Bacteriophage T4 Inhibits Colicin E2-Induced Degradation of *Escherichia coli* Deoxyribonucleic Acid

II. Inhibition by T4 Ghosts and by T4 in the Absence of Protein Synthesis

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The deoxyribonucleic acid (DNA) of *Escherichia coli* B is converted by colicin E2 to products soluble in cold trichloroacetic acid; we showed previously that this DNA degradation (hereafter termed solubilization) is subject to inhibition by infection with phage T4 and that at least two modes of inhibition can be differentiated on the basis of their sensitivity to chloramphenicol (CM). This report deals exclusively with the inhibition of E2 produced by T4, or T4 ghosts, in the absence of protein synthesis. The following observations are described. (i) The stage of T4 infection that inhibits E2 occurs after reversible adsorption of the phage to the bacterial surface, but probably prior to injection of T4 DNA into the cell's interior. (ii) The extent of inhibition increases as the T4 multiplicity is increased; however, the fraction of bacterial DNA that eventually is solubilized is virtually independent of the phage multiplicity. (iii) Phage ghosts (DNA-less phage particles) possess an approximately 15-fold greater inhibitory capacity toward E2 than do intact phage; however, because highly purified T4 (completely freed of ghost contamination) still inhibit E2, we discount the possibility that preparations of "intact phage" inhibit exclusively by virtue of contaminating ghosts. (iv) T4 infection does not liberate an extracellular inactivator of E2. In fact, infection with sufficiently high multiplicities of T4 produces a supernatant factor that protects E2 from nonspecific inactivation at 37 C. This protective factor does not interfere with the colicin's ability to induce DNA solubilization. (v) Inhibition of E2 occurs even when phage are added well after initiation of DNA solubilization by E2, suggesting that a late stage of E2 action is the target of inhibition by T4 infection. (vi) Increasing the CM concentration from 50 $\mu\text{g/ml}$ to 200 $\mu\text{g/ml}$ appears to reduce the inhibition appreciably; however, this can be attributed to an enhancement by CM of the rate of E2-induced DNA solubilization. (vii) The same degree of inhibition of E2 by T4 seen in CM is observed when CM is replaced by puromycin or rifampin. (viii) Others have shown that raising the multiplicity of E2 increases the rate of DNA solubilization. We find that the fractional inhibition (i), $i = (1 - y_i/y_0)$, where y_i and y_0 represent the inhibited and uninhibited rates of solubilization of DNA, respectively, produced by a given T4 multiplicity is independent of the multiplicity of E2 and hence is independent of the rate of DNA solubilization induced by E2.

As previously described (44), the colicin E2-induced solubilization of *Escherichia coli* B deoxyribonucleic acid (DNA) is subject to inhibition by infection with phage T4. When protein synthesis was not blocked by chloramphenicol (CM), an unequivocal demonstration of the inhibition required that the infecting phage contain an amber mutation (e.g., in gene 42) that prevents phage DNA synthesis (19) and a mutation in gene 46, 47 (references 29, 47, 48),

or *denA* (24, 46) that blocks T4-induced degradation of host DNA. In the absence of CM, infection of *E. coli* B with the phage mutated in genes 42, 46, and 47 virtually eliminated the solubilization of DNA induced by E2 alone. In contrast, when protein synthesis was interrupted by addition of CM, the inhibition of E2 still persisted, but at a substantially reduced level. This residual inhibition, referred to hereafter as protein synthesis-independent inhibition, was

further distinguished by its lack of dependence on any particular amber mutations carried by the phage, e.g., a mutant in gene 42 alone inhibited equally as well as the triple mutant (genes 42, 46, and 47).

Since the E2-induced solubilization of DNA is not markedly altered by CM (35), our results suggested the existence of at least two different modes of inhibition of E2 by T4 infection; we detailed some characteristics of the protein synthesis-dependent inhibition and noted that the block to degradation could be occurring at both endonucleolytic and exonucleolytic levels (44). This report presents details of the protein synthesis-independent inhibition and concludes that this type of inhibition is directed primarily at a late (solubilizing) rather than an early (nicking or cutting) activity associated with the E2 effect. In part III of this series (Swift and Wiberg, manuscript submitted for publication), the above conclusions are supported by the results of zone sedimentation analyses of the DNA degradation products generated during both protein synthesis-dependent and -independent inhibition.

