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Cation Fluxes and Permeability Changes Accompanying Bacteriophage Infection of *Escherichia coli*

SIMON SILVER, ELAINE LEVINE, AND PHILIP M. SPIELMAN

Department of Biology, Washington University, St. Louis, Missouri 63130, and Virus Laboratory, University of California, Berkeley, California 94720

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Infection of *Escherichia coli* by bacteriophage T2 was accompanied by a rapid but transient increase in the rate of loss of small molecules from the bacterial cells. This transient leakage was studied with radioactive labels such as ^{42}K and ^{28}Mg. Bacteriophage-induced leakage was dependent on the ratio of phage to bacteria: the higher the multiplicity of infection, the greater the leakage. No leakage occurred at 4 C [when adsorption proceeds but injection of phage deoxyribonucleic acid (DNA) is blocked]. Leakage was caused by heavily irradiated phage as well as by normal phage; therefore, the intracellular functioning of the bacteriophage DNA was not required. This conclusion was supported by experiments which showed phage-induced leakage in the presence of chloramphenicol or sodium cyanide. Leakage could be prevented by infecting the bacteria with phage in the presence of high magnesium concentrations. Phage-induced leakage was terminated by a "sealing" reaction, after which potassium turnover by infected and uninfected cells was very similar. The sealing reaction occurred even in the presence of chloramphenicol, suggesting that the sealing is controlled by bacterial and not bacteriophage genes. We were not able to detect any effect of normal bacteriophage infection on the influx (active transport) of potassium and magnesium into the cells.

Virus infection can affect the permeability properties of cells in either of two ways. First, the virus nucleic acid contains genes whose products can alter the cell surface *from within* (20, 21). Second, the virion itself can alter or damage the cell surface *from without* (3, 16, 17), during the initial steps of infection: the adsorption of the virus particle onto the cellular surface and the "injection" of the viral nucleic acid through the surface into the interior of the cell.

Puck and Lee (16, 17) studied the leakage of ^{32}P from cells of *Escherichia coli* during infection with bacteriophages T1 and T2. They described an early and transient period of accelerated loss of ^{32}P having properties consistent with "hole punching from without," except that the leakage appeared to be independent of the multiplicity of virus particles per bacterial cell. The accelerated leakage of ^{32}P ended less than 5 min (at 37 C) after the beginning of phage infection (16, 17). Puck and Lee (17) postulated that a "sealing" reaction brings about this change in permeability and suggested that the sealing is under bacteriophage control.

We have confirmed and extended the experiments of Puck and Lee, making use of both the technological innovations introduced during the intervening years and of our increased understanding of the infection process. We have found that an accelerated loss of ^{42}K and ^{28}Mg accompanies infection with bacteriophages T2, T4, and T6. This leakage has the characteristics expected of damage from without to the cell surface, and is terminated by a "sealing" reaction which appears to be independent of control by bacteriophage genes.

MATERIALS AND METHODS

E. coli B and bacteriophages T2, T4, and T6 have been used in our other studies of permeability alterations (20-22). The usual bacteriophage techniques and terminology are as described by Adams (1).

Radioisotopes. Sodium radiophosphate (^{32}P) was purchased from Nuclear Consultants Corp., St. Louis, Mo.; spectrographic grade potassium chloride (^{42}K) was from Iso/Serve, Inc., Cambridge, Mass.; and magnesium chloride (^{28}Mg) was from Brookhaven National Laboratory, Upton, N.Y.

Media. For experiments on phage infection and cation fluxes, a medium is required which readily supports phage growth and has a low cation content. Tryptone broth [8 g of tryptone (Difco) and 5 g of NaCl per liter of water] is a very satisfactory medium, which contains only 6×10^{-4} M K^+ and 8×10^{-5} M Mg^{++}. Higher concentrations of cations lead to more

rapid fluxes (5, 8; *in preparation*), which mask the effects of phage infection. ^{42}K and ^{28}Mg were added at a concentration of about 10^{-4} M.

