A SPECIFIC REVERSIBLE INHIBITION OF BACTERIOPHAGE T2

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With most phages, the titer of a lysate measured by plaque count remains stable from the time of lysis or shows a progressive decrease as phage becomes inactivated by a variety of environmental agents. Freshly prepared lysates of bacteriophage T2 of Escherichia coli rise in titer spontaneously with time, the maximum titer being reached in several weeks under refrigeration. Bertani (unpublished data, 1950) noted that this rise could be accelerated greatly by diluting a lysate in distilled water or by adding ZnSO₄ to a final concentration of one per cent. Watson (personal communication, 1951) observed that lysates of T2 to which antibacterial serum was added before lysis did not show any rise in titer after dilution in distilled water. This suggested that normal lysates contained a certain proportion of particles that are prevented from forming plaques because of combination with specific host materials. The present paper provides evidence of the occurrence of such combination and of the properties of the combined phage.

MATERIALS AND METHODS

Cultures. The bacteriophages studied were T2, T4, T6, and their mutants, whose common host is E. coli, strain B (Delbrück, 1946).

Methods. Phage activity was determined by plaque counts using the agar layer method (Gratia, 1936). Stocks of phages T2, T4, and T6 were lysates from "lysis inhibited" cultures (Doerrmann, 1946) clarified by filtration or low speed centrifugation. The organisms were grown in Difco nutrient broth (containing 0.5 per cent NaCl) or in M9 medium to a density of about 1 x 10⁶ cells per ml. The cultures were infected with about 10 phage particles per cell, and a second equal phage input was made 7 to 10 minutes later. The cultures were aerated at 37 C until clear. Clearing generally occurred about 9 hours after infection; it was rapid, being completed within 15 minutes after the beginning of visible lysis.

RESULTS

Spontaneous and induced rise in the titer of T2 lysates. Increase in phage titer. Freshly made lysates of phage show a spontaneous rise in titer with time (see figure 1). The amount of rise is variable, but all T2 stocks show an increase. The extent and rate of the increase can be reduced by suspending the phage in M/15 phosphate buffer plus 0.85 per cent NaCl (Watson, personal communication, 1951).

Diluting fresh lysates of T2 in distilled water causes a rapid increase in titer. Maximum titers were reached in a 1:50 dilution in distilled water at 37 C. The distilled water effect is observed with T2 lysates, T2r lysates, and T2h lysates prepared either in nutrient broth or in synthetic medium. It is observed with T2 lysates prepared on E. coli, strain B/4, or strain B/6, as host cells and in fresh lysates of T2h prepared on E. coli, strain B/2.

Fresh lysates of T4 and T6 phages prepared on E. coli, strain B, do not show any rise in distilled water. Thus, the increase in titer with dilution in distilled water appears to be peculiar to phage T2 and its mutants.

Filtration of inhibited lysates. Filtration of fresh T2 lysates through Mandler filters (tested at 9 lb pressure) did not eliminate nor reduce...
REVERSIBLE INHIBITION OF BACTERIOPHAGE T2

**TABLE 1**

**Plaque titer in parallel one-step and mass lysates**

A culture of *Escherichia coli*, strain B, in nutrient broth (2.1 × 10⁹ cells per ml) was infected with 4 particles of T2 per cell. Seventeen minutes after infection, an aliquot was diluted 1:10,000 (dilute lysate). When the mass lysate cleared, both lysates were assayed before and after treatment with distilled water. Note that these lysates inhibited cultures yield about 500 particles per ml.

<table>
<thead>
<tr>
<th>LYSATE</th>
<th>TITER AFTER LYSIS, REFERRED TO THE CONCENTRATION IN THE MASS LYSATE</th>
<th>TITER AFTER DILUTION 1:50 DISTILLED WATER, 1 HOUR AT 37 C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass lysate</td>
<td>2.6 × 10⁹</td>
<td>1.4 × 10¹³</td>
</tr>
<tr>
<td>Dilute lysate</td>
<td>1.2 × 10¹³</td>
<td>1.3 × 10¹³</td>
</tr>
</tbody>
</table>

*Figure 1.* Spontaneous and induced rise in the titer of T2 lysates. Freshly made T2 lysates show a spontaneous rise in titer with time (broth assay). The extent and rate of this increase in titer are reduced by suspending the phage in m/15 phosphate buffer plus 0.85 per cent NaCl (buffer assay). Dilution 1:50 in distilled water induces a rapid increase in titer, generally to a higher level than is achieved spontaneously with time (water assay).