We have used the term "solubilization" to describe the enzymatic conversion of DNA to a form soluble in cold trichloroacetic acid. "Solubilization" is often used loosely to imply the action of an exonuclease; conversely, "endonucleolytic" activity is often taken to exclude solubilization. However, both of these usages are seen to be incorrect when one considers *E. coli* endonuclease I; this enzyme by itself can solubilize DNA by producing oligonucleotides with an average length of 7 nucleotides (33). Thus we shall use a less ambiguous set of terms, borrowed freely from the literature: a "nick" is a break in only one strand of a double-stranded DNA; a "cut" represents two nearby breaks, one in each strand, such that two double-stranded fragments are produced from a linear molecule; "solubilization" is an operational term that may encompass both exonucleolytic activity and the late stages of extensive endonucleolytic activity. (A preliminary report of this work has appeared: Abstracts, p. 23, 1970 Phage Meetings, Cold Spring Harbor Laboratory of Quantitative Biology.)

MATERIALS AND METHODS

Bacteriophage and bacterial strains. *Shigella sonnei* (ATCC31) was obtained from B. Molholt. Bacteriophage T4 *amN69* was obtained from R. Edgar, and T6 wild type was obtained from R. Russell. All other bacteriophage and bacterial strains used, as well as the procedures for the construction of multiple mutants, the

preparation of phage stocks, and the assay procedures for viable phage and bacteria have been described previously (11, 29, 30, 48-50). Specific amber mutants of phage T4 will be referred to by gene number enclosed in brackets: *amN55x5* (deoxycytidylate hydroxymethylase) = [42]; *amN69* (a component of the phage structural apparatus necessary for irreversible adsorption) = [12].

Growth media. All bacteria except the *Shigella* strains were grown and infected in GCA, the glycerol-Casamino Acids medium of Fraser and Jerrel (21). Both *Shigella* strains were grown in M medium, a mixture of one part dilution broth and one part GCA; dilution broth contains 8 g of nutrient broth (Difco) and 5 g of NaCl per liter.

Chemicals. Micrococcal nuclease was obtained from Worthington Biochemical Corp.; rifampin, B grade, was purchased from Calbiochem. Puromycin dihydrochloride, grade II, and trypsin, type III, were purchased from Sigma Chemical Co. Uracil-6-³H and L-leucine-¹⁴C (U) were obtained from New England Nuclear Corp. All other chemicals used have been described previously (44).

Radioisotopic labeling and determination of the breakdown of DNA. Bacterial DNA was labeled according to the procedure described by Kutter and Wiberg (29, 30). The extent of degradation of DNA was determined by measuring the residual ³H-deoxythymidine (³H-dT) in cold trichloroacetic acid-insoluble material according to the filter paper technique of Bollum (5).

Preparation and purification of phage ghosts. Ghosts of phage T4D were prepared by incubation of equal volumes of concentrated phage suspension (>10¹² particles per ml) and saturated NaCl at 25°C for 5 min, followed by a rapid 100-fold dilution into ice-cold, deionized water. The resulting dilute preparation was then treated with micrococcal nuclease (0.01 mg/ml) and CaCl₂ (0.003 M) for 1 h at 30°C to degrade DNA. The preparation was then centrifuged at 80,000 × *g* for 2 h in a Spinco SW25.1 rotor, and the pellet was suspended in a volume of M9 buffer equal to the original volume of untreated intact phage suspension. A sample of the concentrated ghosts was layered onto a 4.8-ml linear gradient (0 - 99.85%) of deuterium oxide and H₂O in 0.1 M sodium-potassium phosphate buffer, pH 7.7, and centrifuged at 32,000 × *g* (average) for 20 min at 4°C in a Spinco SW50 rotor. The gradients were collected from a hole punctured in the bottom of the centrifuge tube (cellulose nitrate tubes, Beckman) and 28 equal fractions of 15 drops each were obtained. Fractions 16 to 24, containing the ghosts, were pooled and dialyzed against M9 buffer. The resulting ghosts are designated purified with respect to contaminating intact phage which sediment further into the linear gradient. The purified ghost preparation was titered by the method of Duckworth and Bessman, which is based on the ability of ghosts to inhibit β-galactosidase induction with an efficiency comparable

to that of intact phage (15). This ghost preparation contained less than 0.01% contamination by plaque-forming phage.

Purification of intact phage: method A (purified phage). Lysates of the phage were centrifuged at $5,000 \times g$ for 15 min at 4°C to remove bacterial debris. The supernatant fluid was then centrifuged at $18,000 \times g$ for 90 min at 4°C to pellet the phage. The pellet was suspended overnight at 4°C to 1/20th of the original volume in M9 buffer. The suspended phage received an additional slow-speed spin ($1,200 \times g$ for 3 min) at room temperature to remove residual debris. Phage stocks purified in this manner have been shown to contain variable contamination by phage ghosts.

Method B (highly purified phage). To eliminate contamination of the intact phage by ghosts, the above purification procedure for ghosts was used, except that a different zone was collected from the gradient (44).

Determination of the gene 12 particle titer. These particles were prepared and titered (by *in vitro* complementation) by the procedures of Edgar and Lielausis (17). The efficiency of complementation was 80 to 100%, based on the ratio of plaque-formers to ultraviolet absorbance of the particles.