Cation flux. For the *efflux* experiments, *E. coli* was grown for several generations at 37 C, with aeration by shaking in the presence of radioactive ^{42}K or ^{28}Mg until the cells reached a density of 3×10^8 to 6×10^8/ml. The radioactive cells were centrifuged at 20 C, washed once with fresh medium, and resuspended again at about 5×10^8/ml. At this stage, most of the radioactivity was in the cells and little was free in the medium. After 15-ml samples were distributed in a series of 125-ml flasks, and 1-ml samples of each were filtered through HA filters (Millipore Corp., Bedford, Mass.) at time zero, inhibitors or phage, or both, were added and the flasks were placed in a shaking water bath. At intervals, 1-ml samples were removed and filtered. The filters were not washed, as washing did not affect the results appreciably. The filtrates were collected in small "polyvials" (Olympic Plastics Co., Los Angeles, Calif.), and both the filters and 0.5-ml samples of the filtrates were dried and counted in a Nuclear-Chicago gas-flow counter. After correcting for the differences in counting efficiency between the filter and filtrate samples (measured directly in each experiment by comparing early samples, in which about 90% of the radioactivity remained on the filters, with late samples, in which about 90% of the radioactivity was found in the filtrates) and for the decay of ^{42}K ($t/2 = 12.4$ hr) and ^{28}Mg ($t/2 = 21.3$ hr), the data used are the averages of the values from the filters and from the filtrates given as per cent of total radioactivity remaining on the filters ("radioactivity in cells").

For the measurements of *influx*, nonradioactive *E. coli* was grown in broth under similar conditions to those for the radioactive cultures. The cells were distributed into 125-ml flasks, inhibitors or phage, or both, were added at time zero, and ^{42}K or ^{28}Mg was added 1 min later. The flasks were placed in the shaking water bath, and 1-ml samples were removed, filtered, and washed twice with 5 ml each of tryptone broth at room temperature. To remove extracellular radioactivity without leaching radioactivity from the cells, the wash solution should be of at least the same osmotic strength as the growth medium and should not be chilled (7; *unpublished data*). In the influx experiments, only the filters were counted.

In some experiments, 10 μg of deoxyribonuclease per ml was added to reduce viscosity and speed up filtration.

RESULTS

Phage-induced leakage: the effect of multiplicity. Infection of *E. coli* cells with bacteriophage T2 resulted in a rapid loss of cellular potassium (Fig. 1). The extent of this loss was dependent on multiplicity of infection: the more phage infecting a single cell, the greater the loss. After about 5 min at 29 C, the accelerated leakage of potassium stopped, and the leakage from infected cells approached that from uninfected cells. Thus,

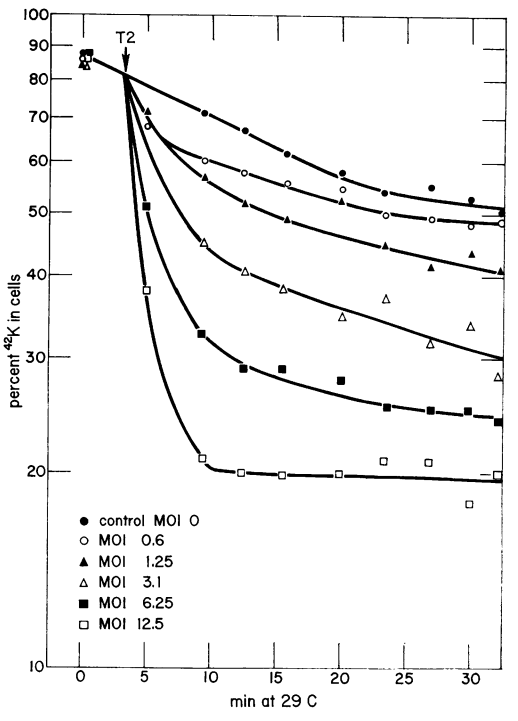

FIG. 1. *Potassium efflux: effect of varying the multiplicity of infection with bacteriophage T2. Bacteria were grown in broth containing 0.3 μc of ^{42}K per ml and 10^{-3}M total potassium, centrifuged, washed, and resuspended in fresh broth containing 6×10^{-4}M potassium. Bacteriophage T2 was added at 3 min.*

the results in Fig. 1 define both the early leakage stage and the relatively impermeable stage after "sealing." The slowing of ^{42}K efflux was not due to a total sealing of the cell surface; the addition of 10^{-2} M nonradioactive potassium 15 min after infection resulted in an additional period of rapid loss of ^{42}K (data not shown), similar to that found when excess nonradioactive potassium is added to uninfected cells (7, 8).

