# FURTHER OBSERVATIONS ON THE MECHANISM OF PHAGE ACTION*

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Several investigators have reported studies on the mechanism of phage production. Among these Krueger and Northrop (1), Clifton and Morrow (2), Lin (3), Northrop (4), and Fischer (5) have employed in the original or modified form an activity method of phage assay developed by Krueger (6). Their results with several different phages and bacteria have agreed on the following points: (*a*) Phage production proceeds logarithmically with time. (*b*) Massive lysis occurs only when a definite critical ratio of phage to bacteria is attained.

D'Herelle (7), Burnet (8), Gratia (9), Ellis and Delbrück (10), and others have utilized the plaque count method for determining phage in analyzing the phenomenon of bacteriophagy. Delbrück (11) in particular has worked extensively with this procedure and has secured much information of interest from its application to one-step growth experiments.

Delbrück's data and the observations of others who have used the plaque count procedure lead to the conclusion that: (*a*) Cellular lysis takes place at intervals throughout the reaction instead of being merely a terminal event. (*b*) Phage formation occurs in steps; each step-like increment in [phage] corresponds to the liberation of newly formed phage as the cells in which it has been formed undergo lysis.

We have reinvestigated the reaction between an antistaphylococcal phage and the homologous bacterium utilizing both the activity and the plaque methods of analysis with the hope of securing a more complete picture of bacteriophagy than the use of either method alone will permit. Employing reduced temperature and lower pH to slow down the process it was observed that: (*a*) There is an initial phase of rapid logarithmic sorption of phage to susceptible cells during which the total phage activity and the loci of phage action (plaque numbers) in the mixtures remain constant. (*b*) When 90 per cent of the phage has been bound there is a sudden very rapid increase in phage activity not paralleled by an increase in plaques; *i.e.*, phage is being

*The experimental work reported here was completed over 5 years ago but was not prepared for publication due to the fact that the senior author has been in the Naval Service.

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formed intracellularly but is retained within cellular confines. (*c*) After a further drop in the extracellular phage fraction there occurs a pronounced increase in the total phage count not accompanied by any increase in total activity. This indicates a redistribution of phage formed intracellularly. At the same time there is a rise in the extracellular phage curves (both activity and plaque).

The number of times this basic cycle is repeated before complete lysis of the mixture takes place depends on the initial ratio of phage to bacteria in the suspension. Under conditions of optimal pH and temperature the step-like phases of the reaction are so brief that their existence is hardly detectable. However, the length of time occupied by each phase can be increased by appropriate adjustment of pH and temperature of the mixtures.

## EXPERIMENTAL METHODS

The following concomitant processes in phage-bacterial mixtures were followed: (1) Bacterial growth. (2) Increase in total phage (activity) and total phage (plaques). These terms are abbreviated below as: [total phage]$_{act.}$ and [total phage]$_{plaq.}$. (3) Changes in extracellular phage concentration (activity) and (plaques), abbreviated as [extracellular phage]$_{act.}$ and [extracellular phage]$_{plaq.}$

The reaction was studied under various conditions of pH and temperature as follows: (*a*) pH 7.2, 36°C., mechanical stirring with oxygenation (Fig. 1). (*b*) pH 6.1, 28°C., mechanical stirring with oxygenation (Fig. 2). (*c*) pH 7.2, temperatures 0°, 23°, and 30°, for rates of sorption (Fig. 3).

### *Methods*

*1. Growth Curves.*—The course of growth in suspensions of staphylococci in broth and in phage was followed at 0.1 hour intervals by turbidity readings both directly as previously described (1) and with a Klett-Summerson photoelectric colorimeter. The latter was calibrated with cell suspensions of known density and the readings of unknown preparations were converted into bacteria per milliliter by reference to the calibration curve.

*2. Total Phage (Activity).*—1 ml. samples at 6 minute (0.1 hour) intervals were diluted for measuring phage activity by the method of Krueger (6).

*3. Total Phage (Plaques).*—Samples used for activity titrations were diluted further for plaque determinations conducted by Gratia's method (14). Both phage activity and plaque titrations were set up immediately upon withdrawal of the samples. The dilutions made for activity estimations, however, were held at 0°C. until the end of the sampling period, at which time the bacterial suspension for titration was added and all samples were titrated at once. This has been demonstrated to be sound experimental procedure; numerous control titrations gave duplicate readings whether the samples were titrated at once or held at 0°C. for several hours.

*4. Extracellular Phage (Activity and Plaque) Determinations.*—30 ml. samples were withdrawn and chilled at the same time as those on which total activity and

plaque determinations were made. A 20 ml. aliquot of this phage-bacterial mixture was passed through a filter mat of Johns-Manville standard super-cel (prepared as described below) and the filtrate discarded. The preliminary filtration served to saturate the filter surface. Finally, 10 ml. were passed through and aliquots of this filtrate were diluted for extracellular activity and plaque determinations as described under sections 2 and 3. All dilutions were held at 0°C. until used.

*Preparation of Filters.*—Filters prepared of Johns-Manville standard super-cel have been found to be most satisfactory for quantitative separation of the active material in the filtrates.

