ions. The yield of progeny phage was substantially increased by the addition of 100 mM MgSO₄.

A transient leakage of small intracellular solutes has been observed after infection with many bacteriophages, but the amount of leakage apparently strongly depends on the experimental conditions used; therefore, the reports vary from laboratory to laboratory (8, 22, 23, 27, 29). One has to consider that a defined ionic intracellular environment might play a role in the efficiency not only of the syntheses of DNA, mRNA, and protein, but also of different steps of phage morphogenesis (13). The observation that the optimum of ionic strength for many phage enzymes *in vitro* is lower than that of the corresponding host enzyme (20, 30) supported on one hand the hypothesis that lowering the intracellular ionic concentrations might be part of the strategy of normal virus multiplication, either immediately upon infection (12, 25) or during the switch from early-late to late protein synthesis (17). On the other hand, abortive T7 multiplication in male cells is correlated with a particularly strong leakage, suggesting as cause for the abortion an even lower level of the intracellular ion concentrations (2, 3).

In the present study we not only investigated different T7-infected hosts for leakage, but also determined the intracellular contents of small solutes before and after infection. For the normal hosts we found no substantial change, suggesting that initial leakage is very minor or immediately compensated for by pumping. However, the male strain K-12-F' and the female strain K-12-M, both of which do not allow

T7 propagation, showed reduced intracellular content of potassium, magnesium, and polyamines, (2, 15). Our primary interest is answering the question of whether the reduced intracellular concentrations might be a cause of T7 abortion for both cases. T7-infected K-12-F' and K-12-M cells differ not only in their extent of membrane permeabilization, but also in their modified intracellular phage development. It has been shown that *o*-nitrophenyl- β -D-galactopyranoside (ONPG) penetrates into male cells shortly after infection (3), but we found that this is not the case with K-12-M cells. In addition, K-12-M allows the expression of the late genes (15), whereas late gene expression is inhibited with K-12-F' (2). As expected, the phage morphogenesis in K-12-M is thus blocked at a late step, namely, at that of DNA maturation and DNA packaging into accumulated preheads (15).

Changes of intracellular concentrations may result not only from leakage but also from inhibited or decreased pumping. Several cation pumps have been characterized for *Escherichia coli* cells (for a review, see reference 26) and lead to the following concept: at the potassium concentration of our medium (about 1 mM) only one specific potassium pump is present, namely, that formed by the *Trk* genes, whereas the expression of the *Kdp* genes is repressed (4). For magnesium, genetic evidence suggests two different pumps, both working under our conditions, which have been defined as the cobalt-resistant and magnesium transport systems (21).

We also have to assume an uptake of putrescine and spermidine, as the growth rate of polyamine-deficient mutants depends on the presence of these two polyamines (10). In the strains we used, the polyamines putrescine and spermidine can be synthesized. For sodium we assume an antiport, promoting efflux of Na^+ against the uptake of protons. This type of pump has been proven to exist in *Streptococcus faecalis* (9). Some evidence for a similar active exchange is available also for *E. coli*: sodium is not actively transported into metabolizing *E. coli* (M. Moncany, personal communication). We assume in addition what we call "spontaneous" leakage. This could be any accidental efflux through pumps and pores of the membrane. A constant efflux of $^{42}\text{K}^+$ -labeled cells has been observed (27). Maintaining any constant intracellular concentration level would thus need active pumping even in cases where metabolizing cells do not increase in size. Pumping would already be needed for compensating such a spontaneous leakage. This would also explain why metabolically arrested (energy-uncoupled or stationary) cells are losing potassium (5).

We compared altered intracellular concentrations with dynamic changes of effluxes induced by the infection process of bacteriophage T7. This will allow us to understand whether a modified intracellular concentration is caused by a decreased pumping or an enhanced efflux of the host or a combination of the two. Furthermore, we prevented the efflux by adding adequate amounts of solutes to the outside medium and followed the effects on the block of T7 maturation in *E. coli* K-12-M.

MATERIALS AND METHODS

Bacterial and phage growth. *E. coli* B (Sup⁺), K-12 (W3110) (*gyrA*), and K-12 (WA921) (*met leu lac supF*) were used as normal hosts. The *E. coli* K-12-M phenotype has been recently described (15). Phage T7-Ha and T3 were obtained from R. Hausmann. For bacterial growth we used shaking or bubbling cultures of tryptone medium (Difco Laboratories, Detroit, Mich.) containing 0.1 M NaCl at 37°C. Phage multiplication was started by infecting cultures at densities of 2×10^8 to 3×10^8 cells per ml with a multiplicity of infection (MOI) of 5. For the determination of the ion contents, 0.2% glucose was added. K-12 [*met leu lac supF* F'(lac)] was obtained from D. Krüger.

Determination of intracellular ions by atomic absorption spectrophotometry on filtration deposits. The technique for atomic absorption spectrophotometry is similar to the one described previously (5). Fresh overnight cultures were diluted 1/50 into tryptone medium containing 0.2% glucose and 0.1 M NaCl and bubbled at 37°C. At different times after infection, 2-ml samples were withdrawn and deposited on a membrane filter (GSWP, 0.22 μm ; Millipore Corp., Bedford, Mass.) under suction. The cells were washed with 4 ml of aerated, prewarmed isotonic solution of 50 mM LiCl-0.2% glucose-7% sucrose, and the deposit

was dissolved in 1 M HNO_3 for 30 min. The solution was then analyzed with a Unicam SP 2900 atomic absorption spectrometer.