Puck and Lee (16, 17) observed a transient phage-induced loss of cellular ^{32}P, but this loss showed little dependence on multiplicity of infection. We have confirmed these observations (Fig. 2; *see below*). The striking multiplicity-dependent leakage occurred only with small molecules, such as potassium (Fig. 1) and magnesium (Fig. 6); therefore, phage-induced leakage is more readily measured with potassium than with a radioactive label such as ^{32}P, which is primarily incorporated into large macromolecular forms [ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and phospholipids]. The early data points after T2 infection were left out of Fig. 2 (because of the scale of the drawing), but signifi-

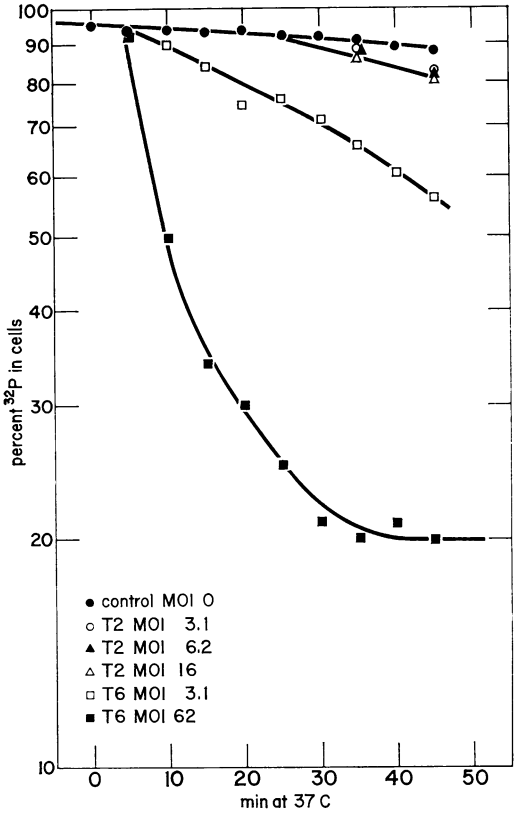

FIG. 2. *Phosphorus efflux: effects of phage infection and lysis from without with bacteriophages T2 and T6. The experiment was similar to that in Fig. 1, except that the cells were grown in broth with added ^{32}P. Bacteriophages were added at 3 min. Infection with T6 at a multiplicity of 62 produced visible clearing of the culture over a 10-min interval (lysis from without).*

cant early leakage of ^{32}P was observed and this leakage showed little dependence on multiplicity of infection. In one set of experiments with multiplicities of infection of about 5 phage per bacterium, the increased early leakage of radioactivity over that from uninfected cells was 0.9% from ^{32}P-labeled cells, 2.0% from cells labeled with ^{14}C-uracil, and 2.4% from cells labeled with ^{14}C-leucine. Although these numbers are small, the phage-induced leakage was readily measured in filtrates which contained from 1 to 5% of the radioactivity from the uninfected cells. Figure 2 also shows the greater loss of cellular phosphorus with bacteriophage T6 infection as compared with bacteriophage T2 infection at the same multiplicity. This was a consistent finding in several experiments, but we are not sure to what extent it resulted from differences between the phage with regard to adsorption and infection or from differences in the number of "dead" phage particles (capable of killing bacteria but incapable of producing plaques) in our phage stocks (*see below*). The 20% of the ^{32}P remaining on the filters in Fig. 2, after lysis from without (1) with 62 T6 per cell, was largely in the cell membrane. Experiments with ^{14}C-labeled nucleic acid precursors showed that more than 90% of the nucleic acid passed through the filters under the experimental conditions used, i.e., after lysis from without in the presence of deoxyribonuclease (19).

Effect of temperature. The phage-induced loss of ^{42}K appeared to be related to the injection stage of phage infection and not to the adsorption of the phage onto the cell surface. Adsorption of bacteriophage T2 can occur at room temperature or in the cold (4 C), but injection of the viral nucleic acid does not take place at 4 C (1, 16, 17). Similarly, the phage-induced leakage of ^{42}K occurs at 25 or 29 C but not at 4 C (Fig. 3).

Although the phage-induced leakage apparently required the injection of viral DNA into the cellular interior, it did not depend on the functioning of that DNA within the cell. This was demonstrated in several ways.

(i) Heavily ultraviolet (UV)-irradiated bacteriophage are able to adsorb to bacteria and inject their DNA, but the functional activity of the DNA is destroyed by the irradiation at the rate of about one "phage-lethal" hit inactivating 5 to 10% of the bacteriophage genes (6). Infection of ^{42}K-labeled bacteria with heavily UV-irradiated bacteriophage T2 resulted in a rapid loss of radioactivity (Fig. 4). This loss was again dependent

