THE INHIBITION OF BACTERIOPHAGE DNA INJECTION BY DYES BOUND TO THE DNA

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ABSTRACT A delay (~10 min) in the appearance of intracellular phage is caused by preincubating the infecting phage $T4o_1$ in proflavine, acridine orange, or ethidium, but not polyamines. No significant delay in attachment is observed. Apparently the presence of the dye is required inside the permeability barrier of the phage at the time of infection. The effect of proflavine is reduced in the presence of polyamines, suggesting that the active site is on DNA. The phage-host complex is sensitive to shear if the infecting phage have been incubated in proflavine or ethidium, indicating that the completion of DNA injection is delayed. Finally no partially injected complexes could be detected after shearing, which suggests that most of the delay occurs near the beginning of the injection process.

INTRODUCTION

It has long been known that certain acridine compounds interfere with the maturation of T-even bacteriophage (Foster, 1948). Recently it has been demonstrated that it is the phage head that is affected (Piechowski and Susman, 1967). Since the demonstration by Lerman (1961, 1963, 1964) that acridines bind to DNA by intercalating between base pairs, a plausible explanation of the inhibition of phage maturation has been that the acridine prevents proper folding of the DNA, since intercalation causes a lengthening and probably a stiffening of the DNA helix. This possibility is supported by the recent finding that intercalation of acridine dyes is suppressed in binding to the DNA of intact phage (Dusenbery and Uretz, 1972), which suggests that there is not sufficient space in the phage head for much lengthening of the DNA.

In view of the above considerations we wondered whether acridines would also inhibit injection of DNA from the phage. This question assumes importance from the possible implications about the injection process and possible uses of the phenomenon. For instance if it were possible to slow down the rate of injection,

methods might be developed to determine the direction in which DNA is injected, if there is a specific direction. In addition it might prove possible to isolate mutant phage in which injection is not inhibited and thus obtain a method for studying the injection process. Finally such an inhibition would provide a new tool for use in studies of the role of DNA in various phage functions during infection.

The methods used in this study were largely determined by consideration of the fact that high concentrations of various dyes frequently lead to an irreversible loss of viability of phage, which is probably caused by a failure to inject. In order to avoid this complication we chose to employ biological assays which would characterize only the viable phage.

MATERIALS AND METHODS

All manipulations involving dyes were carried out under red fluorescent lights (General Electric Co., Schenectady, N. Y.). Proflavine hydrochloride was free of other dyes as determined by paper chromatography in two solvents (Albert, 1966, p. 150). Ethidium bromide was obtained from Boots Pure Drug Co., Ltd. (Nottingham, England). Putrescine dihydrochloride was obtained from ICN Nutritional Biochemicals Div. (International Chemical and Nuclear Corp., Cleveland, Ohio).

Diluted phage buffer (DϕB) contained per liter of distilled water 0.7 g Na_2HPO_4, 0.3 g KH_2PO_4, 0.5 g NaCl, 1.0×10^{-4} M $MgSO_4$, 1.0×10^{-5} M $CaCl_2$, and 0.001 g gelatin. Nutrient broth (NB) contained per liter of distilled water 5.0 g NaCl and 8.0 g dehydrated nutrient broth (Difco Laboratories, Detroit, Mich.). Phosphate buffer (PB) contained per liter of distilled water 6.0 g Na_2HPO_4 and 3.0 g KH_2PO_4.

The phage strains used were $T4Bo_1$ (osmotic shock resistant) and T4rIIB638. Both were grown on *Escherichia coli* B/r in NB and plated on tryptone plates (Chase and Doermann, 1958).

The phage ($T4o_1$) were incubated with proflavine in DϕB for 2 hr at 45°C, which is sufficient to reach equilibrium. In the case of ethidium bromide the incubation was extended to 14 hr. After the proper incubation time the mixture was placed on ice until it was used (within 3 hr). The phage concentration was about 1×10^{10} phage/ml.

In all experiments the host bacteria were *E. coli* B prepared by diluting an overnight culture 1000-fold into cold NB, shaking in a 37°C incubator for 160 min, centrifuging, and resuspending in 0.1 vol of fresh, cold NB. At this point the bacterial concentration was approximately 5×10^8 and the culture was kept on ice until needed (up to 3 hr).

5 min before an intracellular phage experiment was to start approximately 5 ml of the host bacteria was pipetted into a tube in a 37°C shaking water bath. A minute later a portion of the phage incubation mixture was diluted 100-fold into cold NB (or a dilution from the phage stock was made for control experiments). The experiment was begun by adding 0.1 or 0.2 ml of the phage dilution to the host bacteria (multiplicity less than 0.01). 5 min later the infected bacteria were diluted 100-fold into fresh NB also at 37°C. 8 min after infection a sample was diluted into PB containing 0.01 M KCN (PB-KCN). This sample provided a measure of the number of infected cells. At various later times samples were diluted 100-fold into NB saturated with chloroform. These samples provided a measure of the number of mature phage present at the time of dilution.

