LYSIS AND LYSIS INHIBITION WITH ESCHERICHIA COLI BACTERIOPHAGE

A. H. DOERMANN[1, 2]

Department of Biology, Vanderbilt University, Nashville, Tennessee

Received for publication September 27, 1947

The present understanding of genetic principles has emerged only after exhaustive study of numerous organisms characterized by various levels of organization. Recently the trend has been toward investigations with the simpler organisms as is illustrated by work with fungi (Beadle, 1945*a*, 1945*b*; Lindegren, 1945) and several of the protista (Moewus, 1940; Sonneborn, 1946). Analysis of the pattern of mutability has made even the bacteria amenable to genetic investigation (Luria and Delbrück, 1943). Genetic studies have, however, been made with organisms at an even lower level of organization than those mentioned above. Luria (1945) with his experiments on host range mutants and Hershey (1946*a*, 1946*b*) with his analysis of plaque size mutants have cleared the way for a more complete inquiry into the genetics of the bacteriophages. Research into the genetics of these organisms may lead to the solution of genetic problems, which other studies have not yet resolved.

Those bacterial viruses which have been most thoroughly investigated genetically belong to a group of seven phages and their mutant forms that comprise the T system (Delbrück, 1946). These phages, named T1, T2, . . . and T7, fall into several subgroups on the basis of serology, electron microscopy, host range, and certain physiological characteristics. One of these subgroups is of particular interest here, the even-numbered phages, T2, T4, and T6. These phages are closely related serologically and show the same characteristic morphology in the electron microscope (Delbrück, 1946).

Hershey (1946*a*, 1946*b*) has shown that they also have another property in common: all three are capable of mutation from the wild type, r^+, to the r type. The former type is characterized by the fact that it forms small plaques with very turbid halos on agar plates. The mutant type, r, forms large plaques with clear halos. Another characteristic distinguishing r^+ from r type, and probably the basic cause of the plaque size difference, is the time required for lysis of visibly turbid cultures. When highly diluted, suspensions of infected bacteria have the same latent period between infection and lysis whether the phage used for infection be of the r^+ or the r type. When, however, the infected cultures are visibly turbid, a difference in the latent period occurs. An r-infected culture will clear between 21 and 30 minutes after infection. In contrast to this, a visibly turbid culture infected with r^+ phage will not clear between 21 and 30 minutes but will

[1] Aided by grants from the John and Mary R. Markle Foundation and from The Rockefeller Foundation, New York City.

[2] Present address: Department of Genetics, Carnegie Institution of Washington, Cold Spring Harbor, New York.

remain turbid for several hours even though all the bacteria have been infected. This condition will be referred to as "lysis inhibition."

The r mutations appear to offer a unique opportunity to acquire more information about the difference between a wild type gene and its mutant form as well as about the mechanism of action of a specific gene. Therefore the difference between the r^+ and r phages was analyzed more closely. Since lysis inhibition seems to be the cause of the difference in plaque morphology, the study of this inhibition was considered the most promising point of attack. The present discussion deals with experiments designed to give a more complete picture of the mechanism of lysis inhibition.

METHODS

In the experiments described here the progress of lysis was followed by one of two general procedures: either by measurements of turbidity of infected cultures or by plaque count assays of cultures subjected to various conditions. The turbidity measurements were made with a recently described photoelectric nephelometer (Underwood and Doermann, 1947). The bacteria used were grown in 25-ml cultures made in 0.8 per cent nutrient broth (Difco) containing 0.5 per cent sodium chloride. Cultures were made in tubes, 175 mm by 20 mm, fitted with two-hole rubber stoppers, one hole having a short, cotton-plugged glass tube, the other a long tube reaching to the bottom of the culture and plugged with cotton at its upper end. The latter is for aeration and the former for air escape and for the introduction of phage suspensions. The medium is inoculated with 0.025 ml of a 24-hour aerated broth culture in which the bacteria have grown to saturation. The tube is then rigidly fixed in place in the nephelometer, which is housed in an incubator at 37 C. After inoculation the culture is aerated for 5 minutes to ensure thorough mixing before the first reading is taken. The tube is not removed from the nephelometer during the entire course of the experiment.

In taking a reading, the air flow is diverted into a side arm of the aerating system. Thirty seconds are allowed thereafter before a reading is made. This allows the aeration tube to fill up with the culture and disturbances of circulation to come to rest. With this procedure the readings were found to be reasonably reproducible.

The plaque count procedure was patterned after the one-step growth experiments first described by Ellis and Delbrück (1939). When the plaque count increases in an infected culture, lysis is assumed to be taking place. All the experiments were done with bacteria in the exponential phase of growth. The technique as used here involves several steps, and each step may vary from experiment to experiment. The steps used are as follows: (a) Determining the bacterial titer by colony counts—this serves as a check on the number of infected bacteria expected to be present when multiple infections are made. (b) Addition of phage and a short period of incubation to allow for their adsorption on the bacteria—this will be referred to as the primary infection. (c) Addition of antiphage serum, which will inactivate the unadsorbed phage particles but will not affect the adsorbed ones (Delbrück, 1945*a*)—it is sometimes feasible and

convenient to omit this step. (d) Dilutions in nutrient broth for the purpose of diluting the antiserum to the point of relative inactivity and to reduce the titer of the plaque-forming infective centers to a convenient level for plating. (e) Addition of potential inhibitors of lysis in the course of the dilutions in (d)—in many cases these inhibitors are phage suspensions and this step will then be referred to as the secondary infection. (f) Plating samples for plaque count at various intervals to determine the degree of lysis.

RESULTS

Turbidimetric comparisons of lysis. Presentation of the results obtained by using the foregoing procedures is best begun by a discussion of the turbidity curves shown in figure 1. In all these curves two successive drops of the turbidity may be seen, one of these drops occurring immediately after the addition of the phage, and the other one beginning at the end of the latent period of virus multiplication, i.e., 21 to 25 mm after infection. In the case of the *r* mutants the second drop continues uninterruptedly until the culture is clear. This clearing is almost complete within 1 hour after the phage has been added. With r^+ strains, however, the second drop is smaller and is followed by a rise and much later by a third drop. This third drop was not observed in the case of T2r^+. The last turbidity change brings about what appears to the unaided eye to be clearing.

It may be noted here that cultures of T2r^+-infected bacteria do clear after 5 or 6 hours as judged by visual observation. The reason why this is not reflected in nephelometer readings is not clearly understood. It is presumed that the bacteria do not break down as completely as they do with the *r* mutants. As can be seen in figure 1 there is considerable residual scattering with all the r^+ strains. Apparently the particles remaining after lysis in r^+-infected cultures, although too small to give gross turbidity, still retain the property of scattering considerable light. These lysates appear somewhat opalescent. In T2r^+ lysates this incomplete disaggregation seems to be most pronounced. Furthermore, the number of infective centers in filtered T2r^+ broth lysates has in several cases been known to rise by a factor 2 to 3 over a period of several months in the refrigerator (unpublished data of M. Delbrück and the writer). This implies that disaggregation of small particles may be taking place. The fact that the burst size of T2r^+-infected bacteria is generally found to be smaller than with those infected with T4r^+ or T6r^+ by a factor of 2 to 3 lends credence to such a hypothesis (Delbrück, 1946).