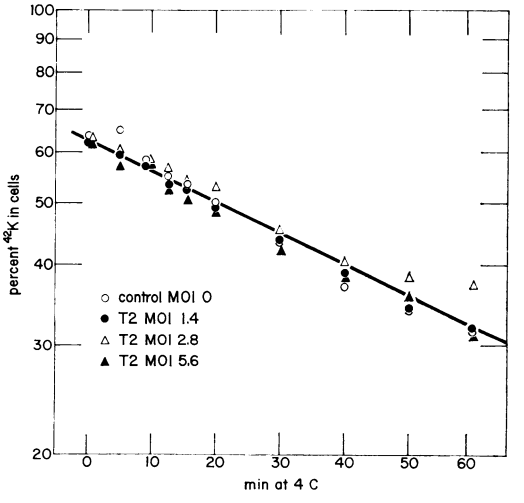

FIG. 3. *Potassium efflux at 4 C. The experiment was similar to that in Fig. 1, except that the radioactive bacteria were resuspended in cold broth and held at 4 C. Bacteriophage T2 was added at 3 min.*

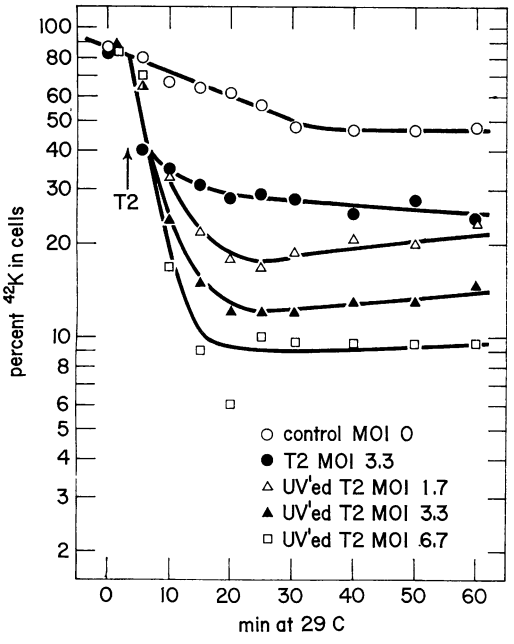

FIG. 4. *Potassium efflux: comparison of leakage induced by live and UV-irradiated phage. The bacteriophage T2 sample was divided in half, and one portion was irradiated for 10 min at 18 inches from a 15-w germicidal lamp. The UV dose was 14 phage-lethal hits per min. The multiplicities of infection were measured with unirradiated phage.*

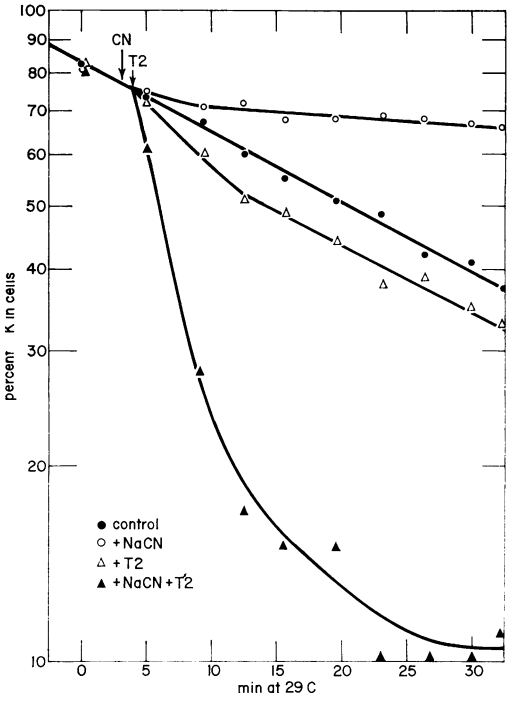

FIG. 5. *Potassium efflux: effect of cyanide on the leakage and sealing reactions. NaCN (10^{-3} M) was added at 3 min and bacteriophage T2 (multiplicity of infection, 3.0) at 4 min.*

on multiplicity of infection, suggesting that both leakage and sealing can occur in these abortively infected cells. UV-irradiated phage caused more leakage per phage particle than unirradiated phage (Fig. 4).

(ii) Phage-induced leakage of ^{42}K could occur in the presence of sodium cyanide (Fig. 5). In fact, in the presence of cyanide, a given multiplicity of phage will cause markedly greater loss of ^{42}K from the cells. With uninfected cells, 10^{-3} M cyanide inhibited the efflux of potassium from the cells (Fig. 5; reference 8). The cyanide effect in accelerating leakage from phage-infected cells was not limited to ^{42}K; similar results were obtained with cells labeled with radioactive magnesium (Fig. 6). Comparison of the data in Fig. 5 with the data in Fig. 6 showed, in addition, that cyanide does not cause the complete lysis of phage-infected cells but that potassium is released more readily than magnesium (and still more readily than phosphorus, *unpublished data*).