Determination of polyamines. The bacterial cultures were deposited by filtration as described above, except that samples of only 0.9 ml were used and dissolved in 3 ml of 1 N HNO_3 . The solutions in nitric acid were treated with 15% trichloroacetic acid overnight and then centrifuged in a tabletop centrifuge. The supernatants were then analyzed on a DC Al resin (Durrum Instrument Co., Palo Alto, Calif.) in an automatic Unicrom analyzer (Beckman Instruments, Inc., Fullerton, Calif.) in the presence of 200 mM sodium citrate and eluted with 90 mM tripotassium citrate, 2.4 M KCl (pH 5.56), and 12 ml of thiodiglycol (Pierce Chemical Co., Rockford, Ill.) per liter. The polyamines were stained with orthophthalaldehyde by the method of Marton and Lee (16). The fluorometer (Gilson) was operating at an excitation wavelength of 340 nm and an emission wavelength of 455 nm.

Cell counts. Titers of exponentially growing cultures were determined by a Coulter Counter model ZB (Coulter Electronics Inc., Hialeah, Fla.). The turbidity of the culture was measured at a wavelength of 550 nm and used as a control for increase of cellular mass. Counting the cells with a Petroff-Hausser chamber was also used as a cursory control.

Continuous flow-through filtration. Fresh overnight cultures were diluted 1/50 into tryptone medium and grown to a density of 10^8 cells per ml at 37°C. A 1-ml sample of the culture was layered on a Millex-GS filter (pore size, 0.22 μm ; Millipore) and rinsed with tryptone medium. Flow was maintained at 37°C (at a speed of 0.5 ml/min) with a peristaltic pump (LKB Instruments Inc., Rockville, Md.). Infection was done by continuing the flow with an appropriate dilution of phage in growth medium for 1 min. The effluent was collected by a fractionator (Gilson) and analyzed for ATP concentration or radioactivity.

ATP determination. The effluent of the flow-through filtration described above was analyzed for ATP before, during, and after phage infection by the luciferase assay FLE-50 (Sigma Chemical Co., St. Louis, Mo.). The luminescence was measured by an XP 2000 bioluminescence analyzer (Skan AG, Basel, Switzerland). The total internal ATP content of uninfected cells was determined by measuring the culture after sonicating for 1 min below 40 W in a B12 sonifier (Branson Instruments Co., Stamford, Conn.).

$^{86}\text{Rb}^+$ release. Fresh overnight cultures were diluted 1/500 into tryptone medium containing 1 μCi of $^{86}\text{Rb}^+$ (Amersham Corp., Arlington Heights, Ill.; 1 mCi/mmol) per ml and shaken at 37°C until they reached a density of 10^8 cells per ml. One milliliter was then deposited on a Millex-GS filter (pore size, 0.22 μm) and washed with 10 ml of nonlabeled, aerated, and prewarmed growth medium. The cultures were then continuously fed and infected by flowing medium as described above. The fractions were collected and assayed for radioactivity. The resulting counts per minute were subtracted from background radioactivity. The total radioactivity of the culture at 2 min before phage infection was calculated and compared with the concentration of $^{86}\text{Rb}^+$ in each fraction after phage infection.

ONPG cleavage. ONPG cleavage was done by the method of Miller (18). A 50-fold dilution of fresh

FIG. 1. Intracellular potassium content of uninfected and T7-infected *E. coli*. Samples (1 ml) of *E. coli* cultures were withdrawn for assay with the filter deposit method at the times indicated on the abscissa. The values obtained by atomic absorption spectrophotometry are expressed relative to the content before infection. Symbols: ▲, *E. coli* B; ■, *E. coli* K-12; ○, *E. coli* K-12-M; ×, *E. coli* K-12-F'.

overnight culture was grown in tryptone medium containing 10^{-3} M isopropyl- β -D-thiogalactopyranoside (Sigma) at 37°C. At a density of 2×10^8 cells per ml the culture was infected with an MOI of 10. Samples (0.2 ml) were then withdrawn and put into 1 ml of reaction buffer (30 mM Na_2HPO_4 , 20 mM KH_2PO_4 , and 1 ml of ONPG (Merck & Co., Inc., Rahway, N.J.) was added. The mixtures were incubated at 37°C for 10 min. The reactions were terminated by the addition of 1 ml of 10% Na_2CO_3 , and the cells were removed by centrifugation. The released *o*-nitrophenol was measured in a spectrophotometer (Gilson) at 420 nm. For control of the total β -galactosidase activity, 1 drop of toluol was added to the culture, and the supernatant was measured as described above.