In order to see whether phage liberation is associated with any of the turbidity changes, plaque count assays were made from a T4r^+-infected culture in which the turbidity was followed nephelometrically. Assays were made by taking samples from the culture by means of capillary pipettes introduced through a side arm in the culture tube. With this procedure it was unnecessary to move the tube or to remove the aerating tube from it. The results are shown in figure 2, in which the plaque titer and the turbidity are plotted against the same time scale. The first point on the plaque titer curve is from an independent

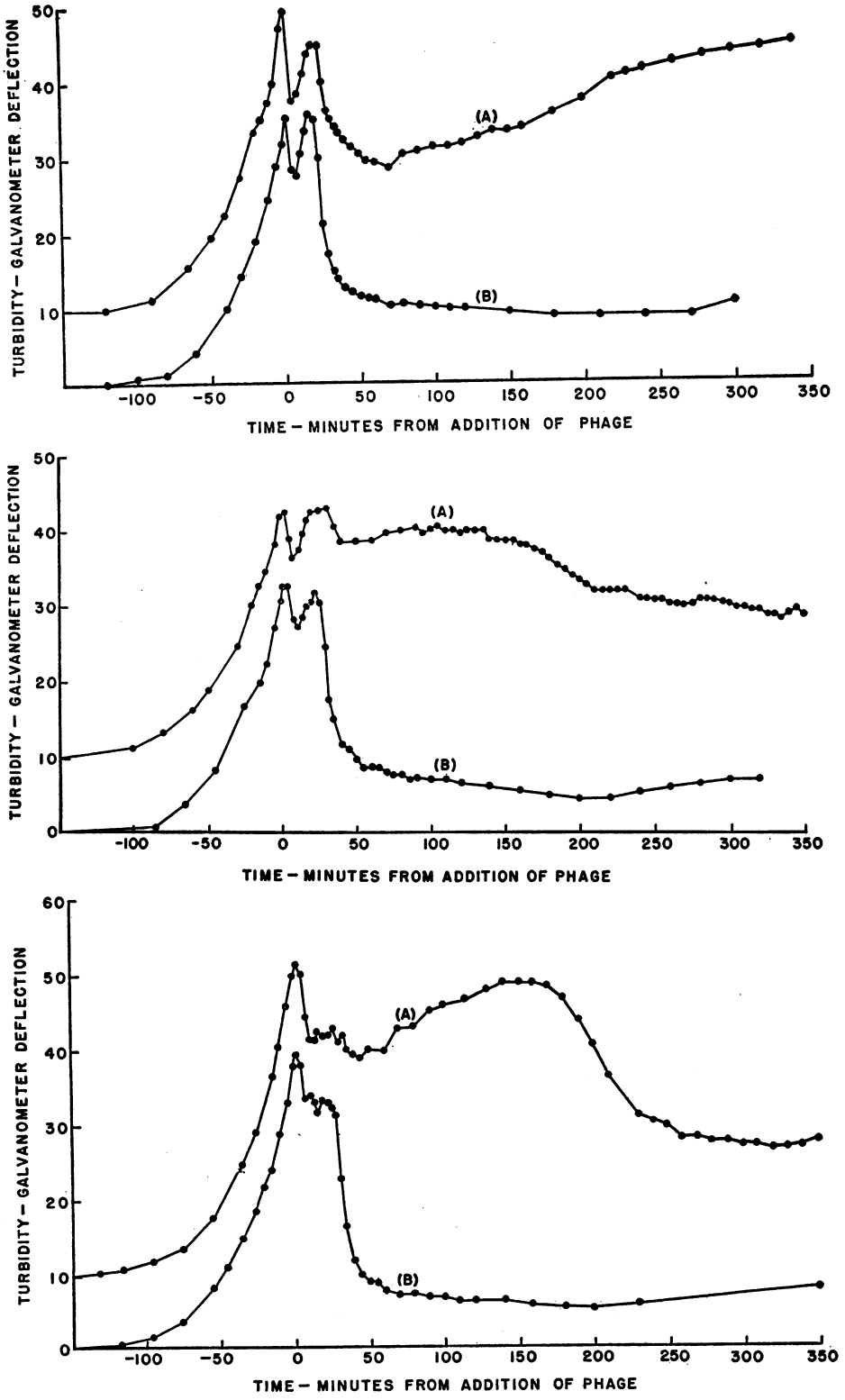

FIG. 1

Top: Turbidimetric comparison of lysis induced by T2*r* and T2*r*$^+$. *Center:* Turbidimetric comparison of lysis induced by T4*r* and T4*r*$^+$. *Bottom:* Turbidimetric comparison of lysis induced by T6*r* and T6*r*$^+$.

In this figure, curve A represents the turbidity of the *r*$^+$-infected culture and curve B that of the *r*-infected culture. In all cases curve A is scaled up ten units on the turbidity axis. The multiplicity of infection in these experiments varied from 3.3 to 5.4.

assay of the phage stock used for infecting the culture. The ratio of phage particles to bacteria at the time of infection was approximately 2.2 to 1.

It is seen in figure 2 that the phage titer dropped slightly in the first 15 minutes after addition of the virus. This is presumably due to the adsorption of several phage particles on single bacteria. At 21 minutes the beginning of a slight rise is noted, which by 30 minutes has reached its maximum with a factor of increase of about 30 over the lowest previous titer. After this time, however, the plaque titer drops again. Apparently only a fraction of the bacteria have lysed and the phage particles liberated from this fraction are being adsorbed on unlysed bacteria. About 50 minutes after this drop, the plaque titer again begins to rise.

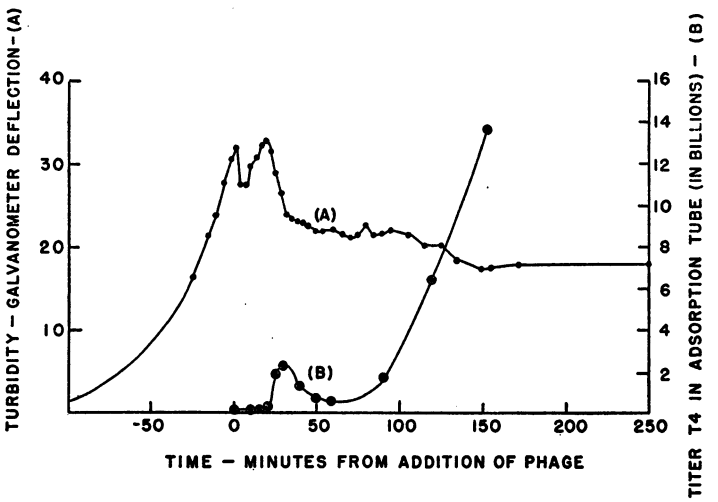

FIG. 2. CHANGES IN TURBIDITY OF A T4r^+-INFECTED CULTURE AS ASSOCIATED WITH THE LIBERATION OF PHAGE PARTICLES

Curve A shows the turbidity of the culture over a period of several hours, whereas curve B shows the changes in phage titer that occurred in the same culture. The multiplicity of infection was about 2.2.

The first rise in plaque titer occurs at the end of the normal latent period of virus multiplication and corresponds on the time scale to the second turbidity drop. The second rise in plaque titer corresponds with the third drop in the turbidity measurements, which, as pointed out, corresponds to visual clearing. The cause of the first drop on the turbidity curve is at present not clearly understood. Its discussion will be relegated to a later section since it is at this time not relevant to the problem of lysis inhibition.