(iii) If the phage-induced leakage does not require a cellular energy supply, then it would not be expected to depend on protein synthesis. This was tested by adding an inhibitor of protein synthesis, chloramphenicol, to the bacterial cells 2 min prior to the addition of phage. Both the phage-induced leakage and the sealing reaction proceeded as usual in the presence of 25 μg of chloramphenicol per ml (Table 1). In this and some similar experiments, the chloramphenicol-treated cells showed slightly more leakage and a somewhat lower level of retention of radioactivity than the untreated cells (Table 1). This chloramphenicol effect generally occurs in uninfected as well as infected cells (8).

Protection by high concentrations of magnesium. Puck and Lee (16, 17) reported that phage-induced leakage of ^{32}P can be reduced or eliminated by carrying out infection in the presence of high (2.5×10^{-2} M) concentrations of magnesium. We have obtained similar results with ^{42}K leakage. Although high magnesium concentrations had no effect on the turnover of potassium in uninfected cells, the addition of a high concentration of magnesium just prior to the addition of bacteriophage completely eliminated the phage-induced leakage (Fig. 7). The phage do adsorb to the bacteria in the presence of a high concentration of magnesium (16–18). Magnesium clearly prevents leakage from cells infected with either live phage or UV-irradiated phage.

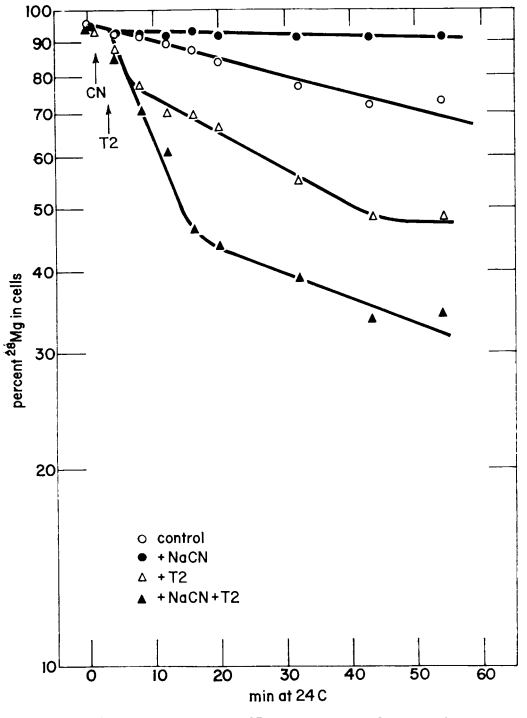

FIG. 6. *Magnesium efflux: effect of cyanide on the leakage and sealing reactions. E. coli was grown in broth containing 0.16 μc of ^{28}Mg per ml (total magnesium = 7 × 10^{-4} M), centrifuged, washed, and resuspended in fresh broth containing 8 × 10^{-5} M magnesium. Bacteriophage T2 and cyanide were added as in Fig. 5.*

Lack of phage effects on cation influx. Whether phage infection alters the functioning of normal cation "pumps" (permeases) in bacterial cells is a question of some interest. Specific effects of magnesium on the growth of T4 r_{II} mutants in *E. coli* K-12(λ) have been reported (4, 11, 18). The cells must maintain and continue to accumulate cations in order to sustain the growth of the bacteriophage. Furthermore, the best indication that the phage-induced leakage of cations reported above does not arise from the lysis of phage-infected cells is the observation of transient efflux in the absence of any changes in influx kinetics. In the experiments in which phage-induced leakage of ^{42}K and ^{28}Mg was measured, no effect of bacteriophage infection on the influx of potassium and magnesium was observed. Figure 8 shows the results of infection with bacteriophage T2, 1 min prior to the addition of ^{42}K. The addition of normal, unirradiated bacteriophage had no effect on the kinetics of accumulation of radioactivity by the cells. However, heavily UV-irradiated phage did influence the kinetics of accumulation of radioactive potassium, but this effect was seen only 5 to 10 min after infection. Occasional stocks of bacteriophages T2, T4, or T6 did have inhibitory effects on potassium influx. These phage stocks appeared to contain large numbers of "dead" particles capable of killing bacteria but incapable of producing plaques. This property was not hereditable, since virus preparations

TABLE 1. *Effect of chloramphenicol (CM) on the sealing reaction after infection with bacteriophage T2*

Multiplicity of infection	^{42}K remaining in cells[a]	
	Without CM	Plus CM (25 μg/ml)
	%	%
0[b]	45 ± 5	48 ± 2
1.25	41 ± 3	38 ± 0
2.5	36 ± 1	33 ± 1
5.0	31 ± 2	27 ± 1

[a] *E. coli* was grown with ^{42}K, centrifuged, and resuspended in fresh broth. CM (25 μg/ml) was added 2 min before the addition of bacteriophage T2. The course of ^{42}K efflux was followed as in Fig. 1. Entries are the average per cent of ^{42}K in the cells ± standard deviation for the five samples in the 20- to 60-min time interval.
[b] Control.

