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Metabolism of T4 Bacteriophage Ghost-Infected Cells

II. Do Ghosts Cause a Generalized Permeability Change?

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Ghosts appear to alter the barrier function of the cell membrane, allowing the release of phosphorylated sugars which normally cannot pass through the cell membrane, whereas phage do not. No increased influx of normally impermeable compounds is observed in the presence of ghosts, indicating that the loss of membrane function after ghost infection cannot be attributed to a generalized breakdown of the permeability barrier.

Although both T4 bacteriophage and their ghosts rapidly inhibit host macromolecular syntheses, evidence has been accumulating that they do so by different mechanisms (3, 4, 21). Ghosts appear to act like energy poisons in that they inhibit the accumulation of thiomethyl galactoside (TMG), α-methyl glucoside (αMG), glucose-6-phosphate, and glycerol (21). Phage, unlike their deoxyribonucleic acid (DNA)-less derivative, have little effect on these processes. Ghosts, but not phage, also cause the rapid release of large amounts of some metabolites from infected cells (3, 21). The release of sugars which require energy to be accumulated can be explained by the fact that ghosts mimic the effects of energy poisons which cause the release of sugars via their normal membrane carriers. The release of other substances, such as organically bound phosphates (8, 11), nucleotides (3), and adenosine triphosphate (ATP) (21; W. A. Cramer, *personal communication*) cannot be explained in this way as the cell has no carriers by way of which these substances can pass through the cell membrane. The exit of these compounds could be explained if ghosts were producing some damage such as a hole or causing a generalized breakdown of the permeability barrier of the cell. If this were the case, one would predict that ghosts would also cause certain compounds to which the cell was previously impermeable to enter the cell. This happens when cells are treated with toluene.

In experiments reported here, we present additional evidence that ghosts do, indeed, alter the the barrier function of the cell membrane in a way which differs from any effects phage may have on the cell membrane (4). To see whether the alteration of permeability in ghost-infected cells can be attributed to damage or a generalized permeability change such as occurs upon treatment with toluene, we have looked for the entry of orthonitrophenyl-β-D-galactopyranoside (ONPG) into lactose permease-negative cells and for the entry of carbamyl phosphate and ATP into cells which are normally impermeable to these phosphorylated compounds. We have found no evidence for a generalized breakdown of the permeability barrier.

MATERIALS AND METHODS

Organisms and media. The source of organisms and their relevant characteristics are listed in Table 1. *Escherichia coli* B DD1 was isolated from a nitrosoguanidine-treated (1) culture of *E. coli* B and selected as a lactose-negative colony on EMB agar. The presence of β-galactosidase in the mutant was determined by the use of toluenized cells. *E. coli* B HW 16 was isolated as an arginine "feeder." The excess arginine produced is usually indicative of derepressed arginine biosynthesis. The level of ornithine transcarbamylase (OTCase) in the mutant is about 50 times that found in *E. coli* B when both are grown in M-9 plus 1% Casamino Acids (CAA).

The bacteria were grown at 37 C in a New Brunswick gyratory shaker in M-9 (7) plus 1% CAA or 0.5% glycerol until they reached a concentration of 5×10^8 cells/ml as indicated by turbidity. M-9 refers to M-9 media with no carbon source. M-9 T is M-9 containing about 200 μg of L-tryptophan per ml.

Phage growth and the preparation and assay of ghosts have been described (3, 21). All phage and ghost infections described herein were at a multiplicity of between 4 and 6.

Exit of phosphorylated sugars. *E. coli* B was grown in M-9 plus 0.5% glycerol. Washed cells were incubated with ^{14}C-αMG (0.5 mM, 0.15 μCi/ml) for 30 min to allow the establishment of an intracellular steady state of αMG and its phosphorylated derivative, αMGP (16, 20). A sample of these cells was filtered and extracted with boiling water. The extract was passed over a column (4 by 0.6 cm) of AG1-X8 formate resin. The neutral and anionic forms were