The rise in titer is due to a dispersal of phage clumps or to a release of phage from combination with some inhibitor. The following experiment was designed to distinguish between the two alternatives. A filtered, freshly prepared lysate of T2 was diluted so that one ml contained about 5 plaque forming units before water treatment. One drop (equals 0.05 ml) of the dilution was added to each of 50 tubes containing 0.5 ml of distilled water. The tubes were incubated at 37 C for one hour. The entire contents of each tube were plated then by the agar layer method. If the low counts before water treatment were due to the presence of phage clumps, each clump being counted as one plaque, and if the rise were due to disaggregation in distilled water, there should be similar numbers of blank plates in both series, if, on the other hand, the rise is due to an activation of inhibited particles, which are presumably distributed at random, the distribution of plaques will, in both series, be random (Poisson); but the average will be higher by the rise factor in the water treated series since the inhibited particles will manifest themselves in this series. The results, illustrated by the experiment in table 2, are in complete agreement with the expectations from the inhibitor hypothesis. The release of

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*The rise in titer as unmasking of inhibited phage particles.*
TABLE 2
Test of the clump hypothesis versus the inhibitor hypothesis

A filtered, fresh broth lysate of T2 was diluted in broth to contain about 3 particles per drop after water treatment. One drop of the broth dilution was added to each of 50 tubes containing 0.5 ml nutrient broth and to each of 50 tubes containing 0.5 ml distilled water. After 1 hr at 37 C the contents of each tube were plated. From the mean number of plaques per plate in the distilled water plates ( = 3.4), the expected number m of plates containing r plaques was calculated from the equation:

\[ P(r) = n \frac{e^{-m}}{r!} \]

where \( P(r) \) is the fraction of plates with \( r \) plaques and \( n \) is the mean number of particles per drop.

<table>
<thead>
<tr>
<th>NO. OF PLATES PER PLATE</th>
<th>NO. OF PLATES EXPECTED IN THE WATER SERIES ASSUMING 3.4 PARTICLES PER DROP ON THE AVERAGE/m</th>
<th>(x - m)/m</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>37</td>
<td>1.65</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>4.50</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>9.55</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>10.80</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>9.25</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>6.25</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>3.55</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>1.70</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0.75</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>0.30</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0.10</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>0.05</td>
</tr>
</tbody>
</table>

\[ \chi^2 = \Sigma (x - m)^2/m = 4.182 \]

\[ P = 0.85 \]

inhibition hereafter will be called activation of inhibited particles.

Rate of activation of inhibited lysates. Freshly prepared phage lysates either in broth or in M9 medium, diluted 1:50 in distilled water at 37 C, reach maximum titer in 15 seconds. At 5 C the maximum titer is reached in 90 to 120 seconds. Because of these rapid rises, a "dump experiment" (Stent and Wollman, 1950) was devised in which phage was diluted with distilled water into several tubes kept at the desired temperature. The reaction was stopped by "dumping" into one tube a large volume of broth. This method is justified by the fact that upon dilution of lysates in broth there is no rise in titer at 5 C nor at 37 C over a three hour period, even when the titer had risen partially in distilled water. The results are shown in figure 2. The rate of rise in titer depends both on the extent of dilution in water and on the temperature. A rapid rise is observed even in broth if the samples are kept at 65 C. The maximum titer is essentially the same after treatment in distilled water at 5 C and at 65 C (figure 2). Rapid activation at 65 C occurs also in the undiluted lysates. The phage in heat activated lysates does not become reinhibited upon cooling.

The properties of inhibited lysates. Heat treatment. The rapid rise in titer of phage T2 when exposed to high temperatures suggested that activation might complicate heat inactivation studies on T2 lysates. In fact, unlike the other T phages, which show exponential rates of heat inactivation, T2 has been reported as exhibiting a multiple-hit type of inactivation curve (Adams, 1949).