Three gm. portions of standard super-cel were weighed into test tubes and sterilized in the hot air oven at 170°C. 30 ml. of sterile broth (1/4 pound beef infused per liter with 0.25 per cent Difco neopeptone and 0.5 per cent NaCl) were added to each portion just before use. The suspension was shaken vigorously and poured into a previously sterilized metal ultrafilter containing supporting disc, washer, and filter paper. The broth was filtered off under 8 cm. vacuum leaving a slight film of moisture at the surface of the filter mat. This moist film is extremely important for if the mass is completely dry, it recedes from the walls of the filter and all the organisms may not be retained. A complete filter was set up for each sample and was used within 30 to 40 minutes from the time of preparation. It was demonstrated repeatedly that 95 to 100 per cent of the phage present in a solution could be recovered in filtrates passed through the mat and that very few staphylococci penetrated it.

## DISCUSSION OF EXPERIMENTAL RESULTS

The analytical data obtained by applying the above methods to reacting mixtures of phage and susceptible staphylococci are presented graphically in Figs. 1, 2, and 3. It will be noted that the plateaus in the phage production curves at pH 7.2 and 36°C. (Fig. 1) are much shorter than those observed at pH 6.1 and 28°C.

*The Reaction at pH 6.1 and 28°C.*—Fig. 2 is a composite graph compiled from the data of four complete experiments and from one separate experiment on the end phase. For convenience in the following discussion the complete process has been divided into seven phases:

*Phase I.*—This phase covers the period from the moment of mixing the reactants ($T_0$) to 0.7 hour. The bacteria do not grow but take up phage from solution very rapidly, binding 90 per cent of the total phage in 0.1 hour. Beginning at 0.2 hour the $[\text{total phage}]_{\text{act.}}$ rises in two well defined steps to a level seventeen times the initial value while the $[\text{total phage}]_{\text{plaq.}}$ remains constant. Thus the amount of phage per bacterium increases without any parallel change in phage distribution. From 0.6 hour to 0.7 hour further sorption of phage occurs until $[\text{extracellular phage}]_{\text{act.}}$ is less than 0.1 per cent of the $[\text{total phage}]_{\text{act.}}$ and $[\text{extracellular phage}]_{\text{plaq.}}$ is 1 per cent of $[\text{total phage}]_{\text{plaq.}}$.

*Phase II.*—0.7 to 1.10 hours. All values remain constant during this period.

*Phase III.*—1.10 to 1.6 hours. While the [total phage]$_{act.}$ remains constant there is a 36-fold increase in the number of plaques. No new formation of phage takes place at this time but that produced during the first phase is now redistributed. At the beginning of phase III, 1 per cent of the [total phage]$_{plaq.}$ is extracellular and 0.5 hour later, at the end of phase III, this percentage has increased to only 6.3 per cent. Consequently, the 36-fold increase in total phage]$_{plaq.}$ represents for the most part an increase in infected organisms.

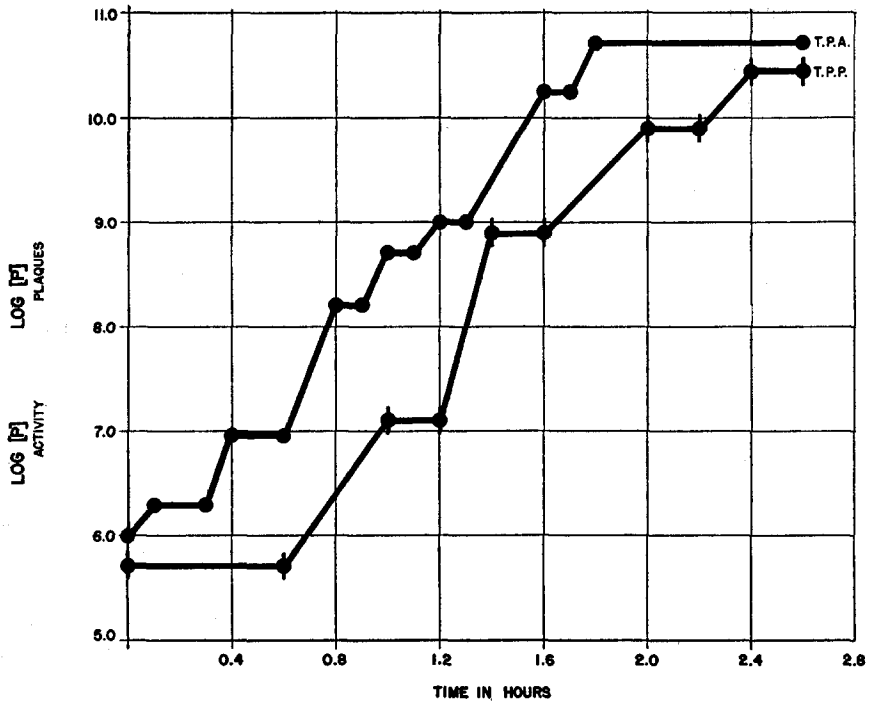


FIG. 1. Phage production in a mixture of staphylococci and homologous phage (three experiments). [Phage]$_0 = 1 \times 10^6$ activity units/ml., [bacteria]$_0 = 5 \times 10^7$/ml. Experimental conditions: pH 7.2, 36°C., $O_2$ stirring. Samples removed every 0.1 hour for activity assay and plaque count. Curves drawn from point to point. T. P. A. = [total phage] by activity assay. T. P. P. = [total ph ge] by plaque count.

