Inhibition of Host Deoxyribonucleic Acid Synthesis by T4 Bacteriophage in the Absence of Protein Synthesis

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The requirement for phage protein synthesis for the inhibition of host deoxyribonucleic acid synthesis has been investigated by using a phage mutant unable to catalyze the production of any phage deoxyribonucleic acid. It has been concluded that the major pathway whereby phage inhibit host syntheses requires protein synthesis. The inhibition of host syntheses by phage ghosts is not affected by inhibitors of protein synthesis.

One question regarding the T-even phage-induced inhibition of host macromolecular syntheses that has not been satisfactorily answered is whether phage protein synthesis is required for the inhibition to occur. There are conflicting reports in the literature regarding this (6). Very early studies showed that there was no deoxyribonucleic acid (DNA) synthesis when phage infected cells in the presence of 5-methyl-tryptophan (1, 2). It was concluded from this that protein synthesis was not required for the phage-induced inhibition to occur. Studies with a variety of inhibitors of normal protein synthesis, including K^+ starvation, amino acid starvation, streptomycin, and chloramphenicol, led to the same conclusion (6). The ability of phage ghosts to cause the inhibition seemed to support this conclusion.

However, as variable effects were observed when phage were used to infect cells in the presence of chloramphenicol, Nomura et al. (11) reinvestigated the problem and concluded that both host DNA and ribonucleic acid (RNA) are synthesized in chloramphenicol-pretreated, phage-infected cells but that all nucleic acid synthesis is inhibited, the degree of inhibition increasing with increasing multiplicity of infection.

But, because chloramphenicol was the only inhibitor observed to prevent the phage-induced "shutoff" of host syntheses, Cohen (3) suggested that chloramphenicol may effect other reactions not involved in protein synthesis. This could explain why chloramphenicol inhibited the phage-induced "shutoff," whereas other inhibitors of protein synthesis did not. Indeed, it has been recently shown that chloramphenicol inhibits teichoic acid synthesis (14).

The present investigation was undertaken to determine whether inhibitors of protein synthesis other than chloramphenicol can prevent the phage-induced "shutoff" of host DNA synthesis. Cells treated with a variety of inhibitors of protein synthesis were infected with a phage mutant unable to catalyze the production of any phage DNA. It was found that in the presence of all inhibitors tested, with the exception of 5-methyl-tryptophan, host DNA synthesis is much greater than after infection of uninhibited cells. Inhibition of host DNA synthesis after phage infection in the absence of protein synthesis was increased by increasing the multiplicity of infection. Thus, chloramphenicol does not cause unusual results, and the major pathway whereby phage inhibit host syntheses appears to require protein synthesis. The fact that phage ghosts can inhibit host syntheses in the presence or absence of protein synthesis may reflect an initial membrane alteration by the phage coat which is almost completely counteracted during phage infection by injection of phage DNA and internal protein.

[MATERIALS AND METHODS]

The organisms used were *Escherichia coli* B, *E. coli* B (his^-), and T4 amE957 phage, a mutant phage which cannot synthesize DNA, late proteins, or phage when used to infect *E. coli* B owing to a defect in gene 1, the deoxynucleotide kinase gene. The origin of the organisms, their growth, and methods of titrating have been previously described (7).

The phage were purified in sucrose density gradients (5) to separate them from any contaminating ghosts. A method for the estimation of the number of ghosts in a phage stock is presented below.

Ghosts were prepared by osmotic shock and assayed as previously described (7).

The cells for the orthonitrophenyl- β -D-galactopyranoside (ONPG) transport assay were grown in the presence of 5×10^{-4} M isopropyl- β -D-thiogalactopyranoside (IPTG) for at least two generations. The cells were then centrifuged and resuspended in the absence of IPTG in fresh medium of the same kind they were grown in. ONPG hydrolysis was measured in a Gilford recording spectrophotometer as an increase in optical density at 420 nm. Reaction mixtures contained 1 ml of 5×10^8 cells, 1.9 ml of 0.025 M sodium phosphate buffer (pH 7.2) containing 0.5% NaCl, and 0.1 ml of 0.03 M ONPG. ONPG and IPTG were purchased from Calbiochem. When infected cells were used, measurements were made from 1 to 12 min postinfection (see reference 7 for further details).