The experiments on the rate of attachment were similar except that samples were taken from 1 to 5 min after infection and diluted into chloroform-saturated NB. The blending experiments involved a similar procedure except for the following modifications. Only 2 ml of host bacteria

were used. At 5, 10, or 15 min after infection a 1.0 ml sample was quickly transferred to the Sorvall Omni-Mixer microchamber (Ivan Sorvall, Inc., Norwalk, Conn.). The blender was run at half-speed for 1 min with ice-water cooling of the chamber. While the blender was running two additional samples were taken from the infection tube. One was diluted 100-fold into PB-KCN for determination of the number of infected cells and the other into NB saturated with chloroform for the number of mature phage. After shearing was completed a sample from the blender was similarly diluted into PB-KCN for determination of the number of infected cells which had survived blending.

The rescue experiments were carried out in an identical manner except for the following. At 2 (or 5) min after infection the culture was superinfected with phage T4r at a multiplicity of 1.7. Half the culture was blended as above 7(10 or 15) min after infection and samples from both halves were diluted 100-fold into PB-KCN. After appropriate further dilution in the same solution the samples were plated with *E. coli* K(λ) as indicator. Under such conditions only complexes which produce at least one r^+ phage will form plaques. The ratios of plaque-forming units in the blended and unblended samples were determined and compared with those in the ordinary blending experiment.

RESULTS

In order to explore the possible effects on the injection of DNA of compounds which intercalate, we first looked at the time-course of production of intracellular phage after infection with phage which had been incubated with certain compounds. Two dyes were tested: proflavine, a common acridine, and ethidium bromide, a similar but somewhat larger dye that is known to intercalate strongly (Waring, 1966; LePecq and Paoletti, 1967). The results are presented in Fig. 1. It may be seen that both dyes cause a delay in the appearance of intracellular phage. This delay is roughly 5 min for proflavine and 10–15 min for ethidium bromide. In further studies we chose to work with proflavine since it more rapidly penetrates the phage coat and thus is more convenient to work with. Acridine orange also produced a delay, similar to that of proflavine.

In order to exclude the possibility that the delay was due to slower attachment of the phage, we examined these rates. The results, presented in Fig. 2, indicate that the phage incubated in proflavine may attach somewhat more slowly than the control, but the difference (about ½ min on the average) is insufficient to account for the delay in appearance of intracellular phage.

The next step in the characterization of the delay was to determine whether or not the active site for binding of the dye was on the DNA. The first question was whether or not it was necessary to incubate the phage in the dye, since it is known that binding to DNA of the intact phage requires incubation. The results, presented in Fig. 3, demonstrate that without incubation the proflavine has no effect, while incubation produces the expected delay, and that subsequent removal of the dye abolishes the delay. This establishes that the delay in appearance of intracellular phage requires the presence of the dye at some site within the permeability barrier of the phage during infection.

We next turned to the question of whether this site was on the DNA. The first

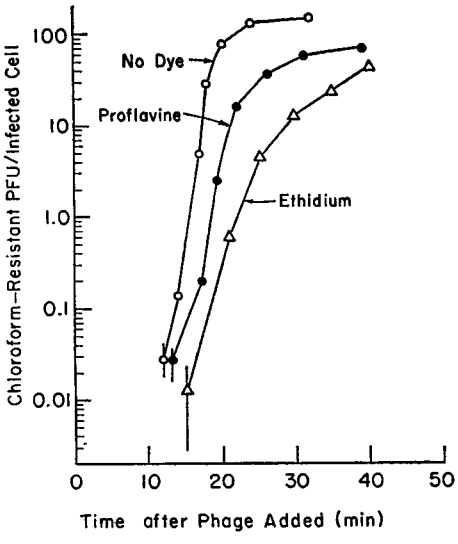

FIGURE 1

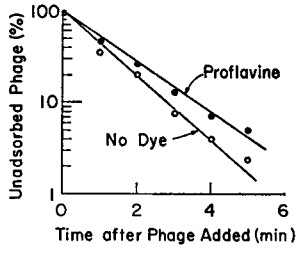

FIGURE 2

FIGURE 1 The time-course of appearance of intracellular phage (chloroform-resistant plaque-forming units [PFU]) after infection with phage incubated in dyes (20 μg/ml).
FIGURE 2 The effect of incubation in proflavine (20 μg/ml) on the rate of adsorption of phage.

approach to the question was to compare the dependence of the delay on the concentration of dye in which the phage was incubated with the dependence of binding to the phage DNA on the concentration of dye. Data on the appearance of intracellular phage after incubation of the infecting phage in various concentrations of proflavine are presented in Fig. 4. It is apparent that the delay in appearance saturates at roughly 10 μg/ml of proflavine. On the other hand binding to intact phage DNA saturates at about 20 μg/ml (8×10^{-5} M) proflavine (Dusenbery and Uretz, 1972). Considering the uncertainties in these measurements, we take this as fair agreement. This comparison is consistent with the hypothesis that the active site is on the DNA.