At the end of phase III the total plaque count per milliliter is $2.5 \times 10^8$ and of this value only $1.3 \times 10^7$ plaques/ml. are extracellular. The difference, approximately $2.4 \times 10^8$, represents phage-containing organisms. Reference to the growth curve (Fig. 4) shows that the value obtained for phage-containing organisms by means of the plaque count approximates the number of bacteria in each milliliter; hence all the staphylococci are infected. Since [total phage]$_{act.}$ and [total phage]$_{plaq.}$ are to all purposes equal, it appears that each cell is infected with a single phage particle.

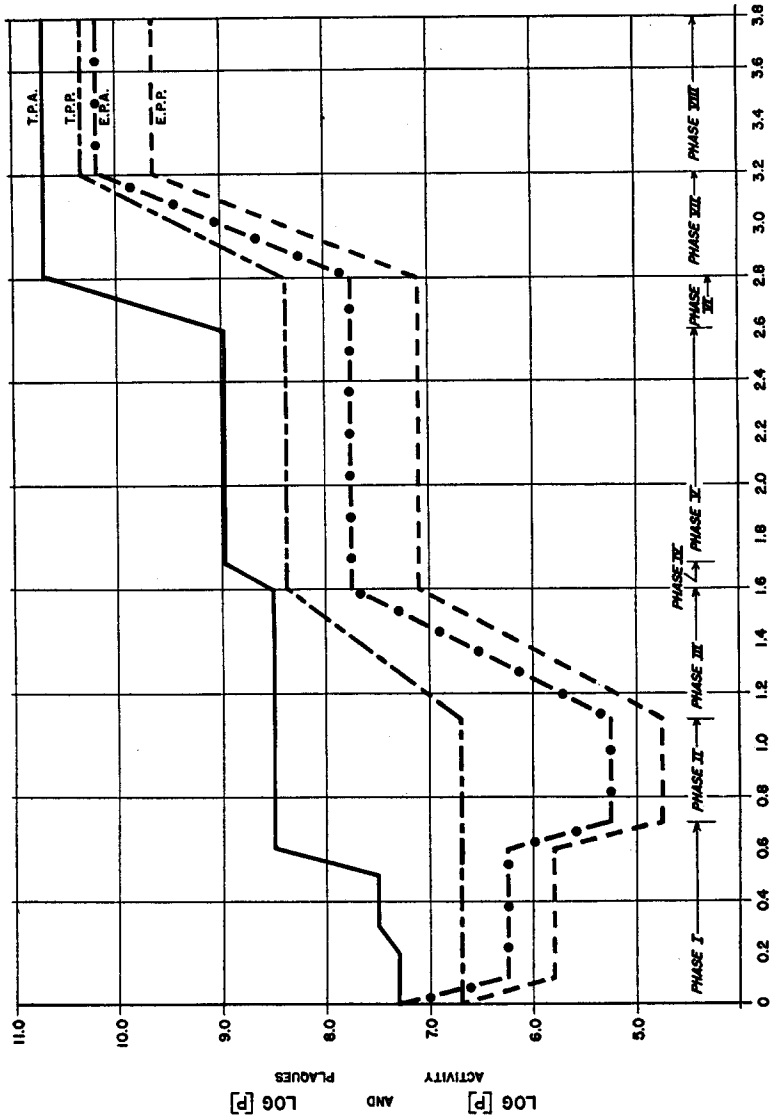


FIG. 2. Phage production in a mixture of staphylococci and homologous phage (four experiments and one separate experiment on end phase). $[\text{Phage}]_0 = 2 \times 10^7$ activity units/ml. $[\text{Bacteria}]_0 = 5 \times 10^7$/ml.

Experimental conditions: pH 6.1, 28°C., $O_2$ stirring. Samples removed every 0.1 hour for activity assay and plaque count. Curves drawn from point to point. T. P. A. = [total phage] by activity assay. T. P. P. = [total phage] by plaque count. E. P. A. = [extracellular phage] by activity assay. E. P. P. = [extracellular phage] by plaque count.