TABLE 1. *Oranisms used*

Organism	Relevant characteristics	Source
Bacteria		
Escherichia coli B	Wild type	M. J. Bessman
E. coli B DD1	Lactose permease-negative	See text
E. coli B HW 16	Derepressed arginine synthesis	See text
E. coli K Lin 8	Lacks α-glycerol phosphate dehydrogenase	E. C. C. Lin
E. coli K X-8	Can produce thiomethylgalactoside phosphate (10)	T. H. Wilson
Phage		
T4 am E957	Restricted growth on *E. coli* B	R. S. Edgar

eluted, and the radioactivity was determined as previously described (19, 20). To determine the efflux, the washed and ^{14}C-loaded cells were diluted 1 to 10 at room temperature in M-9 T containing 10 mM αMG and phage or ghosts as appropriate. At the indicated times, a 3-ml sample was filtered, and 1 ml of the filtrate was applied to a column for separation of neutral and anionic radioactive species as described above. The efflux of phosphorylated TMG, TMGP, in a mutant (10) which accumulates this compound was examined in an analogous fashion except that *E. coli* K X-8 cells grown in M-9 plus 1% CAA plus isopropyl-β-D-thiogalactopyranoside (IPTG) were used.

ONPG transport. The hydrolysis of ONPG by whole cells may be used as a measure of ONPG transport due to the fact that in the following series of reactions, step 1 is the rate-limiting step (15):

$$ONPG_{out} \xrightarrow[\text{permease}]{1} ONPG_{in} \xrightarrow[\beta\text{-galactosidase}]{2}$$

$$ONP^-_{in} \xrightarrow[\text{diffusion}]{3} ONP^-_{out} \text{ (yellow)}$$

E. coli B DD1 cells were grown in M-9 plus 1% CAA. IPTG (5×10^{-4} M) was present for at least two generations. When growth reached 5×10^8 cells/ml, the cells were centrifuged and resuspended at the desired cell concentration in M-9 T without IPTG. Reaction mixtures contained 2.75 ml of cells at the desired concentration and 0.25 ml of 0.1 M ONPG. The reaction was stopped at various times by the addition of 0.7 ml of the reaction mixture to 1.4 ml of ice-cold 1 M sodium carbonate. The cells were then removed by centrifugation, and the optical density at 420 nm of the supernatant fluid was determined.

Uptake of carbamyl phosphate. Carbamyl phosphate uptake was measured by the production of citrulline when whole cells were added to a mixture of ornithine and carbamyl phosphate. HW 16 cells were grown in M-9 plus 1% CAA to 5×10^8 cells/ml. They were centrifuged and resuspended at a concentration of 10^9 cells/ml in M-9 T or 1 M tris(hydroxymethyl)-aminomethane (Tris), *p*H 7.4, containing tryptophan. Reaction mixtures contained 1 ml of cells, 0.4 ml of M-9 or Tris (*p*H 7.4), and 0.6 ml of an assay mixture containing equal volumes of 1 M Tris (*p*H 8.5), 1 M ornithine, and 0.2 M carbamyl phosphate. Incubation was at 37 C. The reaction was stopped by the addition of 0.2 ml of 2 N HCl followed by incubation for 15 min at 90 C. After cooling, the cells were removed by centrifugation, and the citrulline in the supernatant fluid was measured by the colorimetric method of Hunninghake and Grisolia (9). The special reagents for the color reaction were obtained from Eastman Chemical Corp.

ATP uptake. Uptake of ATP was measured by the formation of α-glycerol phosphate (αGP) in *E. coli* K Lin 8, a mutant which cannot metabolize αGP. The cells were grown in M-9 plus 1% CAA to 5×10^8 cells/ml, centrifuged, and resuspended at 10^9 cells/ml in M-9 T. To the cells (1.1 ml) treated in various ways were added 0.1 ml of 1 mM ATP, 0.05 ml of 5% glycerol, and 10 μliters of carrier-free ATP-γ-^{32}P ($\sim 2 \times 10^8$ counts per min per ml), kindly provided by the laboratory of J. Larner. The reaction mixture was incubated at 37 C for the indicated times, and the reaction was stopped by placing 0.5-ml samples in a boiling water bath for 5 min. After cooling, the cells were removed by centrifugation; 0.1 ml of 1 M αGP was added to the cell supernatant fluid. Ten microliters of this was spotted on Whatman no. 1 paper and subjected to electrophoresis for 60 min at 2,000 v in pyridine-acetic acid buffer at *p*H 5.5. The positions of ATP, αGP, and P_i were indicated by standards and by scanning the strips on a Vanguard model 880 automatic chromatogram scanner. The desired regions were cut out and counted in a Packard model 3320 liquid scintillation counter. The per cent αGP was determined from the number of counts in this region as compared to the total number of counts in all three regions.