RESULTS

We originally reported that infection by phage T4 in the presence of CM only partially inhibits E2-induced DNA solubilization in *E. coli* B (44). Further investigation reveals that the magnitude of inhibition is subject to considerable variation, depending on several factors including (i) the multiplicity of phage, (ii) the concentration of CM, (iii) the degree of purity of the phage preparation, and (iv) the multiplicity of E2.

The effect of T4 multiplicity on the extent of inhibition. As shown in Fig. 1, the extent of inhibition elicited in the presence of a constant multiplicity of E2 is markedly dependent on the multiplicity of T4. When the phage multiplicities used to inhibit E2 are plotted against reciprocals of the corresponding rates of DNA solubilization, a straight line with a positive slope is obtained (Fig. 2). The inhibition cannot be attributed to any measurable cell lysis, as optical density (490 nm) did not decrease at any multiplicity of phage tested (data not shown). In contrast to the rate of DNA solubilization, the fraction of *E. coli* DNA solubilized after 180 min of treatment with E2 is virtually independent of the T4 multiplicity (data not shown). Hence, the entire complement of DNA eventually becomes accessible to solubilization regardless of the extent of inhibition imposed by phage infection.

The effect of CM on the extent of inhibition: comparisons with puromycin and rifampin. As shown in Fig. 3A, the extent of

FIG. 1. Effect of phage multiplicity on the inhibition of E2. *E. coli* B (5.5×10^8 cells/ml) was pre-labeled with ^3H -dT as described in Materials and Methods. Portions of the culture were preincubated 5 min with CM (50 $\mu\text{g/ml}$; 37°C) to block protein synthesis, and then treated at 37°C with E2 (multiplicity of 100) and the indicated multiplicities of phage amN55x5 (purified; method A in Materials and Methods). Phage and colicin were added to *E. coli* B simultaneously. Samples were taken at the indicated times for the determination of acid-insoluble ^3H .

inhibition of E2 by T4 infection is appreciably dependent on the concentration of CM. All concentrations of CM used completely blocked the incorporation of ^{14}C -leucine into protein; hence, it is unlikely that residual protein synthesis at the lower concentrations of CM is responsible for the corresponding greater inhibition. A more likely explanation is suggested by the results illustrated in Fig. 3B. Here it is shown that CM initially causes a slight inhibition of DNA solubilization induced by a low multiplicity of E2, followed by a considerable increase in the rate of solubilization. This effect of CM is not detectable at a higher multiplicity of E2 (Fig. 3A) which induces extremely rapid solubilization of DNA. Apparently, high rates of DNA solubilization obscure any effect caused by CM, whereas lower rates permit detection of the CM-induced perturbation. It follows, therefore, that when a normally rapid rate of DNA solubilization is slowed by T4 infection, the modifying effect of CM becomes more pronounced. In conclusion, it seems likely that the counteractive effect of increasing CM concentrations on the E2 inhibition by phage infection is due to an effect directly on the solubilization process itself, rather than to an effect on the synthesis of T4-specific proteins.

The effect of T4 infection on E2-induced DNA

FIG. 2. Effect of phage multiplicity on the inhibition of E2. The various rates of DNA solubilization from Fig. 1 were assigned corresponding numerical values (y) (where y = fraction of DNA solubilized per min, taken from linear portions of the curves, i.e., between 15 and 40 min). Reciprocals of the rate values are plotted as a function of the corresponding phage multiplicity.

solubilization in the presence of other inhibitors of phage expression is illustrated in Fig. 4. Clearly, the inhibition of E2 by T4 infection is no different with either puromycin or rifampin present than it is with CM (at 200 μ g/ml). It was also observed that both puromycin and rifampin enhance the rate of DNA solubilization produced by low multiplicities of E2 alone (data not shown) in a manner similar to that seen with CM (Fig. 3B).

Protein synthesis-independent inhibition requires irreversible adsorption of the phage.

A trivial possibility that might explain the inhibition of E2 by T4 is that the phage particle interacts with and directly inactivates the colicin. To test this possibility, it was first ascertained that E2 is capable of killing the bacterial mutant *E. coli* B/4. This mutant does not adsorb phage T4 (43). When T4 and E2 are incubated together for 5 min at 30 C and then added to *E. coli* B/4, no effect of T4 on the killing efficiency by E2 (Table 1) or the rate of DNA solubilization induced in the presence of CM (data not shown) is observed. From these results, we conclude that direct inactivation of E2 by interaction with T4 does not occur.

