

## INTRACELLULAR PHAGE PRECURSOR\*

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(Accepted for publication, May 3, 1939)

### INTRODUCTION

The early concept of phage production advanced by d'Herelle involved a general host-parasite relationship between the bacterium and the homologous phage. The phage was thought to be an autonomous ultramicroscopic parasite which had the power to break through the cell's outer membrane; once inside the organism it divided into daughter corpuscles and the accumulation of new phage eventually resulted in cellular destruction or lysis. Certainly this description of the essential mechanism of bacteriophagy did fit the facts then available. However, the gradual accumulation of information concerning the reaction that takes place between the phage and bacterium has made it doubtful that this simple picture is correct.

It is now reasonably certain that phage is not an autonomous living agent, at least as judged by the criteria of classical physiology. The experimental evidence obtained by Krueger and his collaborators (1) led to the idea that phage is a protein with many of the properties of an enzyme (Krueger (2)), and Northrop (3) has supplied more direct proof of this concept by isolating a nucleoprotein which possesses all the characteristics of phage. This does not imply that phage occupies an entirely unique position in natural history as the only virus of its kind, for Stanley (4) sometime ago showed that tobacco mosaic virus consists of large protein molecules.

The demonstration of the fact that phage is a protein of high molecular weight assisted immeasurably in approaching the problem of its formation. Previous studies had indicated that bacterial lysis ensues when approximately 100 phage units per bacterium accumulate in

\* This work was supported by grants-in-aid from the Research Corporation and from several interested friends.

the system (1). Why bacterial growth was essential for the formation of phage did not appear. Later it was found that when bacteriophage took place in the presence of 0.25 M NaCl (5) or 0.125 M Na<sub>2</sub>SO<sub>4</sub> (6) there occurred prelytic plateaus in the bacterial growth curves extending from 0.3 hour to 0.8 hour. Despite the fact that there was no bacterial reproduction during this time phage formation went on at a normal rate, suggesting that under certain conditions cell division was not requisite for phage production. Further evidence was supplied by the observation that the temperature and pH optima for the two rate curves differed (7).

These findings lead to the conclusion that cellular reproduction *per se* probably was not responsible for phage formation and suggested that some other cellular reaction operating optimally under nearly identical environmental conditions was accountable. The possibility that phage might be a product of bacterial metabolism had been stressed long ago by Bordet (8) and it now received support from the experiments of Krueger and Baldwin (9). They found that certain cell-free ultrafiltrates of normal staphylococcal suspensions when added to bacteriophage induced the formation of more phage in amounts well beyond the limit of error of the activity titration method. However, the phage-forming fraction appeared irregularly in culture ultrafiltrates and other means were sought for its demonstration.

Krueger and Mundell (10) have recently reported a method for demonstrating what they call "intracellular phage precursor." They observed that staphylococci grown in an oxygenated medium and subsequently maintained under conditions precluding cellular reproduction, had the capacity to increase [phage] very considerably when added to phage-containing solutions. This phage-augmenting characteristic of oxygenated or "activated" cells can be demonstrated with regularity and is not found in normal resting cells. The present paper deals with such properties of the intracellular "precursor" as we have been able to determine. In speaking of it as phage precursor we do so without knowing whether the essential phage-producing reaction is the hydrolytic cleavage of a preformed protein precursor, as seems to be the case with the autocatalytic transformation of inactive enzyme precursors into active enzymes, or whether it involves

completion of a complex protein synthesis by the cell under the stimulus of contact with phage.

#### EXPERIMENTAL RESULTS

Throughout the paper the following terms are used:

- A. [Phage] = Concentration of phage/ml. expressed as activity units.
- B. [Phage]<sub>0</sub> = Initial concentration of phage/ml. in activity units.
- C. P.U. = Phage units or activity units. See Krueger (11) or Northrop (13) for discussion of the activity titration method.
- D. [Bacteria] = Number of staphylococci/ml.
- E. [Bacteria]<sub>0</sub> = Initial concentration of staphylococci/ml.