The second drop in turbidity, which occurs simultaneously with liberation of phage at the end of the latent period, is the criterion of difference between r and r^+ phages. In infections with r strains complete lysis occurs at this time, whereas with r^+ strains lysis begins but is presently arrested. In one-step growth experiments (Delbrück, 1940, 1946; Delbrück and Luria, 1942), in which the infected cultures are highly diluted before the end of the latent period, most of the r^+-infected bacteria do lyse without delay. The failure of the major portion of the

r^+-infected bacteria to lyse in visibly turbid cultures at the end of the latent period must therefore be due to a substance that is released at lysis of the first few bacteria and that inhibits lysis of the remaining ones. Under conditions of the one-step growth experiments lysis of all bacteria would be expected because of the greater dilution of the inhibitor.

Origin and general properties of the inhibitor. To test the foregoing hypothesis the following experiment was done: A standard culture of bacteria in the exponential growth phase was infected with T2r^+, the phage being in excess of the

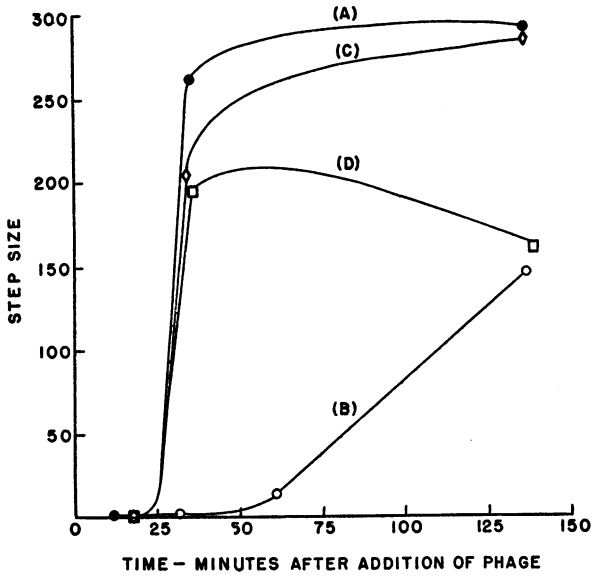

FIG. 3. THE RELEASE OF PHAGE PARTICLES FROM T4r^+-INFECTED BACTERIA AND FROM T4r-INFECTED BACTERIA EXPOSED TO THE SUPERNATANT FROM AN INHIBITED T2r^+-INFECTED CULTURE

	Primary Infection	*Secondary Exposure*
Curve A	T4r^+	Broth
Curve B	T4r^+	Supernatant
Curve C	T4r	Broth
Curve D	T4r	Supernatant

bacteria. Thirty minutes later, at the time when lysis inhibition was presumably being effected, the unlysed bacteria were removed by centrifugation. The supernatant was then tested for its ability to inhibit lysis of bacteria previously infected with either T4r or T4r^+. The test was made in the manner of the one-step growth experiment described earlier. Separate cultures of bacteria were infected with T4r and T4r^+ and, omitting step (c), were diluted after adsorption, the final dilution from each being made into supernatant on the one hand and into broth for the control on the other. Platings were made on an indicator strain resistant to T2, thus making it possible to estimate T4 infective centers exclusively. The results, shown in figure 3, illustrate that the two controls gave normal one-step growth curves. The latent period in both cases ended before 31 minutes. In the case of the cultures diluted in the supernatant, the

latent period of T4*r*-infected bacteria was the same as in the controls. In contrast, the T4r^+-infected bacteria showed only very slight lysis at 31 minutes and only a little more at 61 minutes. Quantitatively it showed less than 2 per cent as many infective centers as the lowest of the other cultures. Several other experiments verified these results. From this experiment it may be concluded that the supernatant contained an agent which can inhibit the lysis of the T4r^+-infected bacteria but not that of T4*r*-infected bacteria.

In order to test whether lysis ultimately occurred because of the breakdown of the inhibitor or whether inhibition was finally overcome in spite of the presence of inhibitor, cleared lysates of T2r^+ were tested for inhibitory power. The tests, conducted in the same way as the previously described tests of the T2r^+ supernatant, showed that lysates do contain inhibitor. Similarly filtrates of cleared T2r^+ lysates were shown to contain inhibitor even after long storage in the refrigerator. In all subsequent experiments, therefore, filtrates were used as inhibitory agent unless otherwise specified.

These results, coupled with those previously described, give rise to the hypothesis that possibly all stock filtrates contain this inhibitor but that only r^+-infected bacteria are susceptible to the inhibitory action. To test this hypothesis, T1 and T2*r* stock filtrates were tested for their ability to delay lysis of T4r^+-infected bacteria. The experiments were performed like those above, with minor modifications. The T1 filtrate showed no difference from broth and the T2*r* filtrate only a very slight effect. T2r^+ filtrate used in precisely the same way again showed inhibitory action. The fact that the T2*r* filtrate did show a very slight effect might, if significant, be ascribed to the action of inhibitor produced by T2*r*-infected bacteria or it might also be due to the presence of a very small fraction of T2r^+ particles which are always present in T2*r* stock because of mutation from *r* to r^+ and selection of the wild type (Hershey, 1946*a*, 1946*b*).

Thus, for inhibition of lysis it is necessary that the bacteria be infected with an r^+ phage. For marked inhibition it is further necessary that the inhibitor be derived from bacteria lysed by r^+ phage.

Characterization of the inhibitor. Several techniques were used in an effort to determine certain characteristics of the inhibitor. In order to obtain some idea of the size of the substance involved, its ability to pass through a cellophane membrane was tested. Five ml of a high titer T6r^+ broth filtrate were placed in a thistle tube whose end was covered with cellophane. The covered end was submerged in five ml of nutrient broth and incubated for 18 hours at 37 C. A sample of the parent T6r^+ filtrate was diluted with nutrient broth by a factor 2 and incubated as a control. The dialyzate was assayed for phage to check against leakage. It was then compared with the incubated filtrate for its ability to inhibit lysis of B infected with T4r^+. The dialyzate showed no inhibitory effect whatsoever while the control showed decided inhibitory action. It was evident that the inhibitor is not a small, rapidly diffusing molecule.

The estimate of the size of the inhibitor was further limited by testing its sedimentability. A high titer broth stock of T6r^+ was centrifuged at ca. 9,000 g for one hour in an angle centrifuge, and the supernatant was removed and

assayed for phage particles. It was found to contain 5.4 per cent of the original titer. This supernatant was compared with a sample of the untreated stock for inhibiting power. T4r^+ on B was the testing agent. Table 1 shows the results as measured by the step size estimated from plaque counts during the expected period of lysis inhibition. It is clear that the inhibitory action was reduced by centrifugation, roughly in proportion to the titer of T6r^+ in the solution tested. Thus the untreated lysate, diluted by a factor 20, showed marked inhibition, almost as much as the undiluted supernatant. The T6r^+ titers were comparable in these cases.