Toluene treatment. In all cases of treatment with toluene, one drop of toluene was added to 1 ml of cells along with one drop of 0.1 hexadecyltrimethyl-ammonium bromide (0.1 mg/ml; Eastman Chemical Co.) to solubilize the toluene. The cells were incubated for 10 min at 37 C with intermittent shaking.

EDTA treatment. Ethylenediaminetetraacetate acid treatment was carried out as described by Leive (12).

RESULTS

Exit of phosphorylated sugars. The phosphorylated derivatives of αMG, αMGP, is formed via

the phosphoenol pyruvate-phosphotransferase system as the parent sugar enters the membrane (16). TMGP is not usually formed by *E. coli*, but in a mutant, X-8, described by Kashket and Wilson (10) it can be formed from intracellular TMG. These phosphorylated compounds can neither enter the cell nor leave the cell under any conditions previously described (19, 20). The complete loss of intracellular radioactivity observed in a cell containing αMGP and αMG in roughly equal proportions has been shown to be due to a fluoride-sensitive phosphatase which hydrolyzes the αMGP to αMG (20). The neutral sugar then leaves the cell.

As shown in Table 2, ghosts, but not phage, cause the exit of TMGP and αMGP from *E. coli*. Cells containing either αMG plus αMGP or TMP plus TMGP were diluted into the indicated medium, the suspension was filtered, and the material appearing in the filtrate was determined. The addition of nonradioactive sugar to the medium prevents the reaccumulation of the exiting neutral sugar so that a large exit is obtained.

Figure 1 shows the time course for the exit of αMGP and also total radioactivity from cells diluted into either αMG or αMG plus ghosts. The initial values in the intracellular pool are shown. In the absence of ghosts an appreciable loss of radioactivity is observed but no αMGP leaves the cell. The addition of ghosts causes a slight increase in the total radioactivity loss, but now under the influence of ghosts more than one-third of the material lost is in the phosphorylated form. The loss is rapid, with 50% of the αMGP efflux occurring in the first minute.

ONPG transport. It had previously been shown that, in lactose permease-positive cells, ghosts depress the level of ONPG transport to that obtained with energy poisons (5, 21). This would suggest that the ghost-infected cell is no more permeable to ONPG than is a normal cell. To test this possibility further, ONPG transport into a lactose permease-negative mutant was tested. Figure 2 shows the effect of toluene, EDTA, and ghosts on such a mutant. It can be seen that ghosts cause a very small increase in the entry of ONPG, equivalent to less than 2% of the total intracellular enzyme becoming freely accessible to substrate. This increase can be accounted for by a small number of lysed cells. EDTA causes a much greater permeability increase, as was shown by Leive (12), and toluene completely removes the barrier to permeability. With the toluene-treated cells, only 7% of the β-galactosidase was found in the cell supernatant fluid, indicating that the increase is due to a cellular permeability increase.

Uptake of carbamyl phosphate. *E. coli* is normally permeable to only one of the two substrate (ornithine and carbamyl phosphate) of the enzyme OTCase. The activity of the enzyme thus cannot be measured in whole cells. Leive (12) showed that by treating the cells with EDTA they can be made permeable to carbamyl phosphate so that OTCase can be measured in whole cells. We have tested the effect of ghosts on the ability of whole cells to synthesize citrulline from ornithine and carbamyl phosphate via OTCase. Figure 3 shows the results of these experiments. It can be seen that whereas

TABLE 2. *Effect of phage and ghosts on the exit of α-methyl glucoside-6-phosphate (αMGP) and thiomethyl galactoside-6-phosphate (TMGP)*