DNA synthesis was measured by the incorporation of ^3H -thymidine (1.25 $\mu\text{Ci}/\text{ml}$; 13 mCi/mmol) into an acid-precipitable product. The conditions were chosen so that incorporation of label in uninfected cells and in T4 amE957-infected cells under several conditions was linear for 12 min. When T4 am $^+$ phage were used, the rate of incorporation was increased 7- to 10-fold between 8 and 12 min. Details are discussed below. In some cases the trichloroacetic acid precipitates were hydrolyzed in 1.5 M KOH for 18 hr at 4°C and then reprecipitated, as it was found that 3 to 4% of the ^3H -thymidine counts were incorporated into an alkali-labile material. This was true for radioactive thymidine from several sources (Calbiochem; Amersham-Searle). The trichloroacetic acid precipitates were collected by centrifugation, suspended in 1.5 ml of 0.1 M NaOH, neutralized with 1.0 ml of tris(hydroxymethyl)aminomethane buffer, and counted with 5 ml of Aquasol (New England Nuclear Corp.) in a Packard model 3320 liquid scintillation counter.

Protein synthesis was measured by the incorporation of ^{14}C -leucine into an acid-precipitable product as previously described (5).

RESULTS

Assay for presence of ghosts in phage stocks. To eliminate the possibility that the inhibition of host DNA synthesis seen in the absence of protein synthesis could be attributable to ghosts in the phage preparation (7), the phage was assayed by a differential assay that can detect ghosts in the presence of whole phage. The assay is based on the fact that ghosts (see reference 6 for an operational definition) or phage-ghost mixtures will cause the inhibition of ONPG hydrolysis (by inhibiting the uptake of ONPG), but phage alone will not (7). Hence, by adding increasing volumes of a phage suspension to a culture of lactose-operon-induced cells, an approximate value for the number of ghosts in a phage suspension can be obtained. A sample assay is shown in Fig. 1. From this it was calculated that the purified phage contained less than 5% ghosts. The maximum inhibition of 75% is observed either with addition of at least five ghosts per cell, so that

FIG. 1. Inhibition of orthonitrophenyl- β -D-galactopyranoside (ONPG) uptake into *Escherichia coli* B by phage and ghosts. Cells were grown in M-9 synthetic medium plus 1% Casamino Acids to a concentration of 5×10^8 cells/ml. Isopropyl- β -D-thiogalactopyranoside (IPTG) at a concentration of 5×10^{-4} M was present for at least two generations. The cells were centrifuged and suspended in M-9 salt solution containing 100 μg of L-tryptophan per ml. Reaction mixtures contained 1 ml of cells; varying amounts of phage, ghosts, or phage-ghost mixtures; 0.1 ml of 0.03 M ONPG and 0.025 M sodium phosphate buffer (pH 7.2) containing 0.5% NaCl to make the final volume 3 ml. Cells were infected for 1 min before addition of ONPG. The increase in optical density at 420 nm was measured in a Gilford recording spectrophotometer. The percentage of ONPG uptake in the infected cells was calculated from the rate of ONPG hydrolysis in those cells as compared to uninfected cells. Symbols: Δ , effect of purified phage; $\bullet$, effect of purified phage to which had been added about 20% ghosts plotted versus the phage multiplicity; $\circ$, effect of ghosts.

every cell is infected, or with addition of 30 mM sodium azide plus 100 mM sodium fluoride to give complete energy poisoning. Inhibition is not complete because the uptake of ONPG is not entirely energy-dependent. The theoretical inhibition at a multiplicity of one ghost per cell was calculated from a modified form of the Poisson distribution: $P(O)_{\text{apparent}} = e^{-n} + (1 - e^{-n}) (P/P_{0\text{max}})$, where $P(O)_{\text{apparent}}$ is the number of cells which appear to be unaffected, e is the base of the natural logarithms, n is the average multiplicity of infection, and $P/P_{0\text{max}}$ is the per cent survival at maximum inhibition. In this case $P(O)_{\text{apparent}}$ at a multiplicity of one ghost per cell should be equal to $0.37 + (0.63)(0.25)$ or 52.5%. It is not known whether the inhibition observed with the purified phage stock results from the presence of ghosts or is a natural consequence of a high multiplicity of phage; but as some preparations give almost no inhibition, it may result from the presence of ghosts.