1. *Demonstration of Intracellular Phage Precursor.*—To demonstrate phage precursor in the strain of staphylococcus which has been used throughout all our previous work on bacteriophage the organisms are grown for 18 hours on nutrient agar in Blake flasks at 37°C. The cells are washed in Locke's solution and are then grown in broth through which oxygen is constantly bubbled. The bacteria are separated from the medium, resuspended in Locke's solution, and are kept for 2 hours at 5°C. in order to inhibit any further cell division. This suspension contains what we call activated bacteria. To 4 ml. of the activated suspension containing  $5 \times 10^8$  bacteria/ml. is added 1 ml. of phage diluted with Locke's solution to contain  $1 \times 10^9$  activity units/ml. The mixture is kept for 5 minutes at 5°C. and is then titrated for phage content by the activity method (11). The final [phage] as shown in Table I is found to be approximately  $2 \times 10^9$  activity units/ml.; that of the control prepared with nonactivated bacteria from the original 18 hour agar culture is  $2 \times 10^8$  activity units/ml.

Certain objections to this experiment at once present themselves and must be answered before the phenomenon can be considered valid. For example, it is quite possible that the bacteria which have been activated by growth in the presence of oxygen have simply acquired an enhanced capacity for reproduction. When added to phage for the purpose of demonstrating precursor they may go on growing and thus may continue to produce phage as a function of cellular repro-

duction; *i.e.*, under the conditions described in an earlier study (12). Another possibility depends upon the fact that when the mixture of activated cells and phage is diluted for titration a certain number of activated bacteria will be present in the final titration mixtures. The activity method for phage titration depends upon ascertaining the time of lysis of a normal bacterial suspension to which dilutions of the phage-containing unknown are added. It is conceivable that the activated organisms constitute a significant proportion of the whole cell population and that they might selectively overgrow the bacteria added for titration purposes. If they grow at a faster rate they will reach the lytic endpoint more rapidly and will give the effect of an increased initial phage concentration.

TABLE I

*Outline of Experiment to Demonstrate the Phage-Augmenting Capacity of Activated Staphylococci*

- 
- a. Staphylococci "activated" by growth in O<sub>2</sub> medium at 36° C. 2 hrs.
  - b. Cells washed and resuspended in Locke's solution ( $5 \times 10^8$  bacteria/ml.).
  - c. Activated cell suspension kept at 5° C. 2 hrs.
  - d. 4 ml. activated suspension added to 1 ml. phage in Locke's solution containing  $1 \times 10^9$  P.U./ml. Mixture kept 5 min. at 5° C. and titrated.  $[\text{Phage}]_0 = 2 \times 10^8$ .  $[\text{Phage}]_{\text{final}} = 2 \times 10^8$
  - e. Control: 4 ml. original 18 hr. agar culture diluted in Locke's solution to contain  $5 \times 10^8$  bacteria/ml. added to 1 ml. phage containing  $1 \times 10^9$  P.U./ml. Mixture kept 5 min. at 5° C. and titrated.  $[\text{Phage}]_0 = 2 \times 10^8$ .  $[\text{Phage}]_{\text{final}} = 2 \times 10^8$
- 

These objections, we believe, have been answered by the following experiments:

*A. Activated Bacteria Carried over into Titration Mixture May Have an Untoward Effect on Accuracy of Results*

Standard phage containing  $1 \times 10^{10}$  activity units/ml. is diluted in three different sets of broth blanks containing respectively  $5 \times 10^5$  activated bacteria/ml.,  $5 \times 10^4$  activated bacteria/ml., and  $5 \times 10^3$  activated bacteria/ml. These concentrations of activated organisms represent the numbers which would be present when the mixture used to demonstrate the presence of precursor is diluted for titration. To 4 ml. of each dilution of phage 1 ml. of normal bacterial suspension ( $12.5 \times 10^7$ /ml.) used for titration is added. The time of lysis of the

various mixtures is determined and it is found (Table II) that the small amounts of activated bacteria present in the mixtures do not affect the titration values in any way.