In the experiment illustrated in Fig. 5, the capacity of phage ghosts (DNA-less phage particles) to inhibit E2-induced DNA solubilization is compared with that of intact phage, in the presence of 200 μ g of CM/ml, with E2 at a multiplicity of 200. When the reciprocals of the various rates of DNA solubilization are plotted as a function of the multiplicity of ghosts on intact phage, a straight line with a positive slope is obtained for both. However, the slope

for the ghost-inhibited cells is nearly 15-fold greater than that for intact phage, indicating a far greater inhibitory capacity for ghosts. Omission of CM from the ghost-treated cells had little effect on the extent of inhibition of E2 (data not shown).

Whereas the experiment illustrated in Fig. 5 used T4 purified by means of zone sedimentation in D_2O - H_2O gradients (method B; Materials and Methods), the one depicted in Fig. 1 and 2 used T4 purified only by means of differential centrifugation (method A; Materials and Methods). The two phage preparations will hereafter be referred to as highly purified and purified, respectively. It was determined that the purified T4 stock contained approximately 20% contamination by non-plaque-forming particles based on the inhibition of β -galactosidase induction, whereas the highly purified T4 contained virtually no excess of particles over plaque-forming units (R. Swift, unpublished observations). If the 20% contamination of the purified stock represents phage ghosts, one would predict that identical multiplicities of purified or highly purified T4 based on plaque titers would possess markedly different capacities to inhibit E2. This prediction is confirmed by the results illustrated in Fig. 6, where it is shown that purified T4 inhibit E2 to a greater extent than highly purified T4. Furthermore, the conditions associated with purification by zone sedimentation are not responsible for the observed difference, since zone sedimentation and fractionation, followed by remixing of all fractions, produced a T4 preparation, termed [42] (control), that was characteristic of the purified T4 (Fig. 6). Further-

FIG. 3. A, Effect of chloramphenicol (CM) concentration on the inhibition of E2 by phage. Procedures and conditions were the same as for Fig. 1 except that the *E. coli* concentration was 5.0×10^8 cells/ml, the E2 multiplicity was 200, the phage multiplicity was 5, and the CM concentration was varied as indicated in the figure. B, Effect of chloramphenicol (CM) concentration on DNA degradation by low multiplicities of E2 alone. As above, except E2 multiplicity was 10 and no phage were added. This effect was reproduced in each of the three experiments in which it was studied.

more, it was determined by resedimentation of highly purified T4, which had been labeled with ^3H -dT and ^{14}C -leucine, that only one resolvable peak occurred, in which ^{14}C -labeled protein completely cosedimented with ^3H -labeled DNA. This result argues that insignificant amounts of phage ghosts are generated during purification by method B. Since inhibition of E2 in CM persists with T4 stocks freed of contamination by ghosts, we conclude that E2 is inhibited by intact phage.

Although ghosts are devoid of DNA, they still adsorb irreversibly to host cells at a rate equal to that observed for intact phage (6, 22). Since ghosts are capable of inhibiting E2, it appears that simply the interaction between the

FIG. 4. Effect of puromycin or rifampin on the inhibition of E2 by phage infection. Procedure and conditions were the same as for Fig. 1 except that the E2 multiplicity was 200, the phage multiplicity was 5, and the CM concentration was 200 µg/ml. Rifampin was prepared in 1% methanol and kept in the dark during preparation, storage, and utilization. Neither the rifampin (100 µg/ml; methanol present at 0.1% vol/vol) nor the puromycin (1 mg/ml) had any effect on the DNA solubilization induced by E2 alone (at multiplicity 200).

TABLE 1. Test for direct inactivation of E2 by interaction with phage T4^a

Additions	Percent survival of <i>E. coli</i> B/4
amN55x5	100
Colicin E2	2.0
Colicin E2 + amN55x5	2.1

^a Colicin E2 and amN55x5 were diluted in GCA medium to 2×10^{11} and 6×10^{10} killing units per ml, respectively. The diluted colicin and phage were incubated, alone or together at 30 C for 5 min, and subsequently tested for killing units by transferring 0.1-ml portions to 2 ml of *E. coli* B/4 at 5.0×10^8 cells per ml at 37 C. After 10 min of incubation at 37 C, samples were taken for determination of percent *E. coli* B/4 survivors. Survivors were determined according to procedures referred to in Materials and Methods.

bacterial surface and the T4 structural protein(s) is adequate to account for the inhibition of E2 in CM. This interaction is known to occur in at least two steps (41, 43), termed reversible and irreversible adsorption, respectively. Our experiments to this point have been done under conditions of irreversible adsorption exclusively. To test the possibility that reversible adsorption alone is capable of inhibiting E2, an amber

FIG. 5. Comparison of intact phage with ghosts as inhibitors of E2. Procedures and conditions were the same as for Fig. 1 except that the bacteria were at 5.0×10^8 cells/ml, the E2 multiplicity was 200, and a concentration of 200 μ g of CM per ml was used for all conditions tested. The intact phage were purified by method B (highly purified phage) of Materials and Methods.