It should be noted here that the low step sizes shown in table 1 cannot be accounted for by the depressor effect described by Delbrück (1945*b*). This is known from the fact that later platings showed the phage yield to be as large

TABLE 1

Effect of sedimentation of the virus particles on the inhibitory power of a T6r^+ lysate

INHIBITOR	TOTAL DILUTION OF INHIBITOR	T6 TITER IN INHIBITION TUBE × 10^6	STEP SIZE*
None, broth control	∞	0	296
Untreated lysate.....	10	240	3
Untreated lysate.....	20	120	10
Supernatant.....	1	130	6
Supernatant.....	20	6.5	306

A broth stock of T6r^+ was centrifuged at high speed and the supernatant removed and assayed for phage particles. It was found to contain 5.4 per cent of the original T6 titer. The supernatant was compared with the untreated stock for inhibiting power on the lysis of T4r^+-infected bacteria.

* Determined between 35 and 40 minutes after the primary infection.

or larger from the inhibited bacteria as from the controls which were not infected secondarily. This consideration also holds true for the experiments described in tables 2 and 3. These are discussed later.

The rough quantitative agreement between the sedimentability of the phage and the inhibitor suggests that the phage and the inhibitor have about the same size and points to the possibility that the phage itself might be the inhibiting principle. This hypothesis was subjected to several tests. First, a refinement of the centrifugation experiment was performed. A sample of phage purified by a differential centrifugation procedure was tested for its ability to inhibit lysis of T4r^+-infected bacteria. The purified phage was prepared by Dr. Seymour Cohen according to the following procedure. Six liters of T2r^+ lysate were centrifuged at 4,000 rpm for 30 minutes. This throws down the large particles of bacterial debris, leaving the phage in the supernatant. The latter was spun at 10,000 rpm for 2 hours, and the pellet taken up in 1 per cent sodium chloride. This suspension was again centrifuged at 4,000 rpm for 1 hour. The phage in the supernatant was sedimented at 10,000 rpm for 2 hours and the

pellet taken up in 100 ml of 1 per cent sodium chloride. The final material contained about 8×10^{11} phage particles per ml (by plaque count assay). For the present experiment a dilution was made in broth to bring the titer to 3×10^8. The latter suspension was used as the inhibitor. The experiment was made in a manner similar to previous experiments and is described in the legend to figure 4. Clearly the purified phage did increase the latent period.

A second test of the hypothesis that the phage itself is the inhibitor is described in table 2, which shows the results of an experiment in which the lysis-inhibiting T6r^+ stock was treated with specific rabbit anti-T6 serum. One aliquot of a filtered T6r^+ stock was incubated for 30 minutes at 37 C with a suitably diluted

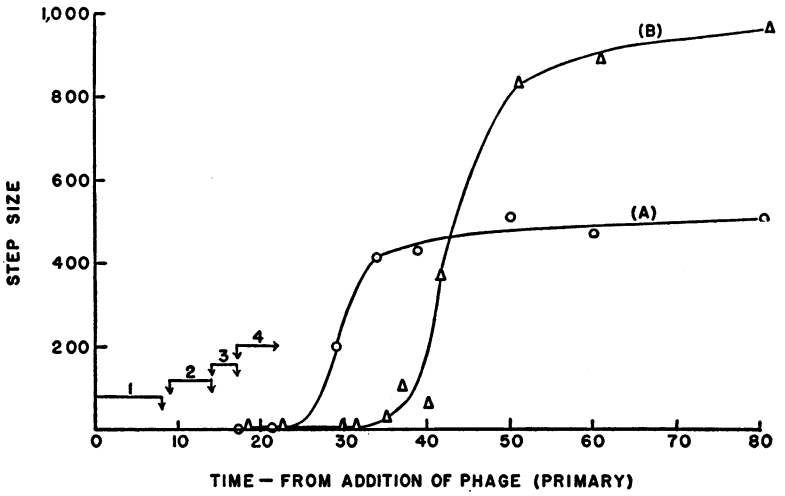

FIG. 4. THE EFFECT OF PURIFIED PHAGE IN DELAYING LYSIS OF T4r^+-INFECTED B

The purification of the phage, based on differential centrifugation, is described in the text. The steps involved in testing this effect are numbered from 1 to 4 above the time scale: step 1, primary infection of B with T4r^+; step 2, dilution into *broth* (curve A) and into *phage* (curve B) (second infection); step 3, further dilutions; step 4, platings for assays.

antiserum. It was then diluted sufficiently to reduce the serum titer to negligible activity and assayed for T6 particles. With T4r^+ on B, the serum-treated T6r^+ stock was compared with an aliquot of the same stock that had been incubated in broth containing no serum. The table shows a close parallelism between phage titer and inhibiting power. This is in agreement with the hypothesis stated above.

A further test made to determine whether the specific properties of the inhibitor agree with those of the phage particles depends on the fact that certain phage-resistant mutants of strain B will not adsorb the phages to which they are resistant (Luria and Delbrück, 1943). For example, the strain B/6, used to assay T4 in the previously described experiments, will not adsorb T6. Therefore, it was reasoned, if the inhibiting agent is the phage itself, T6r^+ mixed with high titer B/6 should lose none of its ability to inhibit lysis; mixing it with B (which will adsorb T6), however, should reduce the inhibiting titer because of

loss of the phage titer. If, on the other hand, some agent other than the phage is responsible for the inhibition, then it seems likely that it should be adsorbed equally well on B and on B/6. To test these alternatives the experiment described in table 3 was made. It can be concluded from this experiment that contact with B reduced the inhibiting potency of the T6r^+ stock, whereas contact with B/6 did not. Within the limits of error of such an experiment the reduction of inhibiting power was proportional to the reduction of the phage titer.

Another result that would be expected if the phage itself is the inhibiting agent is this: if B/6 were infected with T4r^+, then lysis of this system should be inhibited in the highly diluted growth tube by the presence of T2r^+ since it can be adsorbed on such a system; the presence of a T6r^+ lysate, however, should have

TABLE 2

Effect of treatment with specific antiphage serum on inhibiting power of a filtered T6r^+ lysate

INHIBITOR	TOTAL DILUTION OF INHIBITOR	TITER OF ACTIVE T6 INHIBITION TUBE × 10^6	STEP SIZE*
None, broth control.....	∞	0	193
Untreated lysate.....	100	380	9.6
Untreated lysate.....	400	95	33.5
Untreated lysate.....	1,000	38	126
Untreated lysate.....	4,000	9.5	218
Treated lysate.....	10	260	10.4
Treated lysate.....	100	26	231

An aliquot of a T6r^+ lysate was incubated at 37 C with a suitably diluted anti-T6 serum. The factor of inactivation was 14.3. The treated portion and an untreated portion were compared for their ability to inhibit lysis of B by T4r^+.

* Measured between 35 and 40 minutes after the primary infection. Later platings showed that in all cases a step size of 200 or greater was attained.

no effect on the time of lysis The experiment made to check this prediction is outlined in figure 5. It is seen that lysis of the system T4r^+ on B/6 was not inhibited in either broth or T6r^+ suspension. The T2r^+ did, however, increase the latent period, showing the system to be susceptible to inhibition.

From the last two experiments described it seems clear that one common property of phage and inhibitor is the specificity of host range, for the inhibitor will act only on a host capable of adsorbing the phage and is removed from the filtrate by adsorption only on such bacteria as are capable of adsorbing the phage. A second common property is that of specificity of reaction with antiserum. Inhibitor and phage are inactivated at the same rate by specific antiphage serum. A third characteristic common to both is that of size. Neither inhibitor nor phage will pass through a cellophane membrane, but both are sedimented at the same rate in the centrifuge. These results demonstrate that the cause of lysis inhibition is the secondary adsorption of an r^+ phage.