*B. Bacterial Growth May Occur in the Activated Bacteria-Phage Mixtures and This May Be Responsible for the Observed Increase in Phage Titre*

As soon as the activated organisms prepared as described above were suspended in Locke's solution at 5°C. samples were taken at successive intervals for direct microscopic counts and for plate counts. Samples were also taken as soon as the mixture of phage and organisms

TABLE II  
*Outline of Experiment to Test Effect of Activated Bacteria Added to Phage Titration System*

- 
1. 9 ml. broth blanks prepared containing three different concentrations of activated bacteria:
    - $5 \times 10^5$  activated bacteria/ml.
    - $5 \times 10^4$  activated bacteria/ml.
    - $5 \times 10^3$  activated bacteria/ml.
  2. Sets of each concentration used to dilute standard phage for titration (from  $1 \times 10^{10}$  activity units/ml. to  $1 \times 10^7$ ,  $1 \times 10^6$ , and  $1 \times 10^5$  activity units/ml.).
  3. To 4 ml. of each dilution of phage 1 ml. of normal bacteria containing  $12.5 \times 10^7$  cells/ml. was added.
  4. Time of lysis was determined.
  5. Presence of  $5 \times 10^5$ ,  $5 \times 10^4$ , or  $5 \times 10^3$  activated bacteria/ml. in titration system does not affect results.
- 

in Locke's solution had been made. Due to the presence of phage only direct microscopic counts could be done on these latter mixtures. However, no growth was observed in the suspension containing activated bacteria alone or in the mixture containing activated bacteria and phage (Table III). Both preparations were followed for a 2 hour interval.

*C. If Phage Precursor Exists It Should Be Possible to Serially Dilute a Given Amount of Phage in Successive Aliquots of Precursor without Reducing the Original Phage Titre*

One way to accomplish this would be to add sufficient phage to the precursor-containing organisms so that after the precursor had

been converted into phage it would be released by cellular lysis. The lytic threshold for this particular strain of staphylococcus is 100 activity units/bacterium. When enough phage is added to bring about lysis the phage-bacteria ratio is so high that the small increment of phage derived from the intracellular reaction is not detectable. It is possible to circumvent this difficulty by utilizing the fact that small concentrations of  $Mn^{++}$  ions greatly reduce the lytic threshold. We have reported elsewhere (14) experiments in which the serial production of phage from the intracellular precursor of manganese-

TABLE III

*Tests for Growth of Activated Bacteria. Average Values of Three Experiments*

Activated cells were diluted to  $5 \times 10^8$  bacteria/ml. in Locke's solution and were stored at  $5^\circ C$ . At intervals samples were removed for direct microscopic counts, plate counts, and for an activation test conducted as described in Table I.

In addition, direct counts (last column) were made on a mixture containing 4 ml. of activated cell suspension + 1 ml. of phage diluted in Locke's solution to  $1 \times 10^9$  P.U./ml. and kept at  $5^\circ C$ .

Time of sampling	[Bacteria] in Locke's suspension activated cells. Direct count/ml.	[Bacteria] in Locke's suspension activated cells. Plate count	Test for activation of cell suspension. Cells mixed with phage and titrated. [Phage] = $2 \times 10^8$ P.U./ml.	Direct count on mixture of 4 ml. activated cells + 1 ml. phage diluted in Locke's solution to contain $1 \times 10^9$ P.U./ml.
<i>hrs.</i>				
0.0	$4.5 \times 10^8$	$4.10 \times 10^8$	$4.0 \times 10^9$	$3.81 \times 10^8$
0.5	$4.35 \times 10^8$	$4.0 \times 10^8$	$3.5 \times 10^9$	$4.02 \times 10^8$
1.0	$4.61 \times 10^8$	$4.23 \times 10^8$	$4.0 \times 10^9$	$3.92 \times 10^8$
1.5	$4.47 \times 10^8$	$4.06 \times 10^8$	$4.0 \times 10^9$	$3.69 \times 10^8$
2.0	$4.28 \times 10^8$	$3.98 \times 10^8$	$4.0 \times 10^9$	$3.73 \times 10^8$