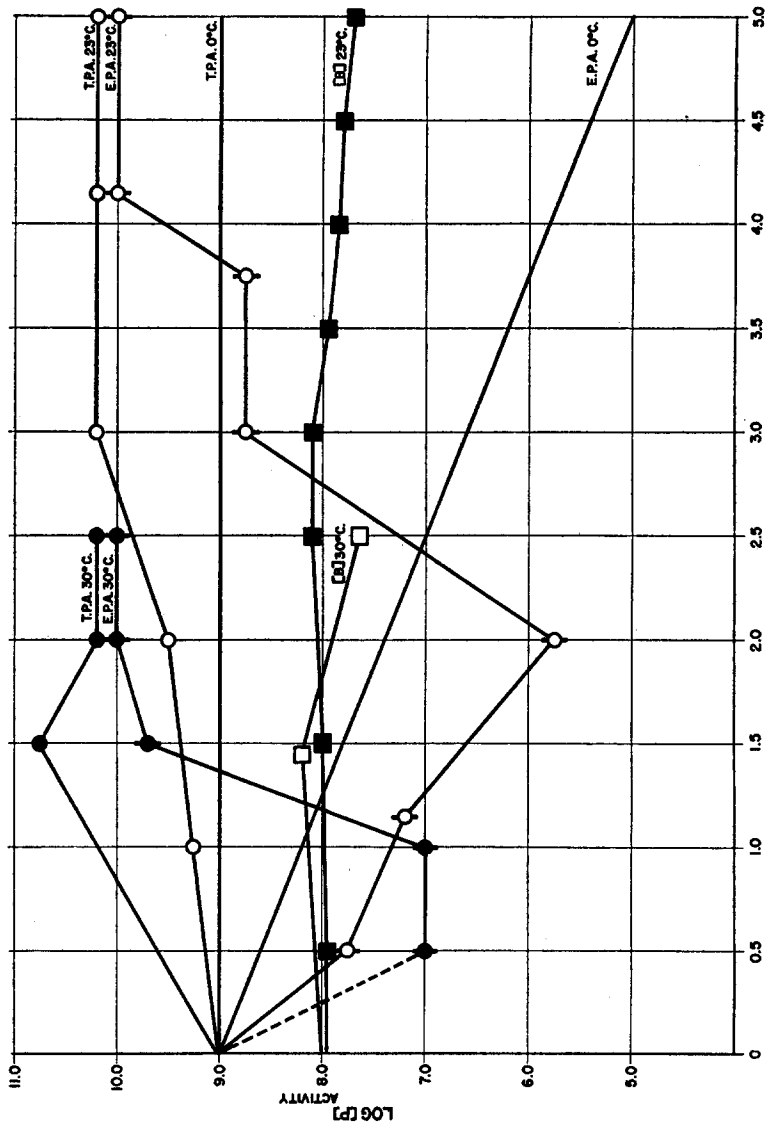


FIG. 3. Sorption of phage by living susceptible staphylococci at 30°, 23°, and 0°C. with pH constant at 7.2. Super-cel filter mats were used for quantitative recovery of extracellular phage fractions. Samples removed at 0.5 hour intervals. T. P. A. = [total phage] by activity assay. E. P. A. = [extracellular phage] by activity assay. [B] = [bacteria].

The slope of the curve for E. P. A. at 30°C. is indicated by a broken line since it is a dubious value; the plateau beginning at 0.5 hour very likely starts earlier but was not detected because of the long sampling interval employed.

*Phase IV.*—1.6 to 1.7 hours. This phase lasts only 0.1 hour but in this short time [total phage]$_{act.}$ increases 3.2-fold while all other values remain constant. Here again the increase in activity implies the formation of phage inside the bacteria without distribution of the newly formed phage to other organisms (*cf.* phase I).

*Phase V.*—1.7 to 2.6 hours. All values remain constant.

*Phase VI.*—2.6 to 2.8 hours. During an interval of 0.2 hour [total phage]$_{act.}$ rises 56-fold due to the development of phage in organisms that are already infected. At the beginning of phase VI [extracellular phage]$_{act.}$ is 5.6 per

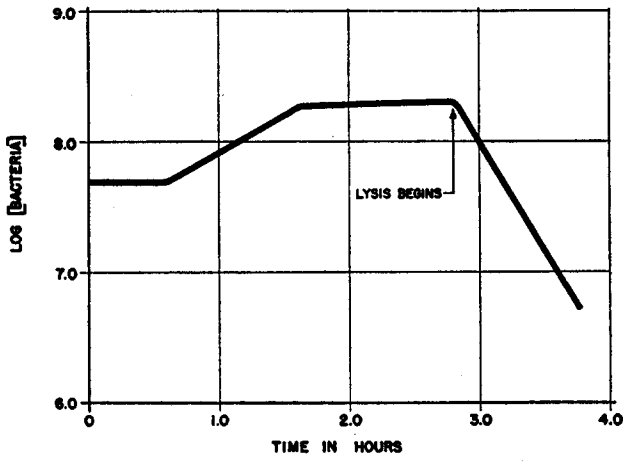


FIG. 4. Growth curve for susceptible staphylococci in presence of phage. [Phage]$_0$ = $2 \times 10^7$ activity units/ml. Experimental conditions: pH 6.1, 28°C., $O_2$ stirring. [Bacteria] determined at 0.1 hour intervals by: (*a*) direct turbidity readings using standards of known [bacteria], and (*b*) photoelectric nephelometer.

cent of [total phage]$_{act.}$ and approximately the same relationship obtains between the extracellular and total phage plaque counts.