A more discriminating approach to the problem was to determine whether a compound which is known to bind to DNA competitively with proflavine but does not itself cause a delay could reverse the delay caused by proflavine. Such compounds are the polyamines spermidine and putrescine. The results of one experiment are presented in Fig. 5. It may be seen that incubation in putrescine does not cause a delay and incubation in putrescine and proflavine results in a delay which is much smaller than that which results from incubation in proflavine alone. Therefore these experiments also indicate that the dye which is responsible for the delay in appearance of intracellular phage is bound to the DNA of the infecting phage.

Thus far we have used the delay in appearance of intracellular phage as a con-

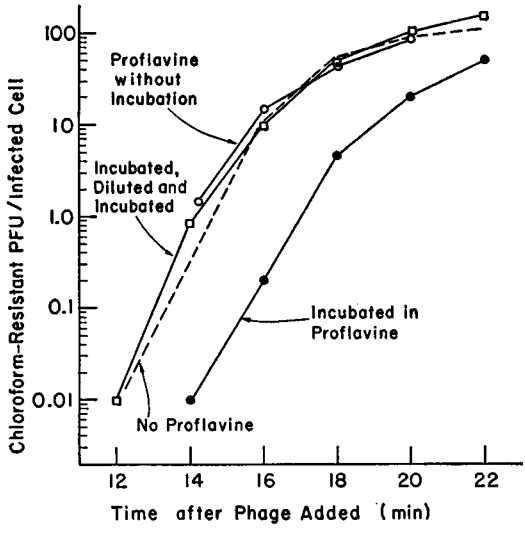

FIGURE 3

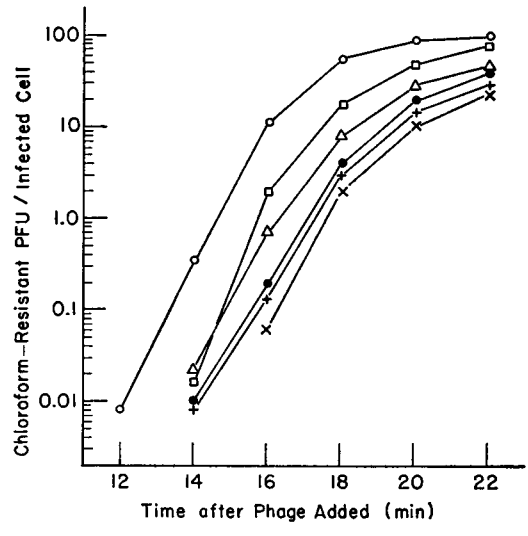

FIGURE 4

FIGURE 3 The time-course of appearance of intracellular phage (chloroform-resistant PFU) after infection with phage exposed to proflavine (20 μg/ml) under various conditions. FIGURE 4 The time-course of appearance of intracellular phage (chloroform-resistant PFU) after infection with phage incubated in various concentrations of proflavine. ○, no proflavine; □, 2 μg/ml; △, 5 μg/ml; +, 10 μg/ml; ●, 20 μg/ml; ×, 50 μg/ml.

venient measure of an effect which is presumed to be a delay in the injection of DNA by the infecting phage. We have demonstrated this presumed delay of infection more directly by determining the sensitivity to shear of the phage-host complex. The results are presented in Fig. 6. It may be seen that complexes formed with untreated phage are resistant to shear but that complexes formed with phage which had been incubated in dye were quite sensitive to shear. This sensitivity was gradually lost after 5 min. The low quantity of mature phage in the experiment eliminates the possibility that resistance to shear resulted from the appearance of mature phage. Incubation in ethidium also produced sensitivity to shear. We thus conclude that the presence of dye bound to the phage DNA inhibits its injection into the host.