Factors influencing the inhibition of lysis. With the phage thus shown to be the inhibitor, the next problem studied concerned the number of phage particles adsorbed per bacterium in the secondary infection necessary for a delay of lysis. In order to set a lower limit on the estimate, an experiment was performed in which T4r^+-infected bacteria were exposed to T2r^+ at several concentrations for a period of 5 minutes in the middle of the T4 latent period. Four simultaneous one-step growth curves of T4r^+ on B were made. The procedure was to mix a rapidly growing culture of B (1.6×10^8 bacteria per ml) with about 10 T4 particles per bacterium. Two minutes were allowed for adsorption, and then a

TABLE 3

Inhibiting power of T6r^+ lysate after exposure to sensitive and to resistant bacteria

INHIBITOR	TOTAL DILUTION OF INHIBITOR	TITER OF T6 IN INHIBITION TUBE $\times 10^6$	STEP SIZE*
None, broth control.....	∞	0	186
Untreated lysate.....	100	400	12.1
Untreated lysate.....	400	100	26.8
Untreated lysate.....	2,000	20	240
Exposed to B/6.....	80	450	10.5
Exposed to B/6.....	320	112	25.5
Exposed to B/6.....	1,600	22	244
Exposed to B.....	40	61	24.8
Exposed to B.....	130	19	336

Aliquots of a T6r^+ lysate were added to cultures of B and of B/6. The bacterial cultures were standard 150-minute cultures in the exponential phase of growth and at a titer of about 5×10^7 bacteria per ml. The phage lysate was added in the volume ratio of 1:40 giving a titer of about 10^9 particles per ml. Adsorption on B was allowed to proceed for 4 minutes, at the end of which time the culture was poured on a sterile, sintered, glass filter. Adsorption on B/6 was allowed to proceed for 11 minutes before filtration was begun. Filtration in both cases was completed in 2 minutes and 30 seconds. The filtrates were assayed twice for their phage titer and then tested for their ability to inhibit lysis of T4r^+-infected bacteria.

* Measured between 35 and 40 minutes after the primary infection.

dilution by a factor 10 was made into specific anti-T4 serum. This reduced the titer of unadsorbed T4 to a level which would not interfere with the significance of the results. After allowing 7 minutes for serum action, a dilution by a factor 40 was made into broth as a control and into three concentrations of T2r^+ as experimentals. The titers of T2 were, respectively, 6×10^7, 3×10^8, and 1.5×10^9 particles per ml. Five minutes were allowed for this secondary infection; then each culture was diluted by a factor 100 in broth, and platings from these tubes were made against an indicator strain resistant to T2. Further dilutions were made from these tubes for convenience in later post-burst platings. Platings were made at intervals and the results are seen in figure 6. In all cases the growth curves from the experimental cultures show a longer latent period than

do those from the controls. In a similar experiment using the same T2 stock at a still higher dilution (1.2×10^7 particles per ml) no inhibition was noted. The growth curve followed the control almost exactly.

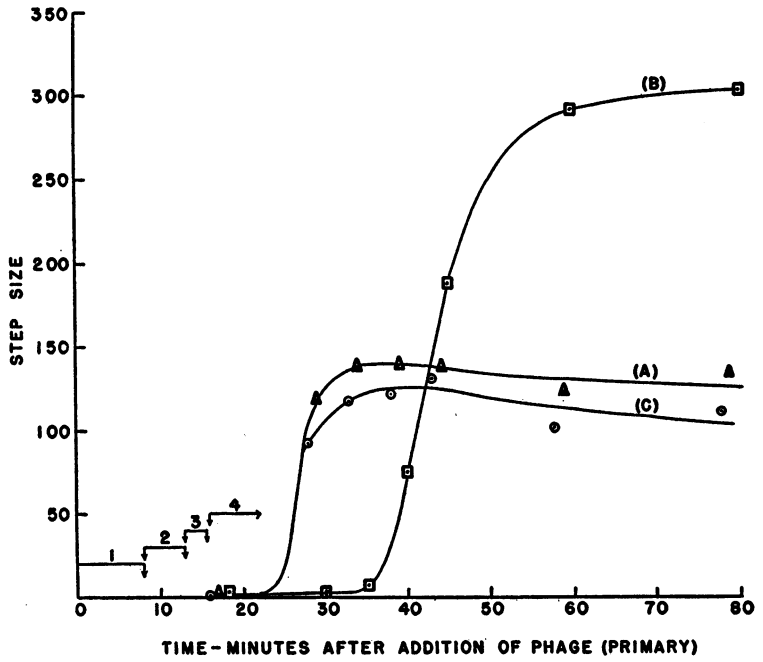

FIG. 5. EXPOSURE OF B/6 INFECTED WITH T4r^+ TO A SECONDARY ADDITION OF T6r^+ IN CONTRAST TO A SECONDARY ADDITION OF T2r^+

B/6 is known to be incapable of adsorbing T6 but adsorbs T2 rapidly. The steps involved in testing whether T6r^+ lysate is capable of inhibiting lysis of T4r^+-infected B are numbered from 1 to 4 above the time scale in the figure. Step 1 shows the time allowed for the primary adsorption of T4r^+. From the adsorption tube a dilution was made into broth for the control (curve A), into a suspension of T2r^+ (curve B), and into a suspension of T6r^+ (curve C). Step 2 indicates the time allowed for the secondary exposure. Step 3 involves further dilutions for convenience in plating, and step 4 indicates the period over which platings are made. These platings determine the points on the curves.

The purpose of curve B (secondary infection by T2r^+) is to show that the system B/6 infected with T4r^+ is capable of being inhibited.

Thus the lower limit of inhibition in these experiments was reached in the range 1.2 to 6.0 $\times 10^7$ particles per ml. The number of phage particles adsorbed under these conditions may be estimated as follows: The amount of T2 adsorbed may be calculated from the expression

$$N/N_0 = e^{-kBt} \tag{1}$$

in which $1 - N/N_0$ is the fraction of phage adsorbed, B is the bacterial titer, t is the time of exposure (in minutes), and k is the adsorption rate constant characteristic for each phage-bacterial system. The value of k may be estimated from experiments with higher concentrations of B. Thus, when T2 is added to a culture of rapidly growing bacteria at 5×10^7 organisms per ml, the fraction of particles adsorbed in 5 minutes is between 75 and 95 per cent. Substituting

these values in equation (1) we find that k for our system lies between the values 5.5×10^{-9} and 1.1×10^{-8}. Again using equation (1) and assuming k to be between the limits just found, we can calculate the number of particles adsorbed in the inhibition experiment. For a T2 concentration of 6×10^7 we thus find this average multiplicity of secondary infection to be between 1.6 and 3.5. The conclusion drawn from this experiment is that one, or at most very few, phage particles are capable of delaying lysis of an infected bacterial cell.