Sugar[a]	Sample	Analysis[c] (counts per min × 10^{-3}/ml)	
		Neutral	Phosphorylated
^{14}C-αMG	Cell pool before dilution	8.1	8.3
	Filtrate after dilution + αMG	10.3	0.4
	Filtrate after dilution + αMG + ghosts	9.5	3.6
	Filtrate after dilution + αMG + phage	10.4	0.4
^{14}C-TMG	Cell pool before dilution	5.1	9.5
	Filtrate after dilution + TMG	3.5	0.0
	Filtrate after dilution + TMG + ghosts	3.2	9.3
	Filtrate after dilution + TMG + phage	2.6	0.1

[a] Cells (4×10^9 cells/ml) were incubated with the indicated sugar (0.5 mM, 0.15 μCi/ml) for 30 min in M-9 T.

[b] The cell pool represents the intracellular pool before a 1 to 10 dilution into medium with the indicated addition of nonradioactive TMG or αMG (10 mM). Phage or ghosts were at a multiplicity of 5.

[c] A sample of the cell suspension extracted before dilution or of a cell-free filtrate after 3 min (αMG) or 5 min (TMG) in the dilution condition was applied to a Dowex column and eluted as described. The material carried over from the loading medium into the 1 to 10 dilution has been subtracted.

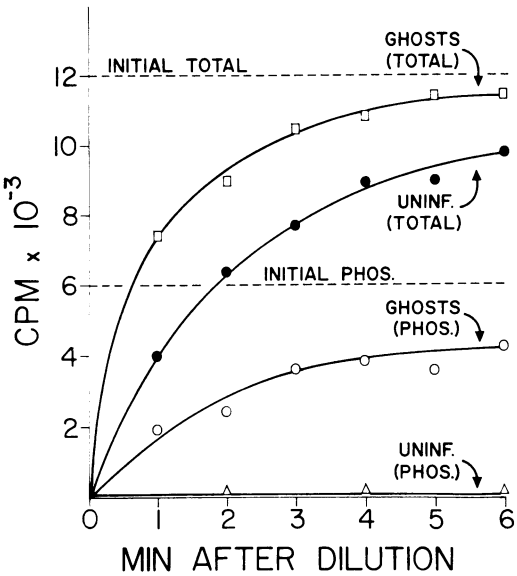

FIG. 1. *Effect of ghosts on the exit of α-methyl glucoside-6-phosphate (αMGP). E. coli B (4 × 10⁹ cells/ml) was incubated with ¹⁴C-αMG (0.5 mM, 0.15 μCi/ml) for 30 min, and a sample of the suspension was analyzed to determine the initial intracellular pool of ¹⁴C-αMGP and total radioactivity. The suspension was diluted 1 to 10 into M-9 T containing 10 mM αMG (nonradioactive) and ghosts as indicated. The suspension was filtered, and the appearance of ¹⁴C-αMGP or total radioactivity in the extracellular medium was monitored by the column technique described in Materials and Methods.*

toluene and EDTA in the presence of Tris cause a very large increase in citrulline production by whole cells, ghost infection, when the cells are in M-9 T, causes only a very slight increase. As in the case of ONPG, the apparent increase in permeability of ghost-infected cells is due to a small number of lysed or lysing cells. This was found by measuring the OTCase in the cell supernatant fluid. With either toluene or Tris-EDTA treatment, from 5 to 20% of the OTCase is found in the cell supernatant fluid, indicating that at least 80% of the apparent increase is due to a true cellular permeability change. Figure 3 also shows that cells incubated with Tris alone become more permeable to carbamyl phosphate and that ghosts enhance this effect. The reason for this is not known.

Uptake of ATP. In these experiments a mutant of *E. coli* K which cannot metabolize αGP was used. Glycerol enters these cells by facilitated diffusion and becomes trapped as αGP (17). In ghost-infected cells the lack of internal ATP prevents this (21). Addition of a high concentration of ATP to the outside of ghost-infected cells should restore the uptake and conversion of glycerol to αGP if ATP can, indeed, enter the cell.