*Phase VII.*—2.8 to 3.2 hours. During this period of 0.4 hour while [total phage]$_{act.}$ remains constant there is a rapid increase in the total number of plaques (88-fold) and also in the quantity of phage which becomes extracellular. The extracellular phage fraction increases 320-fold on an activity basis and 360-fold when measured by plaque count. The events in this phase are comparable to those occurring in phase III where there was no new formation of phage but a redistribution of that already formed took place.

*Phase VIII.*—In phase VIII we have the conditions which exist in the newly cleared lysate. At this time there are about 2.2 activity units for each plaque (calculated from the [total phage]) values. [total phage]$_{act.}$ is 3.3 $\times$ [extra-

cellular phage]$_{act.}$ and [total phage]$_{plaq.}$ 4.8 × [extracellular phage]$_{plaq.}$. The failure of total phage values to agree with the figures for extracellular phage assays is characteristic of phage preparations just after massive lysis has been completed. It is not true of lysates which have been permitted to age. Apparently under the special circumstances obtaining in the finished lysate, our filters remove some of the phage from solution. Probably this is due to the association of phage molecules with bacterial fragments dispersed by the lytic process. It is known that such bacterial debris is formed during lysis; it is microscopically visible, and consequently is of sufficient size to be removed by the super-cel filters. Another explanation may lie in the temporary survival of intact cells which have resisted lysis in numbers insufficient to be microscopically visible (less than $1 \times 10^7$ bacteria/ml.).

### *Sorption*

Fig. 3 presents the data obtained from a series of sorption experiments conducted at pH 7.2 and temperatures of 0°, 23°, and 30°C. In the experiment conducted at 0°C. there was no detectable change in [bacteria] nor in the [total phage]$_{act.}$. [Extracellular phage]$_{act.}$ decreased logarithmically with time until at 5 hours only 0.01 per cent of the total phage was unattached to cells.

At 23°C. a small increase in [bacteria] was noted after 1.5 hours. The rate of sorption of phage to bacterial cells was considerably greater than that observed for the 0°C. mixtures, and at 2 hours approximately only one phage particle in 6,000 remained unattached to the cells present.

At 30°C. the rate of uptake of phage by bacteria was even higher. The peak of sorption appeared to develop at 0.5 hour at which point 0.25 per cent of the phage was extracellular.

The fact that the rate of sorption increases with rise of temperature would support the view that something more than a purely physical adsorption is involved.

### *General Points*

The stepwise nature of the phage production curves is exaggerated at pH 6.1 and 28°C. (Fig. 2), but is not detectable when the reaction proceeds under the conditions normally imposed, *i.e.*, pH 7.2 and 36°C. (Fig. 1), unless samples are taken every 0.1 hour. When 0.25 hour samples are assayed as in earlier studies (1), the titration values plotted as logarithms against time fit a straight line plot, giving no clue to the existence of the plateaus.

A considerable body of earlier experimental work (15) has been interpreted in terms of the production of a hypothetical phage precursor by bacterial cells with subsequent autocatalytic transformation of this material into phage whenever the precursor-containing organisms are brought in contact with

phage. To demonstrate precursor, staphylococci are grown in an oxygenated medium at 36°C. for 2 hours. The organisms are collected by centrifugation, washed, resuspended in Locke's solution, and kept at 5°C. for 2 hours. When these "activated" organisms are added to phage-containing solution, there follows a tenfold increase in $[\text{phage}]_{act.}$ within 0.1 hour. The capacity of activated suspensions to increase $[\text{phage}]_{act.}$ is abolished by concentrations of iodoacetate and methylene blue, which do not destroy the bacteria. The same phenomenon can be demonstrated with sonic waves or heat and the very high temperature coefficient for the thermal inactivation of intracellular precursor suggests that the substance is protein in character.

If one is partial to the precursor theory, the situation presented in Fig. 2, from 1.7 to 2.8 hours, may be interpreted in these terms. For the first 0.9 hour of this period there was no change in any of the four titration values, and then within 0.2 hour $[\text{total phage}]_{act.}$ increased 56-fold. Since no sorption or redistribution of phage occurred during 0.9 hour, it is probable that phage was inside of or attached to the cells which ultimately developed the large increment in [phage].

Two major possibilities may apply to the events described: (*a*) The reaction: precursor + phage → phage is a slow one in its initial phases and only later speeds up to produce measurable quantities of phage. (*b*) The bacterial substrate at 1.7 hours contains no precursor, and it takes 0.9 hour for the formation of precursor to materialize. The synthesis is completed at 2.6 hours, and then this substance reacts with phage already present in the cells to produce more phage.

From what has been said above concerning the nature of the reaction between activated cells and phage, the second interpretation would appear to be the more likely of the two. However, the observed data certainly do not exclude other interpretations; *e.g.*, that phage is an autonomous microorganism, parasitizing bacteria. In this case the phage particles could be looked upon as incubating within the host cells, producing daughter particles at a certain division time (from 2.6 to 2.8 hours), and ultimately inducing massive lysis of the bacterial substrate.