Estimation of the cellular volume. The cell volume was calculated by the dry density, wet density, dry weight, and salt weight per dry weight as described elsewhere (M. L. J. Moncany, F. Borle, H. Jütte, and E. Kellenberger, manuscript in preparation). The dry density (ρ_m) was determined by equilibrium gradient centrifugation of permeabilized cells (4% glutaraldehyde for 10 min) in an IEC A-269 rotor (International Equipment Co., Needham Heights, Mass.) ($120,000 \times g$ for 12 h at 15°C). To consider the influence of hydration (11) the density in different Cs salts was measured. The wet density (ρ_{wet}) was measured by equilibrium centrifugation on a gradient produced by Percoll (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.)—tryptone (54:46, vol/vol) containing NaCl to adequate osmolality (IEC A-269 rotor; $30,000 \times g$ for 20 min at 20°C). The mass of macromolecules (M_m) was obtained by weighing a trichloroacetic acid precipitate (15% overnight) deposited on a glass fiber filter (GF/C; Whatman Ltd., Clifton, N.J.). The weight of K and Mg salts per dry weight (p) was determined on filter deposits of the cells by atomic adsorption spectrophotometry as described above, assuming that phosphates are the main free anions (26, 30).

Experiments designed to prevent leakage. Fresh overnight cultures were diluted 1/50 into tryptone medium at 37°C. At a density of 2×10^8 cells per ml the cultures were infected with phage (MOI of 10). One minute later 1/10 volume of tryptone medium

containing the pertinent components (e.g., 1 M MgSO_4) was added to prevent leakage. The cultures were shaken at 37°C; at 15 min postinfection (p.i.), egg white lysozyme (200 $\mu\text{g}/\text{ml}$) was added, and 5 min later 1 drop of chloroform was added. If lysis was not complete each sample was sonified for 1 min, and then phage titers in the lysates were determined.

RESULTS

Leakage and intracellular contents of small solutes after T7 infection. (i) Potassium content of uninfected and T7-infected hosts. Uninfected and T7-infected cells were gently deposited onto filters and washed to remove the contaminating extracellular fluid. By measuring the atomic absorption of the filter deposits we determined the average potassium content of uninfected *coli* cultures as $3.6 \times 10^{-16} \pm 0.3 \times 10^{-16}$ mol per cell for K-12-M, $3.6 \times 10^{-16} \pm 0.3 \times 10^{-16}$ mol per cell for B, and $3.6 \times 10^{-16} \pm 0.8 \times 10^{-16}$ mol per cell for K-12. After T7 infection of *E. coli* B and K-12, which both produce normal phage yields, the K content did not change (Fig. 1). However, after T7 infection of *E. coli* K-12-M we observed a substantial loss of cellular potassium. Starting about 1 min after the infection of K-12-M with T7, the amount of cellular potassium decreased rapidly for 5 min; it then reached a level of about 1.5×10^{-16} mol per cell, that is, about 40% of the initial content. This level stayed virtually constant from 7 to 20 min p.i. T7 infection of K-12-F' cells resulted in a complete loss of the cellular potassium during the first 5 min.

The loss of potassium in the infected K-12-M and K-12-F' cells was not accompanied by a change of turbidity of the culture. Figure 2a shows that the turbidity remained constant shortly after infection and did not significantly change during the next 2 h.

FIG. 2. Kinetics of mass increase and of phage production after infection of various hosts by T7 and T3. Phage was added at a multiplicity of 5 to 10 to exponentially growing cultures (37°C). The turbidity of the culture was measured by optical density at 550 nm (a and b); samples were withdrawn and sonicated, and PFU titers were determined (c and d).

(ii) **Magnesium content before and after T7 infection of different hosts.** As for determining the potassium content, the cells were gently deposited onto filters and washed by an isotonic solution (see above). During normal T7 multiplication in B or K-12 the infected cell kept its magnesium content at the same level until lysis occurred (Fig. 3). After infection of K-12-M the magnesium content stayed unchanged until 3 min p.i., but then dropped rapidly to reach 60% at 5 min, where it remained constant. The magnesium content of uninfected K-12(W3110) cells was $8.2 \times 10^{-17} \pm 0.2 \times 10^{-17}$ mol per cell, and that of K-12-M cells $8.6 \times 10^{-17} \pm 0.7 \times 10^{-17}$ mol per cell.

(iii) **Polyamine content before and after T7 infection.** We investigated the intracellular content of spermidine and putrescine after T7 infection of K-12 and K-12-M cells by column chromatography of filter deposits (see above). The content of polyamines in K-12 cells was not significantly changed by T7 infection up to the time when lysis occurred. After T7 infection of K-12-M cells, however, the contents of putrescine (Fig. 4a) and spermidine (Fig. 4b) were significantly reduced. Shortly after infection (3 min) the intracellular concentration of putres-

cine started to decrease, to reach a level of about 10% at 5 min p.i. Spermidine decreased to a level of about 60% of the initial value.

(iv) **Reduction of the ATP content after infection of *E. coli* K-12-M, K-12, and K-12-F'.** We measured ATP efflux by the continuous flow-through method where the fractionated eluent was analyzed (see above). For infected K-12 and K-12-M cells, we detected only small transient losses of ATP shortly after infection. The calculated amount of ATP released during the first 10 min p.i. was 3.6% for K-12-M (Fig. 5a) and 0.9% for K-12 (Fig. 5b). Before lysis the subsequent fractions contained ATP in amounts below the limit of detection. For both hosts, the leakage was transient and therefore must have become repaired between 2 and 5 min p.i.