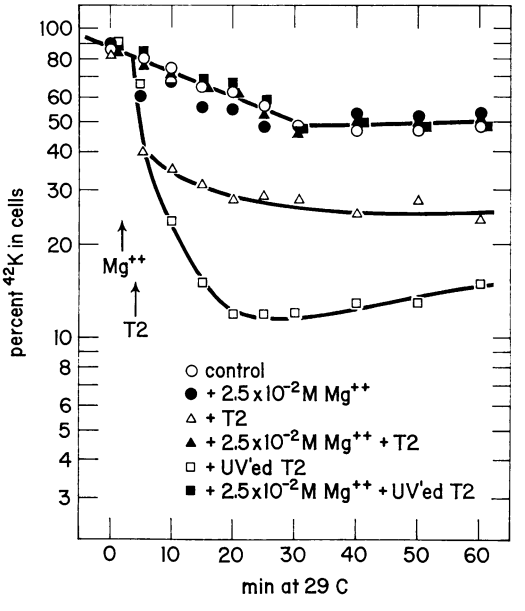

FIG. 7. *Potassium efflux: protection by high concentrations of magnesium. In the same experiment as in Fig. 4, unirradiated or UV-irradiated bacteriophage T2 (multiplicity of infection, 3.3) were added 2 min after the addition of 2.5 × 10^{-2} M $MgCl_2$ to three of the six samples.*

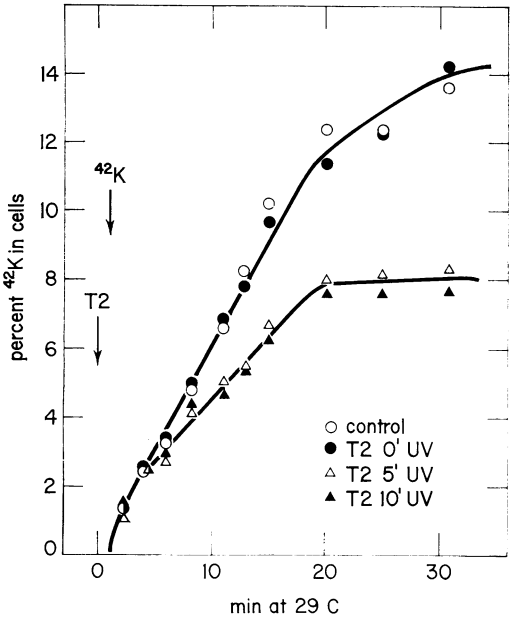

FIG. 8. *Potassium influx: effect of infection with unirradiated or UV-irradiated bacteriophage T2. Unirradiated phage or phage irradiated for 5 or 10 min (as in Fig. 4) were added at a multiplicity of infection of 5.8 to 7.2 × 10^8 cells/ml, and ^{42}K (0.03 μc/ml; 1.3 × 10^{-5} M) was added 1 min later.*

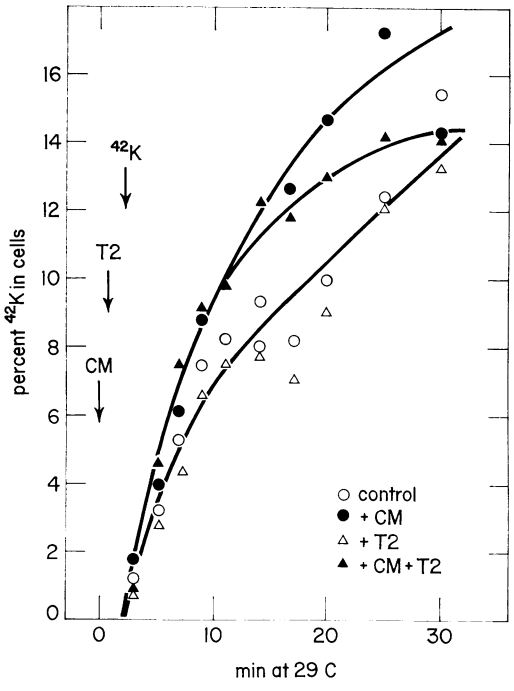

FIG. 9. *Potassium influx: effect of phage infection in the presence of chloramphenicol (CM). CM (25 μg/ml) was added to 4.8 × 10^8 cells/ml, 1 min before the addition of bacteriophage T2 (multiplicity of infection, 6.3). ^{42}K (as in Fig. 8) was added 1 min later.*

grown from the original stocks would often affect efflux of cations without influencing influx. All of the T2 experiments described in this paper were carried out with phage stocks which did not influence cation influx.