Fresh phage lysates were diluted 1:50 in distilled water or in broth. After one hour at 37 C further 1:100 dilutions in nutrient broth were made, and aliquots were placed in separate tubes in a 65 C water bath. The reaction was stopped at various intervals by dumping 9
volumes of chilled nutrient broth into a reaction tube. Each sample was assayed both with and without further treatment in distilled water at 37 °C; the "treated" assay gives the full titer of residual phage, while the "untreated" assay gives the titer of residual noninhibited phage plus residual heat activated phage. The results of such an experiment are shown in figure 3. The activated phage decays exponentially. The nonactivated phage does not; in platings without water treatment there is a rise in titer to a peak at 2 to 3 minutes and then an exponential decay. Nonactivated phage plated in water shows no rise but a plateau with subsequent exponential decay.

Treatment with antiphage serum. Most phages are inactivated by specific antiphage sera at an exponential rate. With phage T2 exponential inactivation is observed with one-step lysates and with activated mass lysates. Nonactivated lysates treated with dilute antiphage serum exhibit a rise in titer followed by a decline at the same rate as for activated phage (figure 4).

Neither normal rabbit serum nor serum against the host bacteria raised the titer of the crude lysates. Antiphage serum subjected to two absorption cycles with young E. coli, strain B (2 × 10⁶ cells per ml; mixture kept at 48 °C for one hour and at 9 °C for twenty-three hours), could still raise the titer of crude T2 lysates. Absorption of the antiviral antibody with ultraviolet inactivated phage T2 (1 × 10¹⁰ phage per ml) completely removed the ability of the serum to raise the T2 titer as well as its ability to inactivate T2. The results suggest that specific antibody can remove inhibitor from the phage particles without necessarily inactivating them.

Ultraviolet inactivation. Nonexponential inactivation of T2 (and T4, T5, T6) is found with both inhibited and noninhibited T2. Both lysates give curves that extrapolate to an initial value above the point of origin (Benzer et al., 1950; Latarjet and Morenne, 1951).

Prevention of inhibition. The results above suggest that inhibition is the result of phage particles combining with surface elements of bacteria. In fact, inhibition is prevented by treatments that prevent attachment. The rise of titer is absent in lysates produced by allowing lysis to occur in salt-free broth or in 10 per cent (NH₄)₂SO₄, where T2 attachment to host bacteria does not occur. Antiserum against the bacterial host partially prevents the formation of inhibited phage.

The properties of inhibited phage particles. Plaque morphology. The most striking chara-
teristic of inhibited T2 lysates is their poor plaque forming ability. The inhibited lysates produce a majority of small, irregular plaques. The more inhibited the lysate, the greater is the proportion of these poor plaques. Distilled water activation, in addition to activating phage, changes all plaques to their normal morphology. This finding suggests that inhibition is not an all or none phenomenon, but that there are transition forms between noninhibited phage and fully inhibited phage. Presumably, the poor plaques stem from partly inhibited phage particles that are still adsorbed by bacteria, although more slowly.

**Adsorption of inhibited and activated T2.** Adsorption was studied by means of streptomycin treated bacteria, which permitted observation over a long period of time. Streptomycin (50 or 100 micrograms per ml) was added to actively growing cultures of *E. coli*, strain B, in nutrient broth, and the cultures were aerated vigorously for one hour; the proportion of survivors was then determined by limiting dilution. These streptomycin killed cells can adsorb phage specifically but do not release new phage.

Aliquots of the cell suspension were mixed with samples of inhibited lysates or of water activated lysates. Assays were made at intervals to determine residual phage. Activated phage is adsorbed rapidly at an approximately exponential rate. The noninhibited fraction of phage present in mass lysates is adsorbed less rapidly. The total phage, noninhibited as well as inhibited, is adsorbed very slowly; even after 24 hours, 43 per cent of the total phage in fresh lysates remains unadsorbed.

**The specificity of the inhibitor for phage T2.** Inactivation of phage T2 by fresh lysates of other phages. Among the T-even phages of *E. coli*, strain B, only T2 exhibits the inhibition-activation phenomenon. Fresh lysates of phages T4 and T6 showed no rise in titer even though the same host cells would have produced inhibited T2 lysates. The following experiment was performed to determine whether inhibitory material was produced in T6 lysates or if the phage in such lysates withstood or overcame inhibitory action. Known quantities of fully active T2 were added to lysates inhibited, T6-infected broth cultures of *E. coli*, strain B, within 15 minutes after visible clearing. The mixture was assayed 15 and 60 minutes later for T2 and T6 activity, before and after water treatment (table 3). The T6 titer showed no increase with water treatment. The added active T2 lost 75 per cent of its titer in 15 minutes. This loss was reversed completely by water activation. If T2 was added to the lysate two hours after clearing, no inhibition of T2 occurred. Lysates of T6 prepared on *E. coli*, strain B/2, which is unable to adsorb T2, fail to inhibit phage T2 but inhibit phage T2h, which is adsorbed by strain B/2.