With T7-infected K-12-F' cells (Fig. 5c) we detected a period of leakage, starting 1 min p.i. It was followed by a drastic loss of 37% of the intracellular ATP without concomitant lysis (Fig. 2).

(v) **Efflux and uptake of K^+ monitored by its analog, $^{86}Rb^+$.** Transient leakage can be monitored by the loss of radioactively labeled intracellular compounds with the continuous flow-through filtration method (Fig. 6). We observed

FIG. 3. Intracellular magnesium content before and after T7 infection of *E. coli* B and K-12-M cells. Assays by the filter deposit method were performed as described in the legend to Fig. 1.

a loss of $^{86}\text{Rb}^+$ after T7 infection of *E. coli* K-12-M cells, whereas *E. coli* K-12 and B cells released the major proportion of intracellular radioactivity only by lysis at the end of the infective cycle. Figure 6a shows the $^{86}\text{Rb}^+$ loss of K-12-M cells. The phage-induced leakage started 2 min after adding T7 and reached its maximum at 3 to 4 min p.i. Between 2 and 6 min p.i., the cells lost 70% of their intracellular radioactivity, whereas uninfected cells lost only about 2% of the actual radioactivity per min.

Figures 6b and c show the $^{86}\text{Rb}^+$ loss after T7 infection of *E. coli* B and K-12 cells. The main proportion of $^{86}\text{Rb}^+$ began to efflux after 10 min p.i. when the cells lysed and phage progenies were liberated (Fig. 6d). In the period from 2 to 6 min p.i. we observed a small leakage of $^{86}\text{Rb}^+$, which was 15% for K-12 and 18% for B of the total radioactivity (total content at 2 min before infection). Consequently, only 56% for *E. coli* K-12 and 65% for *E. coli* B of the initial radioactivity was lost during lysis.

FIG. 4. Intracellular content of polyamines before and after T7 infection. Samples (1 ml) of *E. coli* K-12-M and K-12 cultures were withdrawn for assay by the filter deposit method at the times indicated on the abscissa. Titrers of polyamines were determined, and the values obtained are expressed relative to uninfected cells. The relative content is indicated on the ordinate.

We also analyzed the uptake of $^{86}\text{Rb}^+$ by uninfected and T7-infected cultures. The experimental procedure is described in the legend to Fig. 7. The rate of $^{86}\text{Rb}^+$ uptake into T7-infected cells was similar to that with uninfected cells until 10 min p.i. After 10 min p.i. the uptake was superimposed by $^{86}\text{Rb}^+$ release due to lysis of the host cells. We noticed that the initial rate of uptake of $^{86}\text{Rb}^+$ into K-12-M cells was about the same as that for B cells. K-12-M cells did not lyse (Fig. 2a), and therefore we could follow the uptake of $^{86}\text{Rb}^+$ until 20 min p.i. In the time period between 10 and 20 min p.i. the uptake of $^{86}\text{Rb}^+$ was strongly reduced.

(vi) **The extent of transient potassium leakage of infected K-12-M cells is dependent on the MOI.** Experiments with various multiplicities of T7 and T3 on $^{86}\text{Rb}^+$ -prelabeled cells were made with the continuous flow-through method. At different times after infection the released radioactivity was compared (Table 1). The amount of leakage after T7 infection increased with increasing MOIs; practically all of the internal $^{86}\text{Rb}^+$ was lost at a MOI of 10. After T3 infection, at the same MOI, nearly half of the $^{86}\text{Rb}^+$ content was still retained. Differences of leakage associated with the two phages was corroborated by the kinetics of potassium content of K-12-M cultures (Fig. 8). A substantial loss of potassium was observed after T7 infection. The T3-infected K-12-M cells, however, showed a practically unchanged potassium content until 7 min p.i. The subsequent loss occurred with a significantly slower rate than with T7, but both finally reached about the same reduced internal content of 1.4×10^{-16} mol of potassium per cell, i.e., about 40% of the initial value.

(vii) **Loss of potassium and magnesium from T7-infected K-12-M cells grown in media with different osmolality.** It is well known that a bacterial cell adapts its internal osmotic pressure to outside osmolality by the uptake of potassium salts (5). The intracellular content of potassium ions is therefore directly dependent on the osmolality of the outside medium. For our experiments we chose three different osmotic conditions, namely, tryptone media to which we added 0 mM, 100 mM and 350 mM NaCl. In all three conditions T7 infection of K-12-M cultures resulted in a drastic loss of intracellular potassium and magnesium (Fig. 9). Depending on the osmolality of the medium, the loss of potassium was stabilized at different levels. The amount of intracellular potassium of K-12-M cells, grown in tryptone alone, was 2.2×10^{-16} mol per cell. The transient loss of potassium induced by T7 infection led to a stabilized level of about 6×10^{-17} mol of potassium per cell at about 10 min p.i. Uninfected K-12-M cells grown in tryptone medium containing 350 mM NaCl showed an