treated cells was carried out in the absence of cellular growth (Table IV). As each aliquot of intracellular precursor was converted into more phage the bacteria lysed and released a measurable quantity of phage free in the medium. The new lysate was then diluted to the original titre, more activated bacteria were added, etc. By this means the original phage was diluted to more than  $1/2,000,000$  without any loss of [phage] as determined by both the activity and plaque count titration procedures.

*2. Duration of Activation under Conditions of the Experiment.*—Suspensions of activated organisms in Locke's solution were kept at

TABLE IV  
Serial Production of Phage from Successive Aliquots of Precursor-Containing Bacteria

	Mixture 1	Mixture 2	Mixture 3	Mixture 4	Mixture 5	Mixture 6
Initial [phage]	$6.3 \times 10^8$	$6.3 \times 10^8$	$6.3 \times 10^8$	$6.3 \times 10^8$	$6.3 \times 10^8$	$6.3 \times 10^8$
Initial [bacteria]	$1 \times 10^8$	$1 \times 10^8$	$1 \times 10^8$	$1 \times 10^8$	$1 \times 10^8$	$1 \times 10^8$
Final [phage]	$7.9 \times 10^8$	$9.5 \times 10^8$	$6.3 \times 10^8$	$7.6 \times 10^8$	$5.7 \times 10^8$	$6.3 \times 10^8$
Total phage; <i>i.e.</i> , initial [phage] $\times$ total dilution	$6.3 \times 10^8$	$7.87 \times 10^{10}$	$11.8 \times 10^{11}$	$11.8 \times 10^{12}$	$14.2 \times 10^{13}$	$12.8 \times 10^{14}$

5°C. and samples were removed at intervals. Each sample was mixed with a known amount of phage and after standing at 5°C. for 5 minutes was immediately diluted for titration (Table V).

It was found that the suspensions of activated organisms maintained their activity for 4 hours without change. After this time there was some variation in the different preparations tested. Occasionally, 24 hour samples gave a typical increase in phage titre when added to phage; other suspensions completely lost their activity in 24 hours.

3. *Rate of Reaction between Activated Bacteria and Phage.*—Suspensions of activated organisms were added to phage and samples were removed for titration at brief intervals in order to determine the time required for the typical increase in phage titre. In all cases the full

TABLE V

*Duration of Activation of Staphylococci after Growth in Oxygenated Medium*

$5 \times 10^8$  activated cells/ml. stored in Locke's solution pH 7.4 at 5°C. 4 ml. samples removed at intervals and tested by adding to them 1 ml. of phage containing  $1 \times 10^9$  P.U./ml. Mixture kept at 5°C. for 5 min. and then titrated.

Preparation No.	Total phage formed when activated cells added to phage after storage for				
	0 hr.	1 hr.	2 hrs.	4 hrs.	24 hrs.
1	$3 \times 10^9$	$3.1 \times 10^9$	$2.8 \times 10^9$	$3.3 \times 10^9$	$9.6 \times 10^8$
2	$2.2 \times 10^9$	$2.2 \times 10^9$	$2.2 \times 10^9$	$2.2 \times 10^9$	$3 \times 10^8$
3	$2.0 \times 10^9$	$2.0 \times 10^9$	$2.3 \times 10^9$	$2.0 \times 10^9$	$2.6 \times 10^8$
4	$3.1 \times 10^9$	$2 \times 10^9$	$2.8 \times 10^9$	$2.8 \times 10^9$	$2.0 \times 10^9$

increase in titre was observed after the phage had been in contact with the organisms for 1 to 2 minutes (Table VI).