FIG. 6. Comparison of the inhibition of E2 by purified or highly purified phage preparations. Procedures and conditions were the same as for Fig. 4 except that highly purified phage (method B; Materials and Methods) were compared with purified phage (method A; Materials and Methods) at identical multiplicities based on plaque-forming titers. Symbols: $\circ$, E2 alone; Δ , E2 + [42] (highly purified); $\square$, E2 + [42] (purified); ∇ , E2 + [42] (control). The designation [42] (control) is defined in the text.

mutant of T4 in gene 12 was used. When grown in a nonpermissive host, this mutant produces defective phage particles that undergo reversible adsorption without completing the irreversible step (17, 28, 42). Shortly after the initial ad-

sorption, the particles desorb, apparently because the tail pins fail to anchor to the cell wall (42). These defective phage, referred to as gene 12 particles, were found to be completely incapable of inhibiting the E2-induced DNA solubilization (Fig. 7). This result suggests that reversible adsorption alone is not inhibitory to E2; however, this conclusion should be accepted with caution because reversible adsorption by gene 12 particles is only transitory.

In summary, the preceding results suggest that the E2 inhibitory event occurs after reversible adsorption but prior to entry of T4 DNA into the host and is most likely due to the activities associated with irreversible adsorption.

The effect of E2 concentration on the extent of inhibition by T4. It has been shown (Fig. 1 and 2) that the protein synthesis-independent inhibition of E2 by T4 varies with the T4 multiplicity. To test the possibility of a reciprocal competition between E2 and T4,

FIG. 7. Effect of gene 12 particles on the E2-induced DNA solubilization. Procedures and conditions were similar to those used in Fig. 1 except that the bacterial concentration was 5.8×10^8 cells per ml and the E2 multiplicity was 50. No intact phage have been used in this experiment. The multiplicity of gene 12 particles (g12p) was 20.

we proceeded as follows. E2 at various multiplicities was added to a series of flasks containing *E. coli* B pretreated 5 min with 200 μ g of CM/ml. To one series of flasks, no further additions were made, and incubation with shaking at 37 C was continued over the 60-min sampling period; this series was the source of control rates of DNA solubilization, i.e., with no phage added. To another series containing an identical range of E2 multiplicities, intact phage at a multiplicity of 4 were added to each flask simultaneously with the colicin. In a similar manner, intact phage at a multiplicity of 8, or phage ghosts at a multiplicity of 4, were tested. For each multiplicity of E2, corresponding uninhibited (y_0) and inhibited (y_i) rates of DNA solubilization were determined, as described in the legend to Fig. 2. When the corresponding rates are plotted as a function of E2 multiplicity (Fig. 8), it can be seen that increasing the E2 multiplicity markedly reduces the extent of the protein synthesis-independent inhibition. Further information can be derived more readily from the data of Fig. 8 when it is plotted as in Fig. 9. Here, the fractional inhibition, $(i) = [1 - y_i/y_0]$, is plotted as a function of E2 multiplicity. It is seen that the fractional inhibition remains virtually constant over the range of E2 multiplicities tested. These results indicate that the protein synthesis-independent inhibition of E2 is a reversible process in a qualified sense: although it is clear that inhibited cells of *E. coli* B respond to higher multiplicities of E2 by in-

FIG. 8. Effect of E2 multiplicity on the rate (y) of DNA solubilization at various intact phage or ghost multiplicities. Procedures and conditions were similar to those described in Fig. 5 except for the modifications noted in the text. y = percentage of DNA solubilized per minute.

FIG. 9. Effect of E2 multiplicity on the fractional inhibition (i) by intact phage or ghosts at various multiplicities. From the data presented in Fig. 8, the fractional inhibition (i) = $[1 - y_i/y_0]$ has been calculated and plotted as a function of E2 multiplicity.

creasing the rate of DNA solubilization, a given multiplicity of phage will diminish the rate of solubilization by a constant fraction, regardless of the uninhibited rate determined by the E2 multiplicity.

Adsorption of T4 does not release an inactivator of E2. One explanation for the inhibitory effect of phage infection may be that it causes the release of an E2-inactivating material. On the testable assumption that this material would be liberated into the surrounding medium, we looked for such a substance in the following way. A supernatant fluid was prepared from a culture of *E. coli* B that had been infected with T4 mutant [42]. Details are included in the legend to Fig. 10. This supernatant fluid, referred to as infected cell supernatant fluid, should contain the hypothetical inactivator of E2. In a similar manner a supernatant fluid was prepared from an uninfected culture of *E. coli* B. This will be referred to as uninfected cell supernatant fluid. To each of the supernatant fluids, as well as to fresh GCA medium, E2 was added to yield a final concentration of 2.50×10^{10} /ml. The vessels were then incubated at 37 C. Evaluation of the percentage of E2 activity remaining after specified intervals was achieved by transferring portions from the three flasks to cultures of *E. coli* B/4. The number of E2 killer particles was determined from the percentage of survivors of *E. coli* B/4. The percentage of killing activity of the colicin remaining as a function of the incubation time at 37 C is illustrated in Fig. 10. The nonspecific inactivation of E2 in GCA medium is compatible with the thermostability of E2 reported by others (34). Unexpectedly, the infected cell supernatant fluid protects E2 from this inactivation, whereas the