FIG. 5. ATP leakage of various host cells after T7 infection monitored by continuous filtration. A 1-ml sample of an exponentially growing *E. coli* culture (2×10^8 cells) was put on a Millex-GS filter and continuously nourished by flow through of aerated growth medium. The cells were infected by adding phage to 1 ml of the feeding medium. The effluent was collected in 1-ml fractions and analyzed for its ATP content by a luciferin-luciferase assay (Sigma FLE-50). ATP leakage was analyzed for (a) K-12-M, (b) K-12, and (c) K-12-F' cells. The arrows indicate the eluent fraction collected at the time of phage addition.

internal potassium content of 5.6×10^{-16} mol of potassium per cell. This was lowered to 3.3×10^{-16} mol of potassium per cell, i.e., 60% of the initial content at 10 min p.i. The intracellular magnesium was also lowered in all three conditions. The retained magnesium was stabilized 10 min p.i. at about the same level for all three conditions. We calculated that about 60% of the magnesium was retained in the K-12-M culture grown in all three conditions.

FIG. 6. Leakage of preloaded $^{86}\text{Rb}^{+}$ and liberation of phage after infection by T7 monitored by continuous filtration. *E. coli* cells were preloaded with $^{86}\text{Rb}^{+}$ by growing for 10 generations in the presence of $1 \mu\text{Ci}$ of $^{86}\text{RbCl}$ per ml, deposited on a Millex-GS filter, and washed with 10 ml of unlabeled medium. Infection and continuous filtration were performed as described in the text and in the legend to Fig. 5. The effluent was analyzed for radioactivity in the cases of T7-infected K-12-M (a), B (b), and K-12 (c) cultures and for released PFU in the case of infected K-12 cultures (d). The arrows indicate the fraction collected when T7 phage was added.

(viii) **Determination of the internal K^{+} and Mg^{2+} concentrations in *E. coli* B and K-12-M cells.** For a determination of the intracellular ionic concentrations a precise knowledge of the water content of the cell is a prerequisite. It is well known that the volume of bacteria is depen-

dent on the particular strain as well as on the culture conditions, such as the bacterial concentration of the culture, pH, and salt concentration. The bacterial volume was estimated as will be published elsewhere in more detail (Moncany et al., in preparation). This approach is based on

FIG. 7. Uptake of $^{86}\text{Rb}^{+}$ into uninfected and T7-infected *E. coli* cells. A 5-ml sample of an exponentially growing culture (tryptone, 37°C) was infected by T7 at a density of 2×10^8 cells per ml with an MOI of 5. At 1 min p.i., $0.1 \mu\text{Ci}$ of $^{86}\text{RbCl}$ was added to the cultures, and 0.5-ml portions were withdrawn at the times indicated on the abscissa. The cells were collected on membrane filters and washed with 5 ml of aerated tryptone medium at 37°C , and the filter deposit was analyzed for radioactivity. The resulting counts per minute are expressed per cell. $^{86}\text{Rb}^{+}$ uptake has been analyzed for uninfected B cells (▲), T7-infected B cells (●), and T7-infected K-12-M cells (○).

TABLE 1. Effect of MOI on leakage of $^{86}\text{Rb}^+$

Strain	Phage	MOI	% Retained $^{86}\text{Rb}^+$ cpm at 15 min p.i.	Time interval of the main losses (min p.i.)
M ^a	T7	5	18.4	2-7
K-12-M	T7	2	51.9	1-5
K-12-M	T7	5	23.5	1-5
K-12-M	T7	10	10.3	2-6
K-12-M	T3	5	76.1	4-20
K-12-M	T3	10	59.1	2-18
K-12-M	T3	15	55.1	4-18

^a *E. coli* M is the original strain carrying *groM* (15).

the determination of the "dry" density (ρ_m , of a completely permeabilized cell), the wet density (ρ_{wet}), the dry weight (M_m), and the mass of solutes per dry weight (p). The water volume of a cell V_{H_2O} is calculated as follows: $V_{H_2O} = [M_m(1 - a)]/\rho_m a$ with $a = (\rho_{wet} - 1)/[\rho_m(1 + p) - 1]$.

Our data for *E. coli* K-12, B, and K-12-M are summarized in Table 2. We observed a variation in the volume due to the osmolality of the medium, as is already suggested by the morphology observed in the light microscope.

The calculated intracellular concentrations based on the volume are shown in Table 3. We confirmed earlier findings (5) that the internal potassium concentration increases with increasing osmolality of the outside medium. On the other hand, the magnesium content of K-12-M cells was not significantly affected by the change of osmolality of the medium. All concentrations are expressed relative to the volume and water content of the cells before infection. Reliable experimental data for the volume of infected cells could not be achieved, because these cells are oversensitive toward artificially induced lysis. Observation of the cells in the light microscope suggested no drastic change of the volume, justifying our assumption.

After T7 infection the magnesium concentration was lowered from about 70 mM initially to about 40 mM in all three media (i.e., tryptone containing 0, 100, and 350 mM NaCl). For potassium, the extent of loss after T7 infection was dependent on the osmolality of the growth medium. The level at which the retained potassium was stabilized seemed to directly depend on the osmolality.