4. *The Rôle of Broth and Oxygen in Activation of Bacteria.*—In order to determine whether oxygen and the broth medium were essential for activation, three different sets of bacterial suspensions were prepared:

A. Normal activation mixture with oxygen bubbled through broth suspension of bacteria.

B. Nitrogen instead of oxygen bubbled through broth suspension of bacteria.

C. Oxygen bubbled through Locke's suspension of bacteria.

It was found that very little activation was produced by nitrogen



bubbled through broth suspensions of staphylococci; oxygen bubbled through Locke's solution suspensions of staphylococci produced no measurable activation (Table VII).

TABLE VI

*Rate of Phage Formation by Activated Staphylococci*

16 ml. of a suspension of activated staphylococci in Locke's solution containing  $5 \times 10^8$  bacteria/ml. were added to 4 ml. of phage diluted in Locke's solution to contain  $1 \times 10^9$  P.U./ml. Temperature  $5^\circ$  C.  $[\text{Phage}]_0 = 2 \times 10^8$ . Samples were removed at intervals for titration of total [phage].

Time after mixing phage and bacteria	Preparation 1 [phage]	Preparation 2 [phage]	Preparation 3 [phage]
<i>min.</i>			
1	$4.3 \times 10^8$	$2.0 \times 10^9$	$3.4 \times 10^9$
2	$2.0 \times 10^9$	$2.5 \times 10^9$	$6.7 \times 10^9$
3	$2.2 \times 10^9$	$2.4 \times 10^9$	$6.7 \times 10^9$
4	$2.2 \times 10^9$	$2.5 \times 10^9$	$6.9 \times 10^9$
5	$2.0 \times 10^9$	$2.5 \times 10^9$	$6.6 \times 10^9$
10	$2.0 \times 10^9$	$2.5 \times 10^9$	$6.6 \times 10^9$
20	$2.0 \times 10^9$	$2.5 \times 10^9$	$6.7 \times 10^9$
30	$2.0 \times 10^9$	$2.5 \times 10^9$	$6.6 \times 10^9$

TABLE VII

*Oxygen and Broth in Activation of Staphylococci*

Regular activation procedure carried out as described in Table I (column *b*, below). In columns (*c*) and (*d*) are listed respectively activation values obtained with  $\text{N}_2$  replacing  $\text{O}_2$ , with Locke's solution replacing broth. Column (*a*) lists the values obtained with an untreated 18 hour agar culture suspended in Locke's solution.

Preparation No.	[Phage] formed after mixing 1 ml. of phage ( $1 \times 10^9$ P.U./ml.) with 4 ml. bacterial suspensions prepared in the following ways: ([Phage] <sub>0</sub> in all cases $2 \times 10^8$ P.U./ml.)			
	( <i>a</i> ) Bacterial control Locke's suspension 18 hrs. agar growth	( <i>b</i> ) Growth in $\text{O}_2$ -broth cells resuspended in Locke's solution	( <i>c</i> ) Growth in $\text{N}_2$ -broth cells resuspended in Locke's solution	( <i>d</i> ) Growth in $\text{O}_2$ -Locke's solution cells resuspended in fresh Locke's solution
1	$2.3 \times 10^8$	$5 \times 10^9$	$5.9 \times 10^8$	$2.1 \times 10^8$
2	$1.8 \times 10^8$	$2.1 \times 10^9$	$3.2 \times 10^8$	$2.0 \times 10^8$
3	$1.9 \times 10^8$	$4.6 \times 10^9$	$2.5 \times 10^8$	$1.7 \times 10^8$

5. *Effect of pH during and after Activation of Bacteria.*—The activation process was carried out in broth of various hydrogen ion concen-

trations ranging from pH 5 to pH 9. The organisms were then tested for activation by adding bacteriophage at pH 7.4. No very significant differences were observed over the range studied although the mixtures prepared from organisms activated on the acid side of neutrality produced slightly higher phage titres (Table VIII).