Inhibition of host DNA synthesis. Figure 2 shows the inhibition of DNA synthesis when in-

FIG. 2. Inhibition of deoxyribonucleic acid (DNA) synthesis in *Escherichia coli* B by T4 amE957 phage. Cells were grown in M-9 synthetic media plus 0.4% glucose to a concentration of 5×10^8 cells/ml. For the histidine starvation a histidine auxotroph of *E. coli* B, *E. coli* B his⁻, was used, and cells were grown in the presence of 200 µg of histidine per ml. The cells were treated in various ways and then infected at the indicated multiplicity. L-tryptophan (100 µg/ml) was added prior to the phage except in the case of the 5-methyl-tryptophan treatment. After 2 min of phage infection, ³H-thymidine (1.25 µCi/ml, 15 mCi/mole) was added, and the samples (4 ml) were incubated with shaking at 37°C for 10 min. They were then chilled, centrifuged, washed, and suspended in 0.5% trichloroacetic acid. The trichloroacetic acid precipitates were collected by centrifugation, suspended in 1.5 ml of 0.1 M NaOH, neutralized with 1.0 ml of tris(hydroxymethyl)aminomethane buffer, and counted with 5 ml of Aquasol in a Packard model 3320 liquid scintillation counter. The per cent DNA synthesis was calculated from the amount of ³H-thymidine incorporated into a trichloroacetic acid-insoluble product in the infected cells as compared to the uninfected cells. The method used measures average rates of ³H-thymidine incorporation during 2 to 12 min postinfection, although under several conditions tested the incorporation was linear with time during this period. Symbols: ●, untreated cells; △, cells treated with 2 mg of puromycin per ml for 15 min; ○, cells treated with 50 µg of rifampin per ml for 10 min; □, cells treated with 500 µg of 5-methyl-tryptophan for 10 min; ■, cells (histidine-requiring) starved for histidine for 30 min; ▲, cells treated with chloramphenicol (100 µg/ml or 300 µg/ml) for 10 min. Solid lines indicate phage infection; dotted line indicates ghost infection.

puromycin, or under conditions of histidine starvation greatly decreased the inhibition of host DNA synthesis after phage infection. This effect could be at least partially reversed by increasing the multiplicity of phage infection. Figure 2 shows that a multiplicity of about 8 is needed to inhibit the synthesis by 63% in the presence of inhibitors of protein synthesis, whereas only one phage per cell will inhibit DNA synthesis by 63% in the absence of inhibitors. Nomura et al. (11) previously observed this same phenomenon with pretreatment with 100 µg of chloramphenicol per ml and incorporation of ¹⁴C-adenine and ³²P, and, although Karam reported that there was no effect of phage multiplicity when he used 300 µg of chloramphenicol per ml (quoted in reference 6), I observe no difference in effect between 100- and 300-µg/ml concentrations. Pretreatment with 5-methyl-tryptophan offered very little protection against the phage-induced inhibition, but under the conditions I used, leucine incorporation into protein was inhibited by only 50%, even though the synthesis of β-galactosidase was completely inhibited under these same conditions in uninfected cells. 5-Methyl-tryptophan has been reported to inhibit enzyme synthesis in phage-infected cells (9); therefore, at least part of the leucine incorporation may be into nonfunctional protein. Preincubation for 10 min with either chloramphenicol (100 µg/ml), rifampin (50 µg/ml), or puromycin (2 mg/ml) or with 30 min of incubation of the histidine auxotroph in the absence of histidine caused the uptake of ¹⁴C-leucine to be inhibited by 95 to 99%.

Phage ghosts produced by osmotic shock (6) are seen to cause an inhibition of host DNA synthesis which is slightly less than that caused by whole phage. The difference may be attributable to incomplete inhibition of host nucleic acid synthesis in some cells when the cells are grown in a synthetic medium. Inhibition by ghosts is equal to that caused by phage if cells are grown in broth (6). Chloramphenicol does not affect the level of inhibition obtained with ghosts in either case.