(ix) **The T7-induced change of membrane properties does not allow ONPG to enter K-12-M cells.** The nature of the altered membrane permeability of T7-infected *E. coli* K-12, B, and K-12-M cells was further investigated by monitoring ONPG hydrolysis at various times after infection (Fig. 10). We performed the assays in the presence of excess intracellular beta-galactosidase, such that diffusion of ONPG into cells was the rate-limiting step in the formation of *o*-nitrophenol (19). Figure 10 shows that T7-infected K-12-M cells stayed impermeable for ONPG throughout the period analyzed. The *E. coli* K-12 cells (or B, data not shown) kept their ONPG impermeability until 8 to 10 min p.i.; then the permeability increased sharply. This rise was already associated with cellular lysis, which was premature when this method was used (i.e., incubation for the dye reaction). The K-12-F' cells showed an early permeability to ONPG starting 5 min p.i. without lysis of the cells.

T7 multiplication is partially restored by change of the extracellular milieu to prevent leakage. We prevented the release of small solutes from the K-12-M host after T7 infection by adding adequate concentrations of these solutes to the medium. This "dumping" was done 1 min after the addition of the phage to the culture to avoid unwanted effects on phage adsorption. At 20 min p.i. lysozyme and 1 drop of chloroform were added to liberate intracellular phage.

(i) **Effect of the addition of various potassium and magnesium salts on the production of phage**

FIG. 8. Relative potassium contents in K-12-M cells after infection by T3 and T7. At the times indicated on the abscissa, 1-ml portions of *E. coli* cultures were withdrawn for assay by the filter deposit method. The data obtained by atomic absorption photometry are expressed per cell. The potassium content was analyzed before and after T3 (▲) and T7 (○) infection of K-12-M cells.

FIG. 9. Effect of the osmolality of the growth medium on the intracellular content of potassium and magnesium of uninfected and T7-infected *E. coli* K-12-M cells. Assays were performed as described in the legend to Fig. 1. The osmolality of the media was varied by adding different concentrations of NaCl. The K (a) and Mg (b) contents were analyzed for T7-infected K-12-M cultures grown in tryptone medium (Δ) and tryptone medium with 100 mM NaCl ($\circ$) and 350 mM NaCl ($\bullet$). Each culture was started by the 1:50 dilution of a fresh overnight culture already grown in the corresponding medium. Phage infection was carried out at an MOI of 5, except for the tryptone medium with added 350 mM NaCl. In that case a comparable phage adsorption was only achieved with an MOI of 10.

progeny in T7-infected K-12-M and B-cells. Table 4 shows the PFU we obtained after "dumping" with K^+ and Mg^{2+} salts with various anions. We detected an increase in the number of phage particles by adding KCl to reach 400 mM and a

smaller increase by 400 mM K_2HPO_4 . However, $KHCO_3$ did not overcome the block in multiplication. Most of the added components had a negative influence upon T7-infected B and K-12 cells. We obtained an optimal restoration of yield by adding 100 mM Mg^{2+} salts to the T7-infected K-12-M culture. The increases were observed with $MgCl_2$, $Mg(OOCCH_3)_2$, and $MgSO_4$.

(ii) **Effect of the addition of ATP, polyamines, and compounds that raise the osmolality of the culture medium.** Dumping with ATP had no significant effect on the production of phage progeny (Table 5). Higher concentrations than 10 mM lowered phage production and induced aggregation of the cells. The addition of spermidine also slightly decreased the yield, whereas putrescine had no significant effect. When we increased the osmolality of the medium with glucose or NaCl we observed a minor increase in numbers of PFU. We then tried some different dumping combinations of various components, some of which are shown in Table 5. The T7 yields turned out to be higher with dumping than without, but we were unable to reach more than that obtained with $MgSO_4$ alone.

(iii) **Effect of compensation by $MgSO_4$ added to T3- and T7-infected K-12, K-12-M, and K-12-F' cultures.** As in T7-infected B cultures, the addition of 100 mM $MgSO_4$ 1 min p.i. had no significant effect on the production of T7 or T3 phage progeny in *E. coli* K-12. However, in contrast to T7-infected K-12-M cells, T3-infected K-12-M cells showed only a slight increase in the PFUs per cell, although the levels reached were comparable (9.5 and 14.6). No significant effect was observed for T7-infected K-12-F' cells, where the addition of $MgSO_4$, KCl, or ATP showed no compensating effect similar to that which we observed in T7-infected K-12-M cultures (Table 6).

DISCUSSION

As mentioned above, most phages studied induce, shortly after infection, a leakage of small intracellular solutes. Such a leakage lasts only a few minutes and reflects for some phages, but not all, a decrease of intracellular concentra-

TABLE 2. Estimation of the volume of various *E. coli* cells

Strain	Added NaCl (mM) ^a	Dry density (ρ_m)	Wet density (ρ_{wet})	Dry wt per cell ($g \times 10^{13}$)	<i>a</i>	Vol ($ml \times 10^{12}$)	Water vol ($ml \times 10^{12}$)
K-12	100	1.33	1.096	4.2	0.200	1.58	1.26
B	100	1.33	1.095	4.0	0.195	1.52	1.22
K-12-M	100	1.33	1.098	4.3	0.205	1.57	1.25
K-12-M	0	1.33	1.085	4.9	0.205	1.80	1.43
K-12-M	350	1.33	1.111	3.9	0.196	1.49	1.19

^a Different osmolalities of tryptone medium were achieved by the addition of NaCl.