The effect of hydrogen and hydroxyl ions on suspensions activated at pH 7.4 was tested by adjusting the pH of aliquots to various values and holding the suspensions for 1 hour at 5°C. An aliquot of each

TABLE VIII

*Effect of pH on Activation*

Activation with O<sub>2</sub> carried out by standard procedure (Table I) except pH of broth adjusted with acid or base to various pH's. After activation bacteria centrifuged down and resuspended in Locke's solution pH 7.4. 4 ml. of each bacterial suspension added to 1 ml. phage diluted with Locke's solution to contain  $1 \times 10^9$  P.U./ml. Mixture kept at 5°C. 5 min. and titrated.

$$[\text{Phage}]_0 = 2 \times 10^8 \text{ P.U./ml.}$$

pH of activation	Experiment 1		Experiment 2		Experiment 3	
	[Phage] <sub>final</sub>	$\Delta$ [phage] <sub>final [phage]-initial [phage]</sub>	[Phage] <sub>final</sub>	$\Delta$ [phage] <sub>final [phage]-initial [phage]</sub>	[Phage] <sub>final</sub>	$\Delta$ [phage] <sub>final [phage]-initial [phage]</sub>
5.08	$4.8 \times 10^9$	$4.6 \times 10^9$	$4.2 \times 10^9$	$4.0 \times 10^9$	$2.9 \times 10^9$	$2.7 \times 10^9$
5.55	$4.6 \times 10^9$	$4.4 \times 10^9$	$4.2 \times 10^9$	$4.0 \times 10^9$	$2.8 \times 10^9$	$2.6 \times 10^9$
5.91	$4.8 \times 10^9$	$4.6 \times 10^9$	$3.8 \times 10^9$	$3.6 \times 10^9$	$2.9 \times 10^9$	$2.7 \times 10^9$
6.65	$6.0 \times 10^9$	$5.8 \times 10^9$	$3.1 \times 10^9$	$2.9 \times 10^9$	$1.5 \times 10^9$	$1.3 \times 10^9$
7.02	$6.0 \times 10^9$	$5.8 \times 10^9$	$3.0 \times 10^9$	$2.8 \times 10^9$	$2.0 \times 10^9$	$1.8 \times 10^9$
7.44	$4.1 \times 10^9$	$3.9 \times 10^9$	$3.0 \times 10^9$	$2.8 \times 10^9$	$2.1 \times 10^9$	$1.9 \times 10^9$
8.15	$4.1 \times 10^9$	$3.9 \times 10^9$	$2.8 \times 10^9$	$2.6 \times 10^9$	$1.8 \times 10^9$	$1.6 \times 10^9$
8.40	$3.4 \times 10^9$	$3.2 \times 10^9$	$2.3 \times 10^9$	$2.1 \times 10^9$	$1.8 \times 10^9$	$1.6 \times 10^9$
9.00	$3.6 \times 10^9$	$3.4 \times 10^9$	$1.9 \times 10^9$	$1.7 \times 10^9$	$1.4 \times 10^9$	$1.2 \times 10^9$

suspension was then added to phage and the mixture titrated. As noted in Table IX the alkaline mixtures titrated somewhat lower than those exposed to moderate H<sup>+</sup> ion concentrations.

6. *Inhibition of Precursor-Phage Reaction by Antiserum.*—Rabbits were injected repeatedly with nonactivated live staphylococci and with activated staphylococci. The initial doses used were small and injections were given three times a week using constantly increasing numbers of organisms. The serum of these animals and that of normal rabbits was tested for antibodies active against the phage precursor.