FIG. 10. Test for an E2-inactivating material released by phage infection in the presence of CM. *E. coli* B was grown to a concentration of 5.0×10^8 cells/ml in GCA medium at 37 C. Portions of 20 ml each were preincubated with 100 μ g of CM per ml at 37 C and then either left incubating for 15 min at 37 C for the uninfected cell supernatant fluid, or infected with amN55x5 at a multiplicity of 20 and left incubating for 15 min at 37 C. After incubation, the cultures were chilled on ice and centrifuged at $5,000 \times g$ for 15 min. The resulting supernatant fluids were recentrifuged at $23,000 \times g$ for 2 h to remove any unadsorbed phage. The remaining procedures are described in the text.

uninfected cell supernatant fluid does not. The capacity of phage infection to elicit the protective factor depends on the multiplicity of infection (MOI); a phage MOI of 20, but not 10, will result in the appearance of the protective factor (data not shown). We have not yet attempted to characterize the nature of this protective factor. Suspension of ^3H -dT-labeled *E. coli* B in the supernatant fluid containing the protective factor does not result in a diminished rate of DNA solubilization induced by E2 alone (data not shown). Hence, any interaction occurring between the protective factor and E2 does not interfere with the subsequent capacity of E2 to induce DNA solubilization. It is clear that

irreversible adsorption of intact phage is not releasing E2 inactivating material. It still remains possible that an inactivating material is being produced but remains bound to the surface of the bacterium. We have not tested ghosts for ability to produce the protective factor or an inactivating factor.

Phage T4 inhibits even when added after E2 has initiated solubilization of DNA. As shown in Fig. 11, the inhibition of E2-induced DNA solubilization occurs even when T4 infection is delayed until 17.5 min after E2 addition. Hence, within the interval tested, the normal sequence of events induced by E2 does not proceed to a stage that is refractory to inhibition. For the later times of phage infection, there is an apparent lag period before the inhibition becomes maximal. This may indicate that the mechanism by which T4 inhibits E2 requires several minutes to become fully developed.

Generality of the inhibition by phage. To establish the generality of the E2 inhibition, phages T2H and T6 were tested on *E. coli* B for ability to inhibit E2-induced DNA degradation. As shown in Fig. 12, the inhibition occurs to an extent equal to that seen with T4D. In addition, the ability of T4D to inhibit E2 on *E. coli* K-12 strains CR63 and W3110, as well as on *Shigella dysenteriae* and *S. sonnei* was tested. The results, illustrated in Fig. 13, indicate that all four bacteria support the inhibition by phage.

FIG. 11. Effect of delaying addition of phage on the E2-induced DNA solubilization. Procedure and conditions were similar to those used in Fig. 1 except the E2 multiplicity was 200, the CM concentration was 200 μ g/ml (CM present in all cases). Symbols: no phage added ($\circ$); phage (highly purified amN55x5) added at 0 min (Δ); 2.5 min ($\bullet$); 7.5 min ($\square$); 12 min (∇); and 17.5 min ($\blacksquare$).

FIG. 12. Effect of phage T2H and T6 on E2-induced DNA solubilization. Procedures and conditions were the same as for Fig. 1 except the bacterial concentration was 5.8×10^8 cells/ml, and the E2 multiplicity was 50. Wild-type phage were used in each case.

DISCUSSION

We have observed that infection with phage T4 in the presence of CM inhibits E2-induced DNA solubilization in *E. coli* B, and that an important determinant of the extent of inhibition is the phage multiplicity (Fig. 1). By comparison with intact phage, ghost particles possess a greatly enhanced capacity to inhibit E2 (Fig. 5) that is not markedly altered by the presence or absence of CM (data not shown). Elimination of contaminating ghosts from T4 stocks by zone sedimentation diminishes but does not eliminate the capacity of T4 to inhibit E2 (Fig. 6). We conclude that the inhibition of E2 produced by T4 is not solely attributable to ghost contamination. Although increasing the CM concentration produces a slight decrease in the extent of inhibition of E2 by T4 infection (Fig. 3A), the effect is probably attributable to a direct enhancement of nucleolytic activity (Fig. 3B). It is known that DNA synthesis continues for some time after addition of E2 even though degradation is occurring concurrently (26). This concomitant synthesis might allow re-incorporation of ^3H -dT released from host DNA and produce an apparent reduction in the rate of DNA solubilization measured as acid-insoluble ^3H . Since CM inhibits DNA synthesis (always after a 20- to 30-min lag) to an extent dependent on CM concentration (34, 45), one might expect to see a net increase in the rate of apparent solubilization as the CM concentration is increased.