TABLE 3. Determination of the intracellular potassium and magnesium concentrations before and after T7 infection

Strain	Concn (mM) in growth medium (with tryptone and salt)			Intracellular K concn ^a (mM)		Intracellular Mg concn ^b (mM)	
	Na	K	Mg	Preinfection	12 min p.i.	Preinfection	12 min p.i.
K-12-M	150	0.7	0.04	291	130	69	38
	50	0.6	0.04	154	41	72	50
	400	0.9	0.04	471	277	60	40
B	150	0.8	0.04	293	316	88	90
K-12	150	0.6	0.04	288	290	65	63

^a The potassium content per cell (Fig. 1 and 7) was divided by the water volume of the cell, as given in Table 2. The potassium concentrations of uninfected cells are the average of at least five independent determinations.

^b The magnesium content is expressed as molar concentration of the total volume of the cell. From work in our laboratory (see the text) we know indeed that only about 10% of the intracellular magnesium is in the form of a small molecular solute in the cell sap.

tions. Direct comparative measurements of intracellular contents under identical conditions showed that for λ , P1, T3, T4, and T7 a successful wild-type infection with high progeny yields is not accompanied by a permanent reduction of intracellular concentrations of small solutes (A. Kuhn, Ph.D. thesis, University of Freiburg im Breisgau, Freiburg, West Germany, 1982). Apparently the loss of the transient leakage is normally compensated for by pumping. Therefore, our data from T7-infected productive hosts do not confirm the attractive working hypothesis, that phage-induced changes of concentration levels are part of the normal strategy of phage-controlled regulatory processes (12, 22, 25). However, during infection of male and certain female hosts, in which intracellular concentrations of small solutes remain low, phage yields are greatly reduced (Kuhn, Ph.D. thesis). This correlation obviously does not a priori answer the question of whether this reduction is the causal reason of abortion or whether a common cause leads to both abortion and reduction of the intracellular concentration levels. For T7-infected male cells (F^+ cells), the observed change of membrane permeability and the reduction of progeny yields has already earlier brought up a discussion of whether the two phenomena are causally related (1-3, 22, 24). Until now it has not been determined conclusively whether T7 abortion in male cells is caused by a change of membrane permeability. It is known only that the F-mediated abortion is related to early events, i.e., the switch-on of the expression of the late genes (1).

In contrast to this case, the abortive T7 infection of K-12-M cells leads to a block of morphogenesis at a step just before packaging. Concatemeric DNA is made, which also is engaged into preheads. DNA enters partly into these particles, which therefore remain attached to the

pool of vegetative phage DNA, when this latter is isolated (15). That both the accumulated DNA and the preheads are not abortive per se, but competent for pursuing the morphogenetic pathway, is demonstrated by the in vitro packaging experiments (15) and by our finding, by preventing leakage of magnesium, that the progeny yield can become increased. This is achieved by raising the outside concentration of magnesium in the growth medium to 100 mM shortly after infection. The required amount of intracellular magnesium appears to be very critical. Indeed, without compensation, the intracellular concentration of magnesium is reduced to only 60%. These estimates concern the sum of free and bound magnesium. The results obtained by equilibrium dialysis (M. L. J. Moncan, F. Borle, H. Jütte, H. G. Seiler, Y. Boulanger, J. Brocas, and E. Kellenberger, manuscript in preparation) suggest that in normal, uninfected cells only about 10% of the magnesium is in the form of a small solute in the cellular sap, whereas the bulk is bound to nucleic acids. A reduction by 40% of the total might reflect a near-total depletion of Mg^{2+} in the cellular sap by kinetic reasons. We were not able indeed to determine the actual concentration of free magnesium in infected cells, because the host DNA degradation and phage DNA might alter the proportion of bound and free magnesium at each instance.

We have to consider two possibilities by which a lack of magnesium results in the block of morphogenesis. (i) The packaging process itself needs magnesium; either only Mg-DNA is packageable or free ions are necessary for the activity of a putative packaging enzyme. The stringent need of $MgSO_4$ in vitro for T7 DNA packaging (14) agrees with the observations we made in vivo. (ii) We observed that the final cutting of concatemeric DNA is prevented. In the absence of Mg^{2+} the final cutting of the

FIG. 10. ONPG cleavage by *E. coli* K-12-M, K-12, and K-12-F' cells after T7 infection. Phage infection (MOI of 5) was carried out at 3×10^8 cells per ml. Samples (5 ml) were withdrawn at the indicated times and analyzed for permeability of ONPG. The cleavage product *o*-nitrophenol was analyzed by absorption spectrophotometry. The arrow indicates the moment at which phage was added.

packaged DNA could be inhibited and thus would not allow the formation of a stable, packaged intermediate, from which the DNA would be lost from the capsid again.