One cannot measure the effects of energy poisons on phage-induced changes in cation influx, since the energy poisons inhibit influx directly (8). However, phage-directed protein synthesis is not required for the continued functioning of the potassium pump in phage-infected cells, since uninfected and T2-infected cells in the presence or absence of chloramphenicol all showed approximately the same kinetics of influx of radioactive potassium (Fig. 9). Potassium influx was 25% greater after the addition of chloramphenicol. This result recurred in other experiments but was not completely reproducible (*see* 8). As in the case of potassium influx, phage infection did not affect the accumulation of magnesium by the cells (Fig. 10; reference 18).

DISCUSSION

Virus infection can affect the permeability properties of the cell either *from without* during the course of adsorption and nucleic acid penetration or *from within* by means of phage-directed protein synthesis. Puck and Lee (16, 17) have described a cyclic permeability change during the infection of *E. coli* with bacteriophage T2. They recognized three stages in the process: (i) the formation of localized holes or lesions, followed by (ii) a "spreading" of the "leaky" state leading to loss of small materials from the cell at places distinct from the initial sites of adsorption; and (iii) termination of the generally increased leaky stage by a "sealing" reaction, which also spread over the surface of the cell.

The results described in this paper confirm and extend those of Puck and Lee (16, 17). There are, however, several significant differences, the most important of which is that we could not find any evidence for a spreading of the initial lesion. To study ^{32}P leakage, *E. coli* cells were grown in the presence of ^{32}P, washed, and stored in the cold for various times. Then the phage were adsorbed to the cell surfaces at 0 C. Leakage of ^{32}P occurred when the virus-infected cells were kept for a short time at 37 C, and the cells were then cooled to 0 C to stop the reaction. Leakage was measured by analyzing the ^{32}P content of cell-free supernatant fluids after centrifugation in the cold. By using membrane filtration instead of centrifuga-

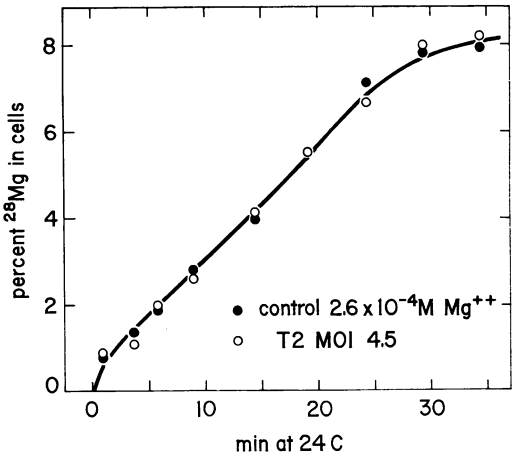

FIG. 10. *Magnesium influx: effect of phage infection. Bacteriophage T2 (multiplicity of infection, 4.5) was added, 1 min before the addition of ^{28}Mg (0.03 μc/ml; 1.1 × 10^{-4} M added magnesium), to 5.5 × 10^{8} cells/ml in broth containing 8 × 10^{-5} M magnesium.*

tion, we were able to measure leakage more rapidly, and the time between the removal of samples and the completion of the filtration step was always less than 1 min. Furthermore, the cells were always under conditions which allowed growth and were never temperature-shocked.

Puck and Lee (16, 17) concluded that phage-induced ^{32}P leakage is *not* dependent on the multiplicity of phage per bacterium, and it is this result which led them to postulate a spread of the initial lesions. We have also found very little effect of multiplicity of infection on ^{32}P leakage (Fig. 2), but ^{32}P leakage from freshly grown cells is very small at all low multiplicities. However, when measuring ^{42}K leakage, we observed that increased multiplicity of infection resulted in increased leakage of ^{42}K (Fig. 1, Table 1). Presumably, since potassium is found within the cells primarily in an unbound and osmotically active form (7, 8), it is a more sensitive measure of generalized leakage. Considering the differences in experimental procedures, we attribute Puck and Lee's result with relatively high multiplicities of infection to abortive infection and lysis from without in an increasing fraction of the cells. We conclude that there is no evidence for spreading of the initial lesions.