Which stage(s) of E2 action is subject to inhibition? We have observed that infection with T4 in the absence of protein synthesis inhibitors E2-induced DNA solubilization even if phage infection occurs well after E2 has initiated solubilization (Fig. 11). The target-specific activities induced by E2 appear to act in three different stages (39). Stage I occurs almost immediately after addition of E2 to a sensitive bacterium and involves the introduction of single-strand nicks into the DNA. After a 4- to 5-min lag, stage II (cutting) converts the DNA to double-stranded fragments. Stage III (solubilization) initiates shortly thereafter. Conceivably then, there are several different functional sites in the E2-induced series that may be susceptible to inhibition by T4 infection. By the time stage III becomes apparent, stages I and II usually have proceeded to completion (39). Because T4 infection stops E2-induced solubilization even after solubilization has begun, we conclude that the inhibition definitely is directed at stage III activity; however, the present evidence does not indicate whether stages I and II are inhibited as well. In part III of this series (Swift and Wiberg, manuscript submitted for publication) we present evidence from zone sedimentation analyses of DNA degradation products that the inhibition by T4 infection in the presence of CM is directed mainly at stage III, thus supporting the indirect evidence discussed above. Furthermore, the fact that the initial stages of E2 action apparently are not subject to impairment by T4 infection in CM is strong evidence that the inhibition of E2 is not due to either a direct inactivation of the E2 molecule by T4 or T4 infection, or to interference by T4 in the ability of E2 to adsorb to the cell. More direct evidence has been presented already against direct inactivation (Table 1 and Fig. 10) and against an effect on adsorption (44); however, the adsorption test was performed only in the absence of CM.

Which stage(s) of phage infection is inhibitory to E2? Since phage ghosts are effective inhibitors of E2, and gene 12 particles are not (Fig. 7), we conclude that the stage of phage infection that is inhibitory to E2 occurs after reversible adsorption of the phage particle to the bacterial surface, but prior to injection of the phage DNA into the host bacterium. Strictly speaking, the use of ghosts to determine which stage of phage infection is involved in the inhibition of E2 depends on the debatable assumption that, in CM, ghosts and intact phage inhibit E2 by means of qualitatively similar mechanisms. Evidence supporting this assumption will be presented later in the discussion. Reversible adsorption of phage T4 involves an

FIG. 13. Inhibition of E2-induced DNA degradation by phage T4 on strains of bacteria other than *E. coli* B. Growth of bacteria, prelabeled of DNA with ^3H -dT, and infection were performed in M medium for the *Shigella* strains and in GCA medium for the *E. coli* strains. The remaining procedures and conditions were identical to those described in Fig. 1. Symbols: $\circ$, E2 alone; $\bullet$, E2 + phage.

interaction between the long tail fibers of the phage and specific lipopolysaccharide receptors on the bacterial surface (51). The next step in the adsorption process is an irreversible attachment of the tail pins of the phage base plate to the bacterial surface. Following contraction of the phage sheath, the tail core (needle) is forced to penetrate the lipoprotein, lipopolysaccharide, and mucopeptide layers of the cell wall (41). Although the needle does not seem to penetrate the plasma membrane (41), it may undergo a physicochemical interaction with the membrane resulting in a DNase-insensitive bridge for the transfer of the phage genome into the cell's interior (3). At a still undefined stage of the adsorption process, a lytic activity inherent in the phage structural apparatus appears to promote localized dissolution of the bacterial mucopeptide layer (18, 27, 37, 38). Clearly there is a

variety of activities occurring on the bacterial surface, as a consequence of irreversible adsorption of T4, that may play a role in the inhibition of E2.

What is the mechanism by which phage infection in CM inhibits E2? A number of bacterial functions have been shown to be impaired by infection with ghosts or intact phage in the absence of protein synthesis. Among these inhibitable functions are permeability and transport properties of the plasma membrane (7, 12, 16, 22, 25, 32, 40, 52) and synthesis of host macromolecules (13, 14, 20, 36). In almost all cases, ghosts exhibit a far greater capacity to inhibit than do identical multiplicities of intact phage in the absence of protein synthesis, thus paralleling our observation with E2.