Table 3 shows the transfer of the γ-³²P of ATP to α-GP. In whole, unpoisoned cells very little of the label is transferred to αGP, whereas 15% is transferred in 10 min if the cells are treated with toluene. Ghost-infected cells show only very slightly more uptake than control cells. If the cells are poisoned with azide and fluoride so that they cannot generate any of their own ATP, the value in the control rises to 3% in 10 min whereas poisoned, toluene-treated cells trap 25% of the γ-³²P of ATP in αGP. In poisoned ghost-infected cells, the value is again slightly higher than in the control, but as in the case of citrulline production the higher level can be accounted for by enzyme in the supernatant fluid. In the poisoned toluene-treated cells about half of the activity of the glycerol kinase is found in the supernatant fluid, indicating that only about half of the apparent uptake of ATP is due to a permeability change.

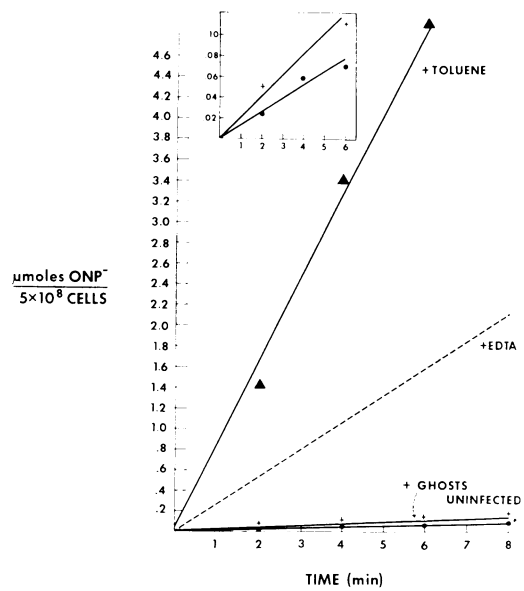

FIG. 2. *Hydrolysis of orthonitrophenyl-β-D-galactopyranoside (ONPG) by whole E. coli B DD1 cells. Cells were grown in the presence of inducer of the lactose operon (IPTG) for at least two generations and then centrifuged and resuspended in M-9 T at the desired concentration. The cells were treated with toluene or infected with ghosts at a multiplicity of 4 to 6. After 10 min of toluene treatment or 2 min of ghost infection, ONPG was added and the reaction was followed as indicated in Materials and Methods. Dashed line represents the data of Leive (12). Insert shows the data for uninfected and ghost-infected cells on an expanded scale.*

In ghost-infected cells none of the apparent uptake can be accounted for by a change in cellular permeability.

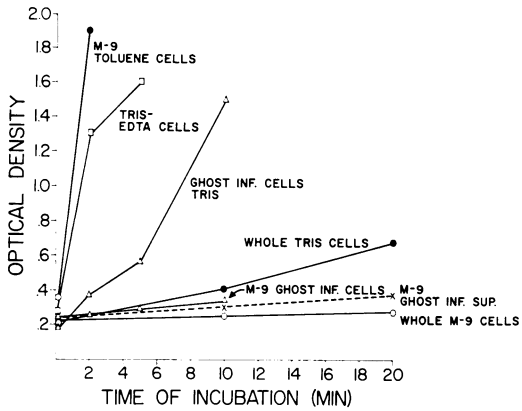

FIG. 3. *Synthesis of citrulline in whole cells from extracellular ornithine and carbamyl phosphate. HW 16 cells were grown to 5 × 10^8 cells/ml and resuspended at a concentration of 10^9 cells/ml in M-9 T or 1 M Tris, pH 7.4, containing tryptophan. Cells were treated as indicated and assayed for their ability to synthesize citrulline as indicated in Materials and Methods. Time of incubation represents the time after addition of ornithine and carbamyl phosphate. Cells were infected with ghosts at a multiplicity of 4 to 6 for 2 min prior to this. The ghost-infected supernatant is the supernatant fluid from a cell suspension in M-9 T which had been infected with ghosts for 5 min prior to centrifugation. Optical density is from a specific color reaction for citrulline and is proportional to the citrulline concentration.*