On the other hand, we cannot exclude effects of the added magnesium other than those due to a reduction of its concentration in the sap, e.g., effects on the membrane. This is suggested by following the reduced potassium content of infected K-12-M after the addition of 100 mM MgSO_4 . In this case the potassium content was only reduced to 80% comparing with 40% without MgSO_4 (data not shown). But a simple magnesium-induced sealing of the membrane is

TABLE 4. Compensating for leakage with various K and Mg salts

Added compound	Final concn (mM)	T7 PFU/cell	
		K-12-M	B
None		0.36	89
KHCO_3	200	0.35	0.58
K_2HPO_4	200	1.1	39
	400	1.4	40
KCl	200	1.2	38
	360	2.2	14
	400	3.6	9
	440	2.9	10
MgCl_2	100	1.3	43
MgSO_4	50	3.0	102
	100	14.6	110
	120	6.9	74
	200	3.3	90
$\text{Mg}(\text{OOCCH}_3)_2$	50	7.2	94
	80	2.7	82
	100	1.8	65

TABLE 5. Compensating for leakage with various small solutes in K-12-M cells

Added compound(s)	Final concn (mM)	T7 PFU/cell
ATP	10	0.46
Spermidine	3	0.10
Putrescine	1	0.28
Putrescine	10	0.36
NaCl	400	1.71
Glucose	550	3.1
MgSO_4	100	5.2
KCl	400	
$\text{Mg}(\text{OOCCH}_3)_2$	80	8.6
KCl	400	
MgSO_4	120	3.1
KCl	400	
ATP	5	
MgSO_4	150	2.4
KCl	400	
ATP	5	
Spermidine	2	
Putrescine	10	

not very likely, since magnesium did not have such a repair effect on T7-infected male cells. Our data are not sufficient to decide definitively between a direct effect of the ion concentration of the cellular sap and an indirect effect of magnesium such as reestablishing pumping functions or repair of leakage. The methods used for determining ion contents do not allow precise measurements of "leaky" cells where the intracellular concentration is kept at a high level only by the outside concentration. By the necessary

TABLE 6. Burst sizes of various *E. coli* strains infected with T3 and T7

Strain	Phage	Added compound (mM)	PFU/cell
K-12	T3	None	65
		MgSO_4 (100)	78
	T7	None	54
		MgSO_4 (100)	63
K-12-M	T3	None	2.8
		MgSO_4 (100)	9.5
K-12-F'	T3	None	51
		MgSO_4 (100)	0.1
	T7	None	0.7
		KCl (400)	0.7
		ATP (10)	0.6

washing where the outside concentration is again lowered, passive—even selective—leakage will change the primary content.

For solutes other than magnesium we could not detect a similar effect of reduced concentration on the T7 multiplication. We conclude that the ATP and polyamine content might not be reduced to a level that is rate limiting for DNA packaging. However increasing the osmolality of the growth medium by the addition of KCl, NaCl, or glucose resulted in a partial overcoming of the maturation block. This suggests that the DNA packaging might be partly entropy driven as DNA is more strongly hydrated at lower ionic strengths.

The lower contents of solutes observed with K-12-M is the consequence of a strong leakage, and uptake pumping seems not to be affected; we observed no significant differences of pumping between uninfected and infected cells. The leakage phase occurs between 1 and 5 min p.i. as judged by our data on $^{86}\text{Rb}^+$ and ATP losses and the extremely rapid reduction of the potassium and putrescine content. However, the reduction of magnesium and spermidine started later, at 3 min p.i. only, and lasted, as for the others, up to 5 min p.i. This delay might be due to a slow release of these components, which initially were bound to the cellular nucleic acids (26). The observed early loss of potassium is easily understood because this ion is mainly free in solution in the cellular sap.

K-12-M has been obtained from K-12 by genetic transfer of the gene *M* (15). We cannot exclude the possibility that other genes are co-integrated. A phenomenon possibly not linked to the changed intracellular ion concentration is the absence of lysis of K-12-M. Lysis of T7-infected cells remained inhibited regardless of dumping. We are presently pursuing a completely independent approach to study the consequences of changed intracellular concentrations. We are searching an adequate ionophore by which we can induce these T7-induced losses directly on the normal host. This is not so simple as it sounds, because *E. coli*, by virtue of the two successive permeability barriers formed by the outer and inner membranes, does not respond to ionophores as well as do gram-positive cells. If these experiments are successful, they will provide complementary data, which would rule out the still-remaining—although very unlikely—possibility of a magnesium-dependent pleiotropic effect of either the gene product *M* itself or of products from other genes that may be cotransferred.

Infection by T3, which is closely related to T7, also caused a loss of internal solutes of K-12-M, but the rate of this leakage differed from that observed after T7 infection (Table 1 and Fig. 8).

The compensation experiments indicate that T3 maturation was inhibited at the same step as with T7. A further comparison of the two phages with respect to their action on the bacterial membrane may elucidate how *groM* interplays with gene products of the phage. In our laboratory other phage-host systems are also being investigated with respect to intracellular concentration levels; for example, we are investigating the well-known observation that T4rII mutants are abortive when infecting K-12(λ), whereas this is not the case with T4 $^+$. The proteins rIIA and rIIB are structural parts of the membrane (28), and the "defect" they cause in case of their absence can be compensated for by the addition of Mg^{2+} or polyamines, but not by the addition of monovalent ions (6, 7). The precise step at which maturation is blocked, has never been demonstrated. We are now investigating if the block and its causes are comparable to what we have observed in the K-12-M cells after T7 infection.

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