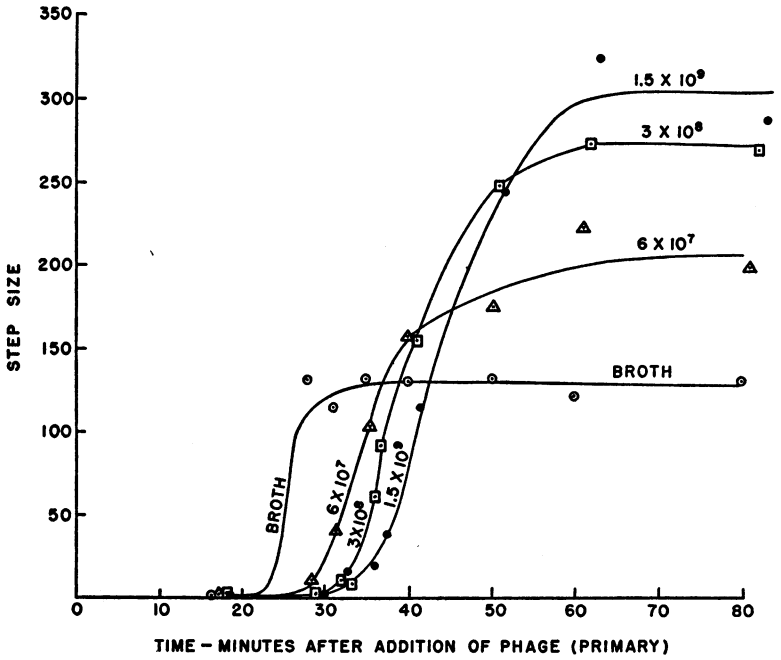

FIG. 6. DETERMINATION OF THE LOWER LIMIT FOR THE NUMBER OF PHAGE PARTICLES WHICH MUST BE ADSORBED ON A BACTERIUM TO DELAY LYSIS

This figure shows the inhibition curves obtained when a T4r^+-infected B culture was exposed to three concentrations of T2r^+ as compared to the control curve where no secondary infection was made. The details of the experiment are described in the text. For the method of determining the average number of phage particles secondarily adsorbed per bacterium, see text.

The number written on each curve shows the titer of T2 per ml in the secondary adsorption tube.

If inhibition is caused by a secondary adsorption of r^+ phage particles and can be effected even though the average multiplicity is as low as 3.5, then one would expect inhibition of lysis in cases in which a sufficient excess of phage particles is given in the primary infection. To test this correlation, four simultaneous one-step growth experiments were made with different multiplicities of T4r^+ infection. The multiplicities used were 0.4, 4, 10, and 33. Five minutes were allowed for adsorption and during this period 94 per cent of the virus particles were adsorbed. Then a dilution of 40 (20 in the case of 0.4) was made into antiserum of sufficient titer to reduce the unadsorbed phage by a factor 8×10^3 in 10 minutes. After the period of serum action a dilution of 500 was

made for the first growth tube. Platings were made from this tube and from subsequent dilutions.

The results, seen in figure 7, show that marked inhibition occurred only in the tube with the highest multiplicity, which was 33. The 10-fold infection showed none, in spite of the fact that, on the average, each bacterium adsorbed 9.4 phage particles. This suggests that the primary and secondary infections must be separated by several minutes. Some estimate of this separation may

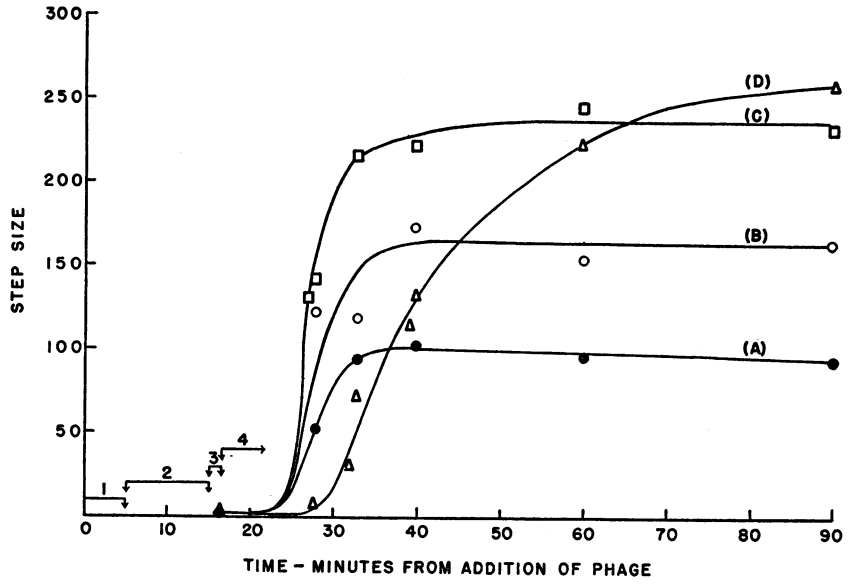

FIG. 7. THE EFFECT OF THE MULTIPLICITY OF PRIMARY INFECTION ON THE LATENT PERIOD OF r^+-INFECTED BACTERIA

Four cultures of B were infected with the following average multiplicities of T4r^+, respectively: 0.4 (curve A), 4.0 (curve B), 10 (curve C), 33 (curve D). Five minutes were allowed for adsorption (step 1). Then a dilution was made into appropriately diluted antiserum to stop further adsorption and to inactivate unadsorbed phage particles (step 2). Dilutions were made from the antiserum tubes (step 3), and platings were made to determine the duration of the latent period and the step size (step 4).

be obtained by determining how many particles will be adsorbed in each minute of adsorption. With equation (1) again, the number of phage particles adsorbed per bacterium in each successive minute was calculated and the results tabulated:

TIME INTERVAL (MINUTES)	% ADSORBED PER INTERVAL	AVERAGE ADSORBED PER BACTERIUM PER MINUTE		
		4×	10×	33×
0–1	43	1.7	4.3	14.2
1–2	24	1.0	2.4	7.9
2–3	14	0.6	1.4	4.6
3–4	8	0.3	0.8	2.6
4–5	5	0.2	0.5	1.6
0–5	94	3.8	9.4	30.9

We have seen that 3 phage particles adsorbed secondarily are sufficient to effect inhibition. Since in the 10-fold multiplicity there were still 5.1 particles to be adsorbed after the first minute and no inhibition occurred, we can conclude that more than 1 minute must separate the primary and secondary infections. Judging from the multiplicity of 33 which did show inhibition, however, the secondary infection could come as little as 2 or 3 minutes after the primary infection.

Several experiments were also done to test at what time an infection with a heterologous r^+ phage could induce lysis inhibition. After primary infection with T4r^+ secondary infections were made with T6r^+. In several experiments 2-minute exposures were made at the following times: 4.5, 10.0, 12.5, 14.0, and 18.0. In all cases lysis was delayed from 20 to 30 minutes over that in the controls. The results did not show a significant difference in the effectiveness of the secondary infection as a function of the time when it was applied. In another experiment 1-minute exposures were made at 1.5 minutes and at 5.0 minutes. In both of these cases inhibition was clearly evident. To test closer intervals than 1.5 minutes is technically difficult, and the results are difficult to interpret because of the "depressor effect" previously described by Delbrück (1945*b*).

Investigation of the first drop in turbidity. The cause of the initial drop in turbidity seen in all the nephelometric curves in figure 1 is, at present, not clearly understood. It could be due to lysis of bacteria, to some alteration in the light-scattering properties of the newly infected bacteria, or to both of these causes. The results shown in figure 1 show only that it is common to all six phage-bacterial systems used in these experiments. Other experiments have shown that it occurs also with T5 but not with T1 (Underwood and Doermann, 1947), T3, or T7. Assays of the number of infected bacteria during the latent period and comparison of this number with the number of bacteria present before infection (from colony count assays) showed little or no loss of infective centers, and hence this dip would not be thought to be due to lysis of the bacteria. This criterion is not very reliable, though, since the magnitude of the difference is not large and since other factors cannot be controlled. One such factor is the division of bacteria in the first few minutes after virus addition and before they have actually adsorbed a phage particle. Another factor is the possibility that infected bacteria might be capable of dividing. Either of these factors might compensate for the infective centers lost by lysis.