Delbrück (12, 13) has advanced the view that the activity assay system is subject to certain inherent errors when used for the determination of bound phage; *i.e.*, phage taken up from solution by bacteria. He found that adsorption rates under optimal physiological conditions might be considerably larger than those measured under the usual conditions in experiments designed to obtain one-step growth curves. He feels that this result may have some bearing on the activity method of assay; to quote (12):

"In this method the time required to lyse a standard batch of bacteria is taken as a measure of phage concentration. The time interval in question is in part spent by

the phage on diffusion preceding the adsorption. It is clear that any slight change in the physiological state of the test sample of the bacteria themselves may greatly influence the adsorption rate and thereby the whole scale of the assay. This is taken care of in Krueger's method by always running a known sample parallel with the unknown in order to establish the scale of the day, but possibly the great day to day fluctuations in this scale are partly caused by this factor.

"It should also be noted that by this method adsorbed phage will be assayed higher than free phage because adsorption of phage is the first stage in the process of lysis. Also, the bacterium to which the phage is adsorbed in the experimental tube will differ physiologically from those used in the assay, and this will alter its growth characteristics and thus its assay value by the activity method. Adsorbed phage and free phage as measured by this method are not comparable. Adsorbed phage may simulate increase in phage where there is only a change in the scale of assay."

Again, in his review (13), Delbrück states:

More serious is the error incurred in the interpretation of a series of experiments by Krueger and his collaborators in which it is claimed that proof has been found for the existence of a virus precursor in 'activated bacteria;' *i.e.*, bacteria in the dividing stage. In these experiments the virus is mixed with 'activated bacteria' in the cold for a few minutes and then titrated in the usual manner. The concentration of the 'activated bacteria' with which the virus was mixed was so high that the virus would be adsorbed in a few minutes. These virus particles will therefore assay higher than free virus particles or virus particles adsorbed to bacteria in the lag phase, and will thus simulate a true increase in virus. In the opinion of the reviewer these experiments serve to illustrate the ambiguities of the particular method of assay employed and do not indicate the presence within the cell of the virus precursor which can be transformed into virus in arresting cell in the cold."

The overlong use of an assay method for any type of laboratory experimentation may make one unduly reliant upon it and inclined to overlook its limitations. The writers are not unmindful of this and feel that they should have utilized both the activity method and the plaque count procedure long ago in approaching the problem dealt with in the present paper. However, certain of Delbrück's criticisms of the activity method and of the experiments dealing with precursor are not in conformance with our own observations.

In the first place there are no "great day to day fluctuations" in the titration curves. The two reacting agents, bacteria and phage, are of course far from being simple in nature and one cannot expect them to be capable of standardization in the same sense that would apply to C.P. chemical solutions. However, it is possible to store phage under conditions permitting very little deterioration and day after day to prepare bacterial suspensions which exhibit remarkably consistent properties with respect to growth rates and phage production rates in the standard titration mixtures. This constancy is reflected in close approximation of times of lysis in the control series.

Our standard, normal, ("non-activated") bacterial substrate is prepared by growing staphylococci at 37°C. in Roux flasks on 2.5 per cent agar made up with infusion broth and 1 per cent neopeptone. After 16 to 18 hours of growth the cells are harvested in Locke's solution, washed once in Locke's solution, and suspended in 1 per cent Difco neopeptone broth containing 0.5 per cent NaCl and adjusted to pH 7.4. Preparation of phage solutions is described elsewhere (1) and the details do not warrant repeating here.

[Total phage] for a mixture of phage and bacteria can be determined accurately by the activity assay method. When phage is added to normal cell suspensions and subsequently the mixtures are titrated by the activity method, the values obtained for $[\text{total phage}]_{act.}$ are the same as those secured by titration of controls made by adding phage to plain broth. This applies to phage-bacteria mixtures held for periods varying from 1 minute to several hours, as long as they are maintained at low temperature to inhibit bacterial growth and phage production.

Three serial tenfold dilutions ordinarily are made of each sample for activity assay. Then bacteria are added to a concentration of $2.5 \times 10^7$/ml. and the tubes are placed in a water bath shaker at 36°C. The time of lysis for each unknown is ascertained, as is the time of lysis of known standard phage concentrations. The latter values provide a titration curve from which there are obtained three separate readings for a single sample. These three values agree well among themselves, even though the titration tubes contain varying concentrations of bacteria from the original phage-bacteria mixture. In practice, dilution of an unknown provides that no more than $1 \times 10^6$ organisms/ml. are carried over into the first of the titration tubes. The second and the third, respectively, contain at most $1 \times 10^5$ and $1 \times 10^4$/ml.