Because the inhibition of E2 appears to be initiated by events restricted to the bacterial

surface, it seems most logical that intracellular effects are attributable, directly or indirectly, to a modification of some structural or catalytic function of the bacterial surface. Possible consequences of a T4-induced modification of the cell surface include the following: (i) a highly specific inhibition of E2-induced deoxyribonuclease(s) (DNase) mediated by a mechanism comparable to that proposed to explain the action of colicins (reference 8; this mechanism is detailed further below), (ii) a disruption of energy metabolism, or (iii) a permeability modification with consequent alterations of intracellular composition.

Does T4 act by mechanism A—colicin-like activity? A widely accepted mode of action proposed for colicins (8) regards the membrane as a structure composed of repeated subunits (protomers) which interact cooperatively with each other; binding of the colicin to a specific receptor on the membrane induces conformational changes in one or more of the protomers, followed by amplification of the change to neighboring protomers. As a consequence of the changes occurring in the membrane, regulatory proteins attached to the inner surface of the membrane undergo functional modification and subsequently produce specific alterations in intracellular activity. By analogy with this model for colicins, the phage particle may induce conformational changes in the membrane which result in the release or activation of a regulatory protein whose specific function is the inhibition of E2-induced DNase(s). We are unaware of any data that can either refute or support mechanism A; at present, such a mechanism is not readily amenable to direct experimental test.

Does T4 act by mechanism B—disruption of energy metabolism? Beppu and Arima (4) presented evidence that a supply of adenosine 5'-triphosphate (ATP) is not required to sustain solubilization of DNA once solubilization has been initiated by E2. In contrast, T4 infection (in CM) inhibits E2 even when added well after solubilization has begun. Consequently, it appears that depletion of ATP by interference with energy production is not the primary mechanism by which the phage particle inhibits E2.

Does T4 act by mechanism C—permeability modification? It was noted that the phage tail core may interact directly with the plasma membrane during the adsorption process. Conceivably, this interaction could result in direct structural damage to the membrane with consequent alterations of permeability.

Alternatively, disorganization of the plasma membrane may result as a secondary consequence of mucopeptide lysis induced by the

phage particle itself. According to this model, the high osmotic pressure within the cell would promote localized evagination and stretching of the plasma membrane into an area of weakened mucopeptide. The resultant stretching of the membrane could increase the permeability by dilation of pores or by brittle fracture (10), with consequent osmotic equilibration of the cell. Barrington and Kozloff (2) and Brown and Kozloff (7) have measured the lytic activity of T-even phage on isolated cell walls of *E. coli* and have observed that freeze-thawing of the phage particles significantly enhances their lytic capacity. This enhanced lytic activity remained associated with the phage particle even after high-speed centrifugation. Further, the enhanced capacity to solubilize cell wall material was ascribed solely to increased availability of a lytic activity qualitatively similar to that in intact phage. Assuming that osmotic shock of phage promotes an analogous increase in lytic activity, the foregoing discussion provides a reasonable explanation for the increased capacity of ghosts to impair host functions. According to the proposed consequences of mucopeptide dissolution referred to above, ghosts would promote greater leakiness of the cell membrane because of their more extensive lytic activity.

How can alterations of membrane permeability inhibit E2-induced nuclease(s)? Presuming that the inhibition of E2 is attributable to alterations of membrane permeability, we must still learn the mechanism by which the altered permeability results in selective inhibition of DNase(s) associated with stage III but not stages I and II of E2 action. The two observations that are probably most relevant are (i) the fraction of bacterial DNA that eventually is solubilized is virtually independent of the phage multiplicity and (ii) the fractional inhibition produced by a given phage multiplicity is independent of the rate of solubilization induced by E2 (Fig. 8 and 9). These results are compatible with the concept of a phage-induced depletion of DNase activity, either through direct loss from the cell of the enzyme itself, or indirectly through loss of essential cofactors or activators of the enzyme. Since some DNases depend on ATP for activity (1, 23), ATP would appear to be a likely candidate for a co-reactant whose depletion might limit DNase activity. However, as mentioned above, ATP apparently is not required for E2-induced solubilization, since shifting succinate-grown bacteria to an anaerobic environment after solubilization of DNA has begun does not interfere with continued solubilization (4). This result also argues against a role for any compound derived from

ATP (e.g., *S*-adenosyl methionine) in the limitation of DNase(s). Alternatively, it is possible that alteration of the intracellular ionic environment is directly responsible for the inhibition of E2. Finally, it cannot be excluded that direct loss of DNase(s) from the cell interior is occurring.

Chapman et al. have reported that the gamma ray-induced solubilization of *E. coli* DNA is susceptible to inhibition by ghosts or intact phage in the absence of protein synthesis (9). In addition, Farrell and Reiter have shown that phleomycin-induced DNA solubilization is also inhibited by infection with phage T4 (personal communication). Hence, it appears that the phage's capacity to inhibit nucleolytic activity is not restricted to that induced by E2.

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