The question of the interpretation of the first drop in turbidity was also approached from a slightly different angle. Turbidity measurements were made of cultures of bacteria infected with varying amounts of T6r, T6r^+, and T4r^+. Several facts can be determined from a study of figure 8, which shows the curves obtained with infections of various multiplicities. In the case of T4r^+ it is noted that the initial dip with a multiplicity of 3.6 is only slightly smaller than with multiplicities of 13 and 16. Also it is seen that the recovery from the dip proceeds at a slower rate as the multiplicity increases. This could readily be interpreted if one assumes that the character of the bacterial surface changes

because of the adsorption of phage particles and that one or a few phage particles are about as effective as 10 to 15 in bringing about this change. Furthermore, one must assume that after most of the phage particles have been adsorbed, recovery of the original surface takes place. One would expect this recovery to be delayed considerably at such high multiplicities as 61, where adsorption continues over a longer period of time. This is seen to be the trend in figure 8-A.

The curves showing the effect of various multiplicities of T6*r* and T6*r*$^+$ show several notable differences, however. Except at the lowest multiplicities, T6*r*

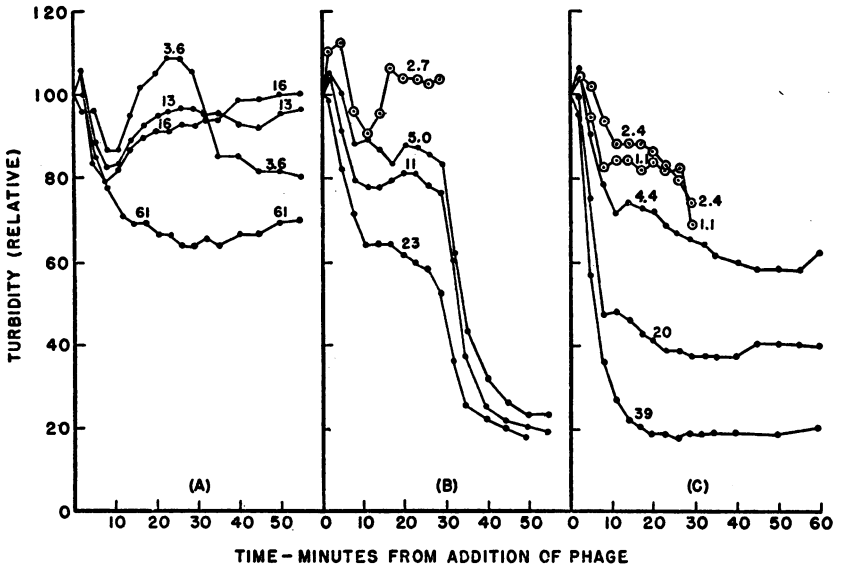

FIG. 8. THE INITIAL DROP IN TURBIDITY AS A FUNCTION OF THE AVERAGE MULTIPLICITY OF INFECTION

(A) infection with T4*r*$^+$; (B) infection with T6*r*; (C) infection with T6*r*$^+$.

The multiplicities of infection are written with each curve. The encircled points have been corrected for the uninfected bacteria present, on the assumption that these bacteria maintain their normal exponential growth rate.

The turbidity is shown from the time of addition of the phage suspension, and for the sake of comparison all turbidities were at this time arbitrarily adjusted to 100. The absolute bacterial titers were 5 to 10 $\times$ 10^7 B per ml which is in the exponential phase of growth. All infections with a given phage were made from the same stock filtrate.

induces a larger dip than T4*r*$^+$ for comparable phage-bacterial ratios. T6*r*$^+$ produces an even larger dip than T6*r*. Another difference, particularly noticeable in figure 8-C, is that the recovery is absent in T6*r*$^+$-infected bacteria. These experiments then suggest that the dip is due to an irreversible loss and probably due to lysis of a fraction of the bacteria. Emphasis for this suggestion comes from the 80 per cent drop in turbidity at a multiplicity of 39 with T6*r*$^+$. Examination of this tube at 20 minutes showed it to be completely clear so far as the naked eye could see. Thus, in T6 infections one would be inclined to suspect lysis from without (Delbrück, 1940) as the cause of part of the initial dip, whereas with T4*r*$^+$ some reversible process seems to cause it.

Lysis from without is probably not the sole cause of the dip even in the T6 curves, since, with a multiplicity of 2.7 of T6*r*, a recovery is very obvious even when the curve is corrected for uninfected bacteria. With the two lowest multiplicities of T6r^+ (1.1 and 2.4), the lower of the two shows a slightly greater drop than does the higher multiplicity. This is not in agreement with the general trend of the rest of the T6 curves in which the amount of lysis from without is directly related to the multiplicity of infection. These two considerations point to the likelihood that there is an initial dip even here that is not caused by lysis of bacteria. The most satisfactory explanation of the initial dip appears to be some change in the light-scattering properties of the infected bacteria, but high multiplicities of infection induce an additional drop that is due to lysis of a fraction of the bacteria.

One further indication that the initial dip in turbidity is due to a change in all the bacteria comes from experiments with T5. It is known that certain phages require that calcium be present in the medium in order for them to complete a cycle of infection, reproduction, and lysis. T5 has this requirement. Calcium is, however, not required for the growth of *E. coli* strain B nor for the adsorption of T5 on the host. When bacteria are infected with T5 in a medium in which calcium is available, a dip and subsequent recovery occur and lysis of the bacteria follows at the expected time (Underwood and Doermann, 1947). However, when T5 infection takes place in a medium where no calcium is available (in these experiments with oxalate present), the dip takes place, but neither recovery nor lysis are observed. It appears from this that recovery from the dip depends on some system which requires calcium to reverse the cause of the dip, rather than on cell division to compensate for the loss of a fraction of the bacteria.

DISCUSSION

The results described above present the following picture of the actual events occurring under conditions of lysis inhibition. Bacteriophages of the r^+ type infect bacteria that reproduce the phage during the latent period, at the end of which lysis begins, first in a few bacteria, but increasing rapidly. Immediately after liberation of the first new phage particles they become readsorbed on those bacteria which have not lysed. The reactions involved in and following this secondary adsorption of an r^+ phage delay the lysis of the secondarily infected bacteria.

The results of growth tube experiments have, however, never duplicated the long delay in lysis shown by nephelometric determinations. The nephelometer experiments indicate a delay of 75 to 150 minutes while growth tube experiments show delays of 15 to 40 minutes. This difference may be due to the fact that the situation in the growth tube is not quite analogous to that in the adsorption tube. First, in growth tube experiments it is not experimentally feasible to make a homologous secondary infection as late in the latent period as it occurs in the adsorption tube, since to wait until some bacteria burst would make it impossible to distinguish between the effects of the products of the burst and

the phage added from the outside. Furthermore, in the adsorption tube a self-regulatory system exists in that inhibitor is produced only if and when the inhibition begins to break down. This type of system could promote extended delay of lysis, since bacteria would lyse until a secondary infection became sufficient for stopping lysis, and more bacteria would lyse only to the extent of maintaining a sufficient level of secondary infection. It is not feasible to imitate this situation in growth tube experiments.