Table 1 shows inhibition of the amount of DNA synthesized after infection of untreated cells and of cells pretreated with inhibitors with phage at a multiplicity of 5. Conditions which inhibit protein synthesis most effectively offer the greatest protection against inhibition of DNA synthesis. Those conditions which are less effective in inhibiting protein synthesis, such as no preincubation or 5-methyl-tryptophan treatment, offer little protection against the inhibition of DNA synthesis. In all cases, the protection against the "shutoff" can be overcome by increasing the

creasing multiplicities of phage are used to infect cells under conditions which inhibit phage protein synthesis. Preincubation of cells with chloramphenicol (three concentrations), rifampin, or

TABLE 1. *Inhibition of amount of deoxyribonucleic acid synthesized after infection with T4 amE957 phage at a multiplicity of 5^a*

Treatment	Preincubation (min)	Per cent inhibition
None		96 ⁺
Chloramphenicol (30 µg/ml)	0	84
Chloramphenicol (30 µg/ml)	10	60
Chloramphenicol (100 µg/ml)	0	73
Chloramphenicol (100 µg/ml)	10	52
Chloramphenicol (300 µg/ml)	10	52
Rifampin (100 µg/ml)	10	44
Puromycin (2 mg/ml)	15	44
Histidine withdrawal (from histidine auxotroph)	0	73
Histidine withdrawal (from histidine auxotroph)	30	60
5-Methyl-tryptophan (500 µg/ml)	10	89

^a In all cases, deoxyribonucleic acid synthesis was measured from 2 to 12 min postinfection.

phage multiplicity. All of the conditions presented in Table 1 have been used by various investigators to inhibit early enzyme synthesis, indicating, not unexpectedly, that the inhibition of synthesis of the early enzymes is less sensitive to small variations in the inhibition of protein synthesis.

DISCUSSION

This investigation was undertaken to resolve the differences in the literature regarding the requirement for phage protein synthesis in the inhibition of host DNA synthesis during phage infection. The results of this investigation show that the major pathway whereby phage inhibit host DNA synthesis does require protein synthesis, as indeed do all the known mechanisms whereby phage can control the switch from host syntheses to viral syntheses (4, 8, 10, 15, 18). The lack of agreement as to the effect of inhibiting protein synthesis on the phage's ability to shut down host macromolecular syntheses is probably due to varying degrees of inhibition of protein synthesis and differences in multiplicities used. It should be pointed out that in all cases where uptake of label into product is measured, the uptake is subject to variations in the internal concentration of substrate due to changes of endogenous substrate and cell water. In this investigation, a relatively high concentration of exogenous thymidine was supplied to protect against such variations.

The reason for the increasing inhibition of host DNA synthesis at higher multiplicities of phage in the absence of protein synthesis is not entirely clear. The results cannot be explained by the presence of ghosts in the phage stock, as the upper limit for the number of ghosts in the phage preparation used could not account for the inhibition seen. In addition, under conditions such as these, simultaneous infection with a mixture of phage and ghosts would have prevented phage synthesis thereby reducing the number of infective centers (7); in the experiments of Nomura et al. (11), the number of infective centers does not decrease at multiplicities where inhibition of host syntheses is clearly seen. Oleson et al. (12) have hypothesized that one of the first steps after phage infection is the binding of host RNA polymerase to phage DNA. They further suggested that the suppression of bacterial RNA synthesis at higher multiplicities of infection and in the absence of protein synthesis could be explained by the binding of increasing amounts of host RNA polymerase at higher multiplicities. The experiments reported here with rifampin, which binds irreversibly to RNA polymerase (13, 16, 17), seem to preclude this explanation as well as the possibility that the effect is due to preferential escape of phage gene function from the effects of the inhibitors at higher multiplicities (11).

Nomura et al. (11) have postulated that phage have two mechanisms for the inhibition of host syntheses, one which requires protein synthesis and one which operates by action of a phage-coat protein. It seems quite clear, however, that the inhibitory properties of empty phage coats are much greater than the inhibitory properties of phage in the absence of protein synthesis, so that if one mechanism of phage-induced inhibition of host synthesis does operate through the phage coat, this mechanism is different from the ghost-induced inhibition of the host. Further, the inhibitory properties of ghosts cannot be explained simply by their inability to catalyze phage protein synthesis.

The results can be explained, however, if it is hypothesized that phage coats cause a specific, lethal change in the cell membrane which is reversed by the injection of the contents of the phage head. This injection of DNA and internal protein restores membrane function at the site of phage attachment but in such a way that the synthesis of host macromolecules is prevented. I believe that the attachment of increasing numbers of phage causes changes in greater proportions of the cell membrane so that host macromolecular syntheses are inhibited even in the absence of phage protein synthesis. At lower multiplicities of

infection, protein synthesis by the phage would be required to produce these changes.

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