The fact that not all the added T2 phage was inhibited in the T6 lysates suggested that the inhibitors may disappear rapidly after lysis. The following experiment confirmed this suggestion.

**TABLE 3**

*Inactivation of phage by fresh lysates and by sonically disrupted cells*

Broth cultures of *Escherichia coli*, strain B (1 x 10^8 per ml), were lysed by phage (lysis inhibited by infection with 20 to 50 particles per cell) or sonic vibration. To aliquots of the lysates, known quantities of fully active phage were added within 15 minutes after the lysates and cleared. After 15 to 60 minutes, incubation at 37 C, the lysates were assayed before and after water treatment.

<table>
<thead>
<tr>
<th>ADDED PHAGE</th>
<th>HOST CELL LYSED</th>
<th>LYSING AGENT</th>
<th>INPUT TITER</th>
<th>LOW POINT TITER</th>
<th>FINAL TITER AFTER DISTILLED WATER TREATMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
<td>B</td>
<td>T6</td>
<td>1.6 x 10^8</td>
<td>6.2 x 10^4</td>
<td>1.5 x 10^6</td>
</tr>
<tr>
<td>T2</td>
<td>B</td>
<td>T6</td>
<td>1.3 x 10^8</td>
<td>2.5 x 10^4</td>
<td>1.1 x 10^6</td>
</tr>
<tr>
<td>T2</td>
<td>B/2</td>
<td>T6</td>
<td>1.2 x 10^8</td>
<td>1.2 x 10^4</td>
<td>1.3 x 10^6</td>
</tr>
<tr>
<td>T2h</td>
<td>B/2</td>
<td>T6</td>
<td>1.0 x 10^8</td>
<td>7.4 x 10^4</td>
<td>1.0 x 10^6</td>
</tr>
<tr>
<td>T2r</td>
<td>B</td>
<td>T2</td>
<td>1.2 x 10^8</td>
<td>1.2 x 10^4</td>
<td>1.2 x 10^6</td>
</tr>
<tr>
<td>T2h</td>
<td>B</td>
<td>T2</td>
<td>1.4 x 10^8</td>
<td>1.3 x 10^4</td>
<td>1.3 x 10^6</td>
</tr>
<tr>
<td>T2</td>
<td>B (filtered)</td>
<td>sonic waves</td>
<td>5.2 x 10^8</td>
<td>3.8 x 10^4</td>
<td>6.5 x 10^6</td>
</tr>
<tr>
<td>T2</td>
<td>B (filtered)</td>
<td>sonic waves</td>
<td>5.2 x 10^8</td>
<td>5.0 x 10^4</td>
<td>5.1 x 10^6</td>
</tr>
</tbody>
</table>
Known quantities of phage T2r or T2h were added to T2-infected, lysed inhibited cultures within 15 minutes after complete lysis. Fifteen minutes later the mixtures were assayed for T2, T2r, T2h, with and without water treatment. There was an eightfold rise in T2 titer but no change in T2 or T2h titer. Inhibitory material had been present as shown by the rise in T2 titer, but it had become unavailable. Either the free inhibitory material is unstable, or it is removed by combination with the phage.

**Phage inactivation by sonically disrupted cells.** The experiments of the preceding sections substantiated the bacterial origin of the inhibitors present in fresh lysates. Bacterial extracts obtained by methods other than phage lysis might also contain such inhibitory material. Young cultures of *E. coli*, strain B, in broth were disrupted by sonic vibration (25 minutes at 9 kilocycles) leaving $5 \times 10^6$ survivors out of a population of $2 \times 10^8$ cells per ml. Unfiltered aliquots of this suspension were mixed with known quantities of active T2 and incubated for 30 minutes at 37°C. Unfiltered cell debris inactivated over 99 per cent of the added phage T2. Distilled water treatment reactivated 12 per cent of this, a fifteenfold rise from the lowest titer. Filtration of the sonicated cell suspension through a Mandler candle removed the inactivating material; filtration of a mixture of phage and disrupted cells removed all the inhibited phage. Thus, phage inhibited by sonically broken bacteria differs from phage inhibited in natural lysates, which can pass bacterial filters.