The difference between the results of the two techniques may also be due to the fact that they measure two different things. It is very difficult to determine from turbidity measurements the exact time when the inhibition of lysis begins to break down in the adsorption tube, since this drop in turbidity is a very gradual one. On the other hand, the growth tube experiments measure the beginning of phage liberation more accurately. It might also be noted that in at least one experiment in which both the primary and secondary infections were made with the same phage (T4r^+), the inhibition did last longer. Perhaps there is a difference in the effect of heterologous and homologous secondary infections.

Considering these conditions and differences it is not unreasonable to make the assumption that the condition of lysis inhibition in the adsorption tube is an extension of the phenomenon observed in the growth tubes.

Although the experiments discussed here reduce the phenotypic difference between r^+ and r from a mass culture effect to one concerning individual bacteria, nevertheless they do not elucidate the mechanism underlying the difference. It is clear that the mechanism involves the new phages to be liberated or some by-product of their production. It is also seen that the type of phage which is secondarily adsorbed determines whether or not lysis will be delayed. It might then be said that both of these reactions are type-specific since they both depend on the type of phage involved. Thus it is requisite that the bacteria be both primarily and secondarily infected with the r^+ type for inhibition of lysis. But we have no clue from these experiments as to what r^+ accomplishes inside the bacterium that the r does not accomplish or vice versa. And we do not know what r^+ prevents from happening when it is secondarily adsorbed.

Even though these results do not explain the mechanism, they do point to several types of experiment that might be done in an effort to learn more about it. For we can now break the mechanism down into effects caused by the primary infection and those caused by the secondary infection. Since it is known, for example, that the secondary infection is one of the determining factors, it can be investigated further using mutant phages which require adsorption cofactors (Anderson, 1945). Early experiments with synthetic media gave complicated results, presumably because of the lack of adsorption cofactors in the medium, but now, with additional experiments, we may be able to interpret them. It might also be possible to duplicate the phenotypic difference by exposing the infected bacteria to specific respiratory poisons shortly before lysis. In this way one might learn something about the chemical nature of the reactions involved. Still another approach might be that of treating the phages to be

adsorbed secondarily in an attempt to remove the inhibition property from the r^+ phages or to add it to the r phages.

One further point that deserves mention is the fact that there is a decided increase in the number of phage particles liberated per bacterium after lysis inhibition has been effected. Figures 4, 5, and 6 show that the step size is higher when T4r^+-infected bacteria are inhibited than when they are not inhibited. Hershey (1946*a*) has noted that T2r^+ has a selective advantage in cases in which it is competing with T2r for survival in mixed cultures. This is true in spite of the fact that the mutation rate from r^+ to r is considerably higher than is the reverse rate. Since r-infected bacteria do not respond to lysis inhibition, the r^+ burst size becomes effectively larger than the r burst size. Whether this difference is an important factor in maintaining the wild type cannot be decided without closer analysis, but the results are suggestive of that hypothesis.

ACKNOWLEDGMENTS

The author is indebted to Dr. Max Delbrück for suggesting the problem and for advice and discussions concerned with its investigation. The author also wishes to thank Mr. William Hudson for assistance in carrying out the nephelometer experiments.

SUMMARY

Lysis caused by r^+ and by r strains of the even-numbered phages active on *E. coli* strain B is studied both by means of plaque count methods and by turbidimetry. From the turbidimetric studies it is seen that one characteristic common to all the lysis curves discussed is an initial dip in turbidity, which occurs immediately after addition of the phage. Present evidence indicates that it is due to a reduction and recovery in the light-scattering properties of bacteria resulting from the phage infection. With high multiplicities of infection immediate lysis of a fraction of the bacteria is also induced, and this adds to the magnitude of the initial drop. A second drop occurs in all cases at the end of the normal latent period. This drop is, however, quite different with r^+ infection from what it is with r infection. With the r phages it continues to complete clearing, but with the r^+ phages it is small and is succeeded by a rise in the curve. This difference is the basic difference between r^+ and r infections, and it has here been called lysis inhibition. After the period of inhibition a third drop in turbidity occurs, except with T2r^+, and this drop represents complete clearing.

The further analysis of lysis inhibition has yielded the following picture: the phenomenon requires that a bacterium be infected twice by r^+ phages of the same or similar type; the second infection, if it is an infection by the same type of phage as the first, may come any time after the primary infection has been established, which takes about 3 minutes; present evidence indicates that a second infection by only a single phage particle will accomplish the inhibition.

Lysis inhibition has the effect of increasing the burst size.

REFERENCES

ANDERSON, T. F. 1945 The role of tryptophane in the adsorption of two bacterial viruses on their host, *E. coli*. J. Cellular Comp. Physiol., **25**, 17–26.

BEADLE, G. W. 1945*a* Biochemical genetics. Chem. Revs., **37**, 15–96.

BEADLE, G. W. 1945*b* Genetics and metabolism in *Neurospora*. Physiol. Revs., **25**, 643–663.

DELBRÜCK, M. 1940 The growth of bacteriophage and lysis of the host. J. Gen. Physiol., **23**, 643–660.

DELBRÜCK, M. 1945*a* Effects of specific antisera on the growth of bacterial viruses (bacteriophages). J. Bact., **50**, 137–150.

DELBRÜCK, M. 1945*b* Interference between bacterial viruses. III. The mutual exclusion effect and the depressor effect. J. Bact., **50**, 151–171.

DELBRÜCK, M. 1946 Bacterial viruses or bacteriophages. Biol. Revs. Cambridge Phil. Soc., **21**, 30–40.

DELBRÜCK, M., AND LURIA, S. E. 1942 Interference between bacterial viruses. I. Interference between two bacterial viruses acting upon the same host, and the mechanism of virus growth. Arch. Biochem., **1**, 111–141.

ELLIS, E. L., AND DELBRÜCK, M. 1939 The growth of bacteriophage. J. Gen. Physiol., **22**, 365–384.

HERSHEY, A. D. 1946*a* Mutation of bacteriophage with respect to type of plaque. Genetics, **31**, 620–640.

HERSHEY, A. D. 1946*b* Spontaneous mutations in bacterial viruses. Cold Spring Harbor Symposia Quant. Biol., **11**, 67–77.

LINDEGREN, C. C. 1945 Yeast genetics. Bact. Revs., **9**, 111–170.

LURIA, S. E. 1945 Mutations of bacterial viruses affecting their host range. Genetics, **30**, 84–99.

LURIA, S. E., AND DELBRÜCK, M. 1943 Mutations of bacteria from virus sensitivity to virus resistance. Genetics, **28**, 491–511.

MOEWUS, FRANZ 1940 Die Analyse von 42 erblichen Eigenschaften der *Chlamydomonas eugametos*-Gruppe. Z. Induktive Abstammungs Vererbungslehre, **78**, 418–522.

SONNEBORN, T. M. 1946 Experimental control of the concentration of cytoplasmic genetic factors in *Paramecium*. Cold Spring Harbor Symposia Quant. Biol., **11**, 236–255.

UNDERWOOD, N. U., AND DOERMANN, A. H. 1947 A photoelectric nephelometer. Rev. Sci. Instruments. *In press*.