The previous experiment demonstrated that the completion of the injection process is delayed, but it did not distinguish between a delay in the initiation of injection and a decrease in the rate of injection. In order to decide between these possibilities we sought to determine the chance of shearing a complex during the injection process. The general method was to attempt to rescue the partially injected complexes by superinfection with mutant phage. The appearance of wild-type phage indicated that the complex had injected the gene for which the superinfecting phage was mutant, since the particular mutant used was a deletion and thus could not revert to wild type. If a significant fraction of the phage which were inactivated by blending succeeded in injecting part of their DNA then some of these partial injections should

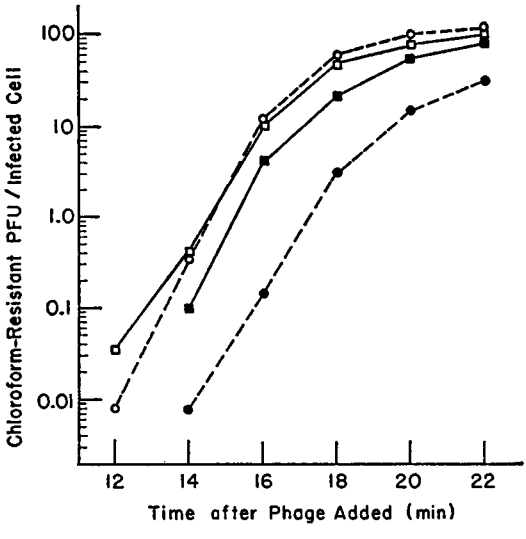

FIGURE 5

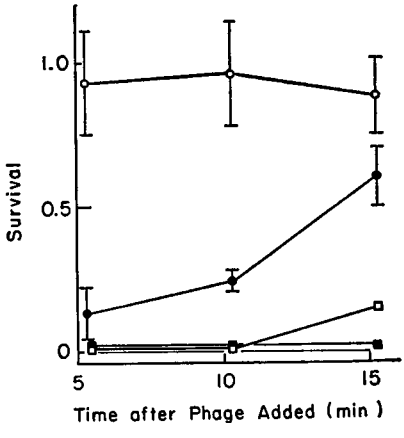

FIGURE 6

FIGURE 5 The time-course of appearance of intracellular phage (chloroform-resistant PFU) after infection with phage incubated in proflavine and/or putrescine. ○, not exposed to either; □, 0.01 M putrescine; ■ 0.01 M putrescine and 10 μg/ml proflavine; ●, 10 μg/ml proflavine.

FIGURE 6 The sensitivity of phage-host complexes to shear. ○, survival after blending of complexes formed with phage not exposed to dye; ●, survival after blending of complexes formed with phage incubated in proflavine (20 μg/ml); □, survival after exposure to chloroform of complexes formed with phage not exposed to dye; ■, survival after exposure to chloroform of complexes formed with phage incubated in proflavine. Means ±95% confidence limits. Each point represents four to six experiments.

TABLE I

PER CENT SURVIVAL AFTER BLENDING

Type of experiment	Time after infection of blending		
	7.3 min	10.3 min	15.3 min
Ordinary	18 ±5*	24 ±3(6)	60 ±9(6)
Rescue	22 ±2(5)	33 ±4(5)	65 ±2(5)

* Calculated by interpolation between five 5-min points and six 10-min points.

Means ±SD (number of experiments).

supply the r^+ gene in the rescue experiments resulting in a significantly higher survival after blending in these experiments compared with the ordinary blending experiments.

The results are presented in Table I. It may be seen that the rescue experiment resulted in only slightly higher survival values which are probably not significantly different from those in the ordinary blending experiment. Thus we are unable to de-

tect any partially injected complexes and must conclude that the delay in injection is mostly at the start and that once initiated injection proceeds rapidly. A more quantitative analysis of this data with comparisons with different models of the time-course of injection is available elsewhere (Dusenbery, 1970).

DISCUSSION

The results indicate that proflavine (and probably ethidium) inhibits the beginning of injection when large quantities are bound to the DNA of the phage. In the Introduction we discussed some reasons for expecting that acridine dyes might inhibit injection; however there was no anticipation that the beginning of injection would prove more sensitive than the over-all rate of injection. The explanation of this fact is not clear, but it could be taken as an indication that during normal injection the initiation is rate limiting and that once started the injection proceeds rapidly, perhaps because increased space inside the phage head allows more rapid uncoiling of the DNA.

We previously discussed the possibility that acridines might inhibit injection because they intercalate. This explanation is consistent with the findings that proflavine and ethidium bromide inhibit injection but that spermidine and putrescine do not; however, the observed dependence of the inhibition on dye concentration indicates that it may be dye bound in the cooperative fashion that is most effective in causing the delay. If this is correct then the relevant properties of the dye may be more closely related to their ability to precipitate DNA or simply to fill up space inside the phage head than to intercalate. Clearly further work will be necessary in order to explain properly the mechanism of the delay. Even without such an explanation the effect reported here may provide a new and useful tool for studying various properties of the phage.

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