**DISCUSSION**

Bacteriophage T2 is present in fresh lysates of *E. coli*, strain B, in an inhibited form, from which it is slowly released. Several problems are of interest in relation to this inhibition: the nature of the inhibitor, the mechanism of phage-inhibitor combination, the difference between T2 and other related phages.

In favor of the bacterial origin of the inhibitor and of its combination with phage after lysis are the observations that inhibition occurs only in mass lysates and that it can be prevented by treatments that prevent phage-bacterium combination (removal of NaCl, addition of an excess of (NH₄)₂SO₄, addition of antibacterial serum). The specificity of the inhibition, shown by experiments on the inhibition of one phage in fresh lysates of another, is similar to the specificity of phage adsorption.

Inhibited phage is present in lysates in the form of single particles. This indicates that the inhibitor, which is filtrable, is of such a nature as not to bind together several particles. Instead, sonic extracts of bacteria give a partially reversible inhibition, but these inhibitors are not filtrable.

The inhibitor in fresh lysates acts by preventing or retarding the adsorption of phage T2 onto the susceptible cells. Schlesinger (1932), Delbrück (1940), and Puck et al. (1951) have compared the adsorption rate of phage with the maximum possible rate (100 per cent collision efficiency). Under optimum conditions, the two rates are similar. In the inhibited lysates the phage appears to be in a variety of forms with collision efficiencies ranging down to zero.

Puck and his co-workers (Puck et al., 1951; Garen and Puck, 1951) have analyzed the role of various ions in phage-host combination. In a medium that by itself does not promote phage attachment, the monovalent ions (Na⁺, K⁺) are most effective in promoting adsorption of phage T2 at a concentration of 0.1 M. The rate of adsorption diminishes below this concentration and is negligible in distilled water. Higher ion concentrations beyond the optimum level retard adsorption. These ionic effects are concerned with the first, reversible reaction of adsorption.

Inhibition of phage T2 in fresh lysates is prevented by ammonium sulfate in a concentration equivalent to 0.76 M and can be reversed by a 1:50 dilution of broth in distilled water, lowering the sodium ion concentration to about $2 \times 10^{-5}$ M. Slow activation occurs upon dilution of sodium ions to a concentration around $1.7 \times 10^{-3}$ M, which is below the optimum for adsorption.

We have no explanation of the mechanism of activation by heat treatment. Foster et al. (1949) noted a rise in titer in phage T2 after heating and proposed the following interpretations: (a) breaking up of clumps; (b) increased enzyme activity on the part of the phage; (c) some form of heat activation of the phage itself, as in spore germination. Our experiments appear to disprove the first and third hypothesis and do not support directly the second one. Studies on the activation of inhibited phage T2 and on the
disappearance of inhibitors from lysates of other phages may indeed reveal some enzymatic actions of phage.

An unexpected result is the activation of inhibited phage T2 by antiphage serum. The inhibited phage might be combined with inhibitor at many places or spots, each combination being individually reversible; the over-all combination might be almost irreversible because of the multiplicity of simple reversible attachments. Antibody molecules capable of combining with the phage, without inactivating it, could reduce the number of sites available to the inhibitor and, in effect, pry the inhibitor off the phage. It would be desirable to test whether activation is due to neutralizing antibody or to nonneutralizing antibody which combines with phage heads only (Lanni and Lanni, 1953).

SUMMARY

Fresh lysates of bacteriophage T2 exhibit a spontaneous rise in titer (activation) which can be accelerated by a variety of treatments: dilution in distilled water, heating, treatment in dilute antiphage serum.

Dilute T2 lysates, in which lysis has occurred at low bacterial concentrations, do not show the rise in titer; their titer corresponds to the maximum titer obtainable from mass lysates. Evidence is presented indicating that the activation is due to removal of an inhibitor of bacterial origin with which the phage becomes combined following lysis. Partially inhibited particles of phage T2 are adsorbed slowly by host bacteria and form poor plaques. Active phage T2 can be inhibited reversibly by fresh lysates of other phages produced on T2-sensitive bacteria. It is inhibited in a partially reversible way by sonically disrupted noninfected bacteria. The inhibition of phage T2 and its reversal by heating account for the apparent anomalies of the kinetics of thermal inactivation of this phage. They do not account for the nonexponential rate of ultraviolet inactivation.

REFERENCES