Further, if Delbrück's objection to the determination of total phage (intracellular plus extracellular) by the activity method were valid, we should not have the situation which obtains during the sorption of phage. For example, during the first 0.2 hour of the phage-bacteria reaction at 28°C. and pH 6.1 (Fig. 2) while $[\text{extracellular phage}]_{plaq.}$ and $[\text{extracellular phage}]_{act.}$ drop rapidly due to sorption of phage onto bacteria, $[\text{total phage}]_{act.}$ remains constant. According to Delbrück $[\text{total phage}]_{act.}$ should rise as the bacteria remove phage from solution and induce an artificial shortening of the time of lysis in the titration mixture. This would apply also to the sorption experiment conducted at O°C., although reference to Fig. 3 shows that $[\text{total phage}]_{act.}$ does not rise during the 5 hour period of observation.

So far as phage precursor is concerned, only the isolation of this hypothetical substance can establish final proof that it exists. Even as a working concept many objections can be brought to bear against it, especially from the biological viewpoint. On the other hand, it is an attractive possibility supported by certain experimental evidence.

Krueger and Baldwin (16) observed that cell-free ultrafiltrates of normal staphylococcal cultures added to phage induced the formation of more phage in amounts beyond the limit of error of the activity titration method. The phage-forming fraction appeared irregularly in culture ultrafiltrates and at no time was present in large amounts. For this reason we turned our attention to "activated" bacteria, which regularly and rapidly increased $[\text{phage}]_{\text{act.}}$ when brought in contact with phage.

Delbrück, as noted above, feels that the "activated" bacteria do not actually increase $[\text{phage}]_{\text{act.}}$ but appear to do so by taking up phage rapidly and later speeding up lysis in the titration mixtures. This explanation probably does not hold, for the following reasons:

1. A mixture of normal bacteria, $4 \times 10^8$/ml. and phage, $2 \times 10^8$ activity units/ml., kept at 5°C. for 5 hours and subsequently diluted for titration of $[\text{total phage}]_{\text{act.}}$ gives a titer of $2 \times 10^8$ activity units/ml. in all three dilutions.

2. A mixture of activated bacteria, $4 \times 10^8$/ml. and phage, $2 \times 10^8$ activity units/ml., kept for a minimum of 5 minutes at the same low temperature, gives a titer of approximately $2 \times 10^9$ activity units/ml. in all three dilutions.

We know that only a trace of phage is left free in solution in the first mixture so that the difference in titers cannot be ascribed to the degree to which phage has been taken up.

The suspensions studied in experiments with "activated" bacteria consist of only one part of broth added to four parts of Locke's solution. When the mixtures are held for as long as 5 hours at 5°C. before assay, one cannot consider the bacteria to be in the dividing stage. They have been maintained too long in an environment unfavorable for cell division to assume that their physiological state will influence the titration mixture.

Again, as in the case of the "normal" bacteria-phage mixtures titrated for $[\text{total phage}]_{\text{act.}}$ one would predict that the degree of aberration ascribable to organisms carried over into the titration system would be roughly proportional to the numbers of such cells introduced. The three assay tubes contain respectively $1 \times 10^6$, $1 \times 10^5$, and $1 \times 10^4$ "activated" bacteria/ml. yet the titration values of all three are in agreement.

Similarly, it has been demonstrated that "activated" cells retain their capacity to increase $[\text{phage}]_{\text{act.}}$ even when first stored in Locke's solution at 5°C. for 4 hours (15). In later experiments the storage period was 6 hours without the activation effect being abolished. The bacteria, as shown by actual counts (15), were not in a phase of cell division, but rather were in something of a basal metabolic state, for storage at 5°C. without nutrients over a period of several hours certainly does not favor cellular activity of any sort. Delbrück apparently concurs in our analysis of the effect of low temperatures for he has stated in connection with a discussion of adsorption (12) that "Krueger's measurements with live bacteria were done at 10°C., when they were truly resting,. . . . ."

It is possible to avoid in another way the postulated error introduced by the presence of some "activated" bacteria in the phage titration system. The cellular substrate participating in the "activated" bacteria + phage reaction can be completely lysed so that the ultimate assay proceeds with no interference from this source. To accomplish this it is necessary to utilize the fact that small concentrations of $Mn^{++}$ ions greatly reduce the lytic threshold; otherwise, when enough phage is added to bring about lysis, the phage-bacteria ratio will be so high that the small increment of phage derived from the intracellular reaction cannot be detected.

To make use of the manganese effect, organisms are activated in the presence of $Mn^{++}$, a mixture of phage and bacteria is made in such proportions that lysis begins before there is an opportunity for cell growth, and the characteristic tenfold increase in $[phage]_{act.}$ is obtained but under these circumstances in a lysed preparation rather than in one containing large numbers of intact cells. This reaction may be carried on seriatim by appropriate dilution of the lysate to the original titer, adding more organisms, determining the end titer of the lysate, etc. In experiments recorded elsewhere (17) the original phage used has been diluted beyond 1 to 1,000,000 without any reduction in activity or plaque count. The reaction was interpreted as indicating that the activated bacteria contain some sort of a substance (phage precursor) which is transformed into active phage by phage itself and that the newly formed phage is dispersed into solution when the bacteria lyse.

From the material presented above it would appear that Delbrück's indictment of the activity assay method for determining [total phage] and his criticism of the experiments in which "activated" bacteria were used as a source of precursor are not in accord with the experimental data.