All three sera produced no inactivation when mixed with phage and subsequently titrated. Likewise none of the sera had a demonstrable bactericidal effect on normal or activated bacteria. However, it was found that the antiserum against activated bacteria and that produced against normal living staphylococci when mixed with activated organisms prevented the increase in [phage] usually observed upon the addition of phage to the activated organisms. This inhibiting effect could be demonstrated up to a 1/20 dilution of both sera. Normal

TABLE IX

*Effect of Storage at Various pH's on Activated Cells*

Activation carried out by standard method (Table I). Cells centrifuged down and resuspended in broth of various pH's. Suspensions kept at 5° C. 1 hr. 4 ml. of each suspension added to 1 ml. of phage diluted in Locke's solution to contain  $1 \times 10^9$  P.U./ml. Mixture kept at 5° C. 5 min. and titrated. [Phage]<sub>0</sub> =  $2 \times 10^8$  P.U./ml.

pH of bacterial suspension for 1 hr. after activation at pH 7.4	Experiment 1		Experiment 2	
	[Phage] <sub>final</sub>	$\Delta$ [phage] <sub>final [phage]-initial [phage]</sub>	[Phage] <sub>final</sub>	$\Delta$ [phage] <sub>final [phage]-initial [phage]</sub>
5.08	$3.5 \times 10^9$	$3.3 \times 10^9$	$4.2 \times 10^9$	$4.0 \times 10^9$
5.35	$3.7 \times 10^9$	$3.5 \times 10^9$	$4.2 \times 10^9$	$4.0 \times 10^9$
5.91	$3.7 \times 10^9$	$3.5 \times 10^9$	$4.0 \times 10^9$	$4.0 \times 10^9$
6.65	$3.1 \times 10^9$	$2.9 \times 10^9$	$3.7 \times 10^9$	$3.5 \times 10^9$
7.02	$2.0 \times 10^9$	$1.8 \times 10^9$	$3.7 \times 10^9$	$3.5 \times 10^9$
7.44	$1.9 \times 10^9$	$1.7 \times 10^9$	$3.7 \times 10^9$	$3.5 \times 10^9$
8.15	$1.9 \times 10^9$	$1.7 \times 10^9$	$3.2 \times 10^9$	$3.0 \times 10^9$
8.40	$1.8 \times 10^9$	$1.6 \times 10^9$	$3.2 \times 10^9$	$3.0 \times 10^9$
9.00	$1.8 \times 10^9$	$1.6 \times 10^9$	$2.1 \times 10^9$	$1.9 \times 10^9$

rabbit serum did not prevent the reaction between activated cells and phage (Table X).

It is known that antibacterial sera readily form deposits on the surface of homologous organisms and it may well be that this surface film prevents the access of phage to the precursor-containing portions of the activated cells. The data cannot very well be interpreted for or against the existence of the precursor as a distinct antigenic component.

7. *Heat Inactivation of Phage Precursor.*—In experiments reported elsewhere (15) the rate of heat inactivation of phage precursor was

determined at several different temperatures. It was necessary to carefully control the time and temperature during heat inactivation of intracellular precursor so that no bacterial deaths would occur. If any appreciable number of cells died during the experiment they would adsorb phage irreversibly (2) when the latter was added to the suspension as a test for presence of precursor. The adsorbed phage would not participate in the titration reaction and the result would be an artificially reduced end titre giving false evidence for the inactivation of phage precursor.

TABLE X

*Inhibition of the Precursor-Phage Reaction by Antiserum*

Antisera prepared by injecting rabbits with live activated and nonactivated staphylococci. 1 ml. of serum dilution added to 1 ml. activated bacteria ( $1 \times 10^9$  cells/ml. in Locke's solution) pH 7.4. After 1 hr. at  $5^\circ$  C. each mixture was added to 1 ml. of phage diluted in Locke's solution to  $1 \times 10^9$  P.U./ml. This was kept 5 min. at  $5^\circ$  C. and was promptly titrated. Control experiments showed that the three sera had no bactericidal properties under the conditions of the experiment nor did they have any direct action on phage alone.