DISCUSSION

These results demonstrate that ghosts, but not phage, cause the cell membrane to become permeable to αMGP and TMGP, compounds to which it is impermeable under other conditions, including the presence of energy poisons (19, 20). The exit of other compounds from ghost-infected cells is well documented (3, 4, 8, 11, 21; W. A. Cramer, *personal communication*). Although an increased influx of material to which the cell is normally impermeable would be expected according to previous hypotheses (4) after ghost infection, we have not been able to demonstrate the expected increase. Three compounds, ONPG, carbamyl phosphate, and ATP, have been tested for their ability to enter cells after ghost infection. The entry of these compounds into cells is greatly increased by either EDTA treatment or toluene treatment or both. One of the compounds, ATP, has been shown by a specific assay to be released rapidly from ghost-infected cells. We have been unable to show any increase in the influx of these compounds after ghost infection—any apparent increase can be accounted for by a small per cent of lysing cells which release enzyme into the supernatant fluid. In an experiment in the laboratory of M. J. Bessman, we also could detect no DNA synthesis in whole, ghost-infected cells when the four deoxynucleoside triphosphates and Mg^{2+} were supplied to the cells. DNA synthesis would have occurred if there had been a generalized breakdown of the cells' permeability barrier or some damage such

TABLE 3. *Uptake of ATP-γ-^{32}P by Escherichia coli K Lin 8*

Cells	Time[a]	Per cent total counts in αGP[b]	No. of expts
Whole	5	0	1
Whole	10	1.3	3
Toluene-treated	5	8	6
Toluene-treated	10	15	15
Ghost-infected	5	0.8	5
Ghost-infected	10	1.9	5
Whole, poisoned[c]	5	2	5
Whole, poisoned[c]	10	3	5
Poisoned, toluene-treated	5	11	3
Poisoned, toluene-treated	10	25	3
Poisoned, ghost-infected	5	3.6	4
Poisoned, ghost-infected	10	4.5	4
Supernatant from poisoned, ghost-infected cells	5	4	1
Supernatant from poisoned, ghost-infected cells	10	5	1
Supernatant from poisoned, toluene-treated cells	5	6	1
Supernatant from poisoned, toluene-treated cells	10	11	1

[a] Time after addition of ATP-γ-^{32}P.

[b] ATP concentration = .08 mM; counts/min = 20,000. Percentages are average of the indicated number of experiments. αGP = α-glycerol phosphate.

[c] 30 mM azide + 100 mM fluoride.

as a "hole". We conclude, therefore, that the effects of ghosts cannot be attributed to a nonspecific permeability change. The results also suggest that the initial events of phage and ghost infection are different. The hypothesis of active repair of a membrane defect by the phage seems very unlikely, as any phosphorylated sugar lost before the phage repaired the defect could have been detected because once the αMGP or TMGP leaves the cell there is no mechanism to regain it.

We do not have an explanation for the apparent "one-way" permeability change caused by ghosts, but the observed effect is similar in some respects to the action of levallorphan on *E. coli* (6, 18). This drug, which is an analogue of morphine, causes a rapid drop in the level of intracellular ATP. ATP, as well as the products of hydrolysis of ATP, ADP and AMP, appear rapidly in the medium. The rate of entry of ONPG into lactose permease-less cells is not increased after levallorphan treatment (6), nor are the cells sensitized to actinomycin (18), indicating that the leakage of ATP is not caused by a generalized breakdown of the permeability barrier. The action of levallorphan is reversible (6, 18) as is the action of ghosts under some conditions (4).

Colicins have been thought to kill the cells to which they attach by a membrane-mediated reaction (13, 14), and the action of phage ghosts has been compared to the action of colicins (4). But the possibility now exists that colicins enter the cells and kill by an intracellular reaction (2). The effects of ghosts, however, appear to be exclusively on the cell membrane, making them an invaluable tool for the elucidation of the general problem of the functional organization of cellular membranes. We are continuing to study the unusual effects of ghosts on the cell membrane with this aim in mind.

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