So far as the experiments cited are concerned [total phage] for mixtures of phage and bacteria can be measured accurately by the activity assay method. The bacteria from such a suspension will constitute at most $< 4$ per cent of the bacterial substrate used in the titration system (changing the intitial [bacteria] from $2.5 \times 10^7$/ml. to $2.6 \times 10^7$/ml.). It has not been observed that a variation of this order of magnitude exerts any noticeable effect upon the subsequent growth curve or upon the time of lysis. This small fraction of the total bacterial population may carry $> 90$ per cent of the total phage, and it would be very desirable to know exactly how this intracellular fraction is released to infect the organisms of the assay system for it seems to participate in the titration just as though the same amount of free phage had been introduced.

Lacking experimental evidence on this point, perhaps the mechanism can be visualized in a general way from the events depicted in Fig. 1, noting that there may be some discrepancies due to differences in the experimental conditions. Specifically, in conducting a titration $O_2$ is not used and $[bacteria]_0$ is $2.5 \times 10^7$/ml. instead of $5 \times 10^7$/ml.

In the mixture analyzed in Fig. 1, 0.6 hour elapses before the first redistribution of phage occurs, and during this period there is a tenfold increase in $[phage]_{act.}$ Whether this production of phage takes place within the small fraction of cells infected by addition of free phage to the assay system or whether it proceeds within the relatively few organisms carried over from a mixture of phage and bacteria, the newly formed phage will be dispersed into a bacterial suspension which has been sustained under conditions favoring the production of "activated" cells. The released phage is bound at once to such precursor-containing organisms and the $[total\ phage]_{act.}$ rises as the precursor is converted into phage. The net results at 1.0 hour (Fig. 1), *i.e.*, infection of approximately 20 per cent of the cell population and a rise in $[total\ phage]_{act.}$ of 500-fold, apparently can be obtained either when the original phage added is in solution or when it is present in a small number of susceptible cells.

## SUMMARY AND CONCLUSIONS

1. The reaction between an antistaphlycoccal phage and the homologous bacterium has been studied, applying the following experimental technics not used in earlier work reported from this laboratory: (*a*) Both the activity assay and the plaque count were utilized for determining [phage]. (*b*) Sampling was done at short intervals; *i.e.*, every 0.1 hour. (*c*) Extracellular phage was separated from the cell-bound fraction by a filtration procedure permitting passage of $>$ 95 per cent of free phage.

2. Using these technics, the reaction was followed: (*a*) with pH maintained at 6.10 and temperature at 28°C. to slow the process; (*b*) with pH maintained at 7.2 and temperature at 36°C.

3. In addition separate experiments were performed on the sorption of phage by bacteria at 30°, 23°, and 0°C.

4. At pH 6.10 and 28°C. the phage-bacterium reaction proceeds in the following sequence: (*a*) There is an initial phase of rapid logarithmic sorption of phage to susceptible cells, during which the total phage activity and the plaque numbers in the mixtures remain constant. (*b*) When 90 per cent of the phage has been bound, there is a sudden very rapid increase in phage activity not paralleled by an increase in plaques; *i.e.*, phage is formed intracellularly, but is retained within cellular confines. (*c*) After a further drop in the extracellular phage fraction there occurs a pronounced increase in the total phage plaque count not accompanied by any increase in total activity. This indicates a redistribution of phage formed intracellularly. At the same time there is a rise in the extracellular phage curves (both activity and plaque). (*d*) With the concentrations of phage and bacteria used in the experiment carried out at pH 6.1 and 28°C. there are two further increments in $[phage]_{act.}$ before massive lysis begins. (*e*) During terminal lysis there are sharp rises in the curves for $[total\ phage]_{plaq.}$, $[extracellular\ phage]_{act.}$, and $[extracellular\ phage]_{plaq.}$. (*f*) Immediately after the completion of lysis there is a considerable dis-

parity between measurements of total phage and extracellular phage, probably occasioned by the association of phage molecules with cellular debris, the latter being of sufficient size to be removed by the super-cel filters.

5. At pH 7.2 and 36°C. the steps in the phage production curve as determined by activity assay and plaque count are much less prominent than those observed at pH 6.1 and 28°C. However, the plateaus described by Ellis and Delbrück (10) for *B. coli* and coli phage can be detected also in the present case if frequent samples are taken.

6. The sorption experiments show a significant rise in the rate of phage uptake with increase in temperature, again supporting the view that the reaction involves more than a purely physical adsorption.

7. Delbrück's objections to: (*a*) the use of the activity assay for determining [total phage] in mixtures of phage and susceptible cells, and (*b*), to the demonstration of phage precursor in "activated" bacteria have been analyzed.

8. The activity assay has been demonstrated to be an accurate procedure for determining either phage free in solution or phage bound to living susceptible cells, under the conditions of the experiments reported here and in earlier work.

9. The titration values obtained in the experiments designed to exhibit intracellular phage precursor are not the result of artifacts as Delbrück has inferred. The data can be interpreted in terms of the precursor theory, although other explanations are not ruled out.

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