In addition to its dependency on multiplicity of infection, the leakage associated with phage infection has the other attributes expected of damage from without. The phage-induced leakage does not require protein synthesis or a cellular energy supply (Fig. 5 and 6; Table 1). Nor is it necessary for the phage DNA to be functionally intact, since heavily UV-irradiated phage induce leakage.

We do not know precisely what stage of the infection process results in the leakage. Irreversible adsorption at 4 C is not sufficient to cause leakage. It is possible that the leakage accompanies the injection of the phage tail needle through the cell wall (23), or that leakage is associated with enzymatic digestion of the cell wall at the site of adsorption (2, 3). The enzymatic digestion of the cell wall occurs at 26 C, but not at 4 C, and is also multiplicity dependent (2, 3). We have been unable to measure leakage of ^{42}K from cells attacked by DNA-free osmotically-shocked phage ghosts (9, 13), because of technical reasons associated with residual live phage and because of difficulties in determining multiplicities of infection with ghosts. Experiments with ghosts would determine whether injection of DNA is necessarily associated with leakage.

High magnesium concentrations prevent phage-induced leakage both from normally infected cells and from cells abortively infected by UV-irradiated phage (Fig. 7). This could be due either to the magnesium stopping the infection before the stage where leakage occurs or to the magnesium aiding and accelerating the sealing reaction. Phage adsorption, injection, and growth occur readily in the presence of high magnesium concentrations (1, 16–18), so that either the stage resulting in leakage can be bypassed or magnesium helps to plug the breach and aid in resealing.

The sealing reaction follows the leaky stage by 5 to 10 min at 25 C, both in normally infected cells and in cells infected with UV-irradiated phage (Fig. 1 and 4). Sealing also occurs in the presence of chloramphenicol and is therefore not dependent on phage gene-controlled processes. Perhaps bacterial proteins are enzymatically active in a sealing process which involves biosynthesis of new membrane material. For example, the incorporation of lipids into cell membrane materials continues after infection, even in the presence of chloramphenicol (4, 10). Or, alternatively, no biosynthesis may be involved, and the cell may be like a "self sealing inner tube" (22).

Recently, it was proposed that a bacteriophage gene might be involved in the resealing phenomenon (21). This gene, called *ac* in T4 and *pr* in T2, functions during the first few minutes of infection (21) in such a way as to change the permeability properties of the cell surface to *ac*ridine dyes such as *pro*flavine (12, 20, 21). It was proposed that the increased permeability to acridines might be a chance, secondary consequence of the

sealing reaction. However, this theory must now be rejected since the *ac* gene function is eliminated by UV-irradiation of the extracellular phage and is inhibited by 25 μg of chloramphenicol per ml (21).

UV-irradiated phage cause more potassium loss than do unirradiated phage and also inhibit potassium influx. We do not know what the underlying mechanism of this added damage is, but similar inhibitions of metabolism and active transport in abortive infections have been found by a number of investigators (9, 13–15). The transient nonspecific loss of small molecules from phage-infected cells could also provide an explanation for the chloramphenicol-insensitive inhibition of host nucleic acid synthesis that Nomura et al. (15) found after infection with T4 bacteriophage. As in the case of early leakage, this inhibitory effect was strongly dependent upon multiplicity of infection.

We can see no effect of normal phage infection on the functioning of the bacterial potassium and magnesium pumps. This is not surprising since, if cellular cations were lost by leakage and phage-induced permeases were needed after infection to restore them, the low cation content of the cell could inhibit the synthesis of these permeases and infection would be abortive. Turning this argument around, the inhibition of bacterial permeases by alterations in the cellular membrane can provide an explanation for some of the characteristics of abortive infection. The one report of a specific effect of bacteriophage infection on active transport is that of Sekiguchi (18), who showed that magnesium influx in r_{II}-infected K-12 (λ) stops abruptly about 6 min after infection at 37 C. We have not studied magnesium transport in K-12 (λ) cells, but we think it unlikely that the r_{II} gene determines directly a part of the magnesium active transport system. It seems more reasonable that the r_{II} gene product causes a change in the bacterial membrane which results in a cessation of active transport and other cellular functions unless the membrane structure is stabilized, e.g., by high magnesium concentrations in a manner analogous to that seen in Fig. 7 (*see* 4). Further experiments on potassium and magnesium influx in K-12 (λ) cells infected with r_{II} mutants in the presence of chloramphenicol should clear up this question.

ACKNOWLEDGMENTS

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