Serum dilutions used	Activated bacteria treated with serum. 2 ml. bacterial suspension added to 1 ml. phage ( $1 \times 10^9$ P.U./ml.) [Phage] <sub>0</sub> in all cases $3.3 \times 10^8$ P.U./ml.		
	Total [phage] formed from suspension treated with anti-activated bacteria serum	Total [phage] formed from suspension treated with anti-nonactivated bacteria serum	Total [phage] formed from suspension treated with normal rabbit serum
1/2	$2.3 \times 10^8$	$3.0 \times 10^8$	$2.9 \times 10^9$
1/10	$3.1 \times 10^8$	$3.8 \times 10^8$	$2.9 \times 10^9$
1/20	$3.1 \times 10^8$	$3.1 \times 10^8$	$3.8 \times 10^9$
1/40	$3.9 \times 10^9$	$3.8 \times 10^9$	$3.9 \times 10^9$
No serum	$3.9 \times 10^9$	$3.9 \times 10^9$	$3.9 \times 10^9$

The critical thermal increment for the heat inactivation reaction was found to be about 90,000. Since figures of this magnitude are uniquely characteristic of protein denaturation reactions in general it would seem likely either that phage precursor is a protein or that the synthetic system which produces precursor contains a protein as an essential component. However, this does not exclude another possibility, namely, that the high temperature coefficient applies to heat induced changes in some entirely separate protein constituent of the

cell which when denatured may alter permeability conditions with the result that phage no longer has access to the precursor.

8. *Quantitative Relationships.*—Various concentrations of activated staphylococci were made in Locke's solution. To 4 ml. amounts of each bacterial suspension 1 ml. aliquots of different phage concentrations were added. The phage was run into the bacterial suspension drop by drop with constant stirring. The time of mixing was 1 minute after which the mixture was stirred for another minute. After standing for 4 minutes at 5°C. the preparations were immediately diluted for titration.

With [phage]<sub>0</sub> constant as [bacteria]<sub>0</sub> increases the total [phage] rises to a maximal limiting value beyond which no further yield of phage is obtained. When the initial concentrations of phage are low the maximal values are reached with relatively small numbers of bacteria; with greater [phage]<sub>0</sub> more bacteria are needed for the production of the maximal yield of phage. As would be expected with large initial phage concentrations and very small numbers of bacteria there is no detectable increase in [phage] concentration.

A detailed account of the quantitative relationships obtaining between activated cells and phage will be published elsewhere.

#### DISCUSSION

The approaches to the problem of bacteriophagy have been beset by constant differences of opinion regarding the interpretation of experimental facts as well as by difficulties in establishing the facts themselves. Probably more divergent views have been developed on the nature of phage and the mechanism of its formation than for any other point in the phenomenon. According to d'Herelle phage is an autonomous living parasite which invades the body of the bacterium and once inside reproduces, synthesizing its substance from material available within the cell. Not all investigators have accepted d'Herelle's concept; many have agreed with Bordet (8) that phage probably is a by-product of cellular metabolism and this view was strengthened by experimental evidence linking phage production with bacterial growth (12). It is undoubtedly true that bacterial growth and phage production go hand in hand under ordinary conditions but it is equally true that the two reactions may be separated. Krueger

and Fong (7) have shown that the pH and temperature optima for cellular reproduction and phage formation differ; in the present paper the evidence for phage production by activated cells in the absence of bacterial growth is summarized. Northrop also has obtained experimental results which can be interpreted in no other way (16).

If phage formation is an intrinsic part of the cell's metabolic activities a possible mechanism would entail the elaboration of an inactive precursor within the cell and its subsequent transformation into active phage. An ideal proof of this mechanism would be the isolation of the precursor in cell-free solution and the experimental demonstration that the precursor-phage reaction could be carried out in the total absence of the mother cell. Krueger and Baldwin (9) found that the addition of phage to ultrafiltrates of growing staphylococcus cultures resulted in a 100 per cent increase in phage titre. However, the results were irregular and it was not possible to get promising amounts of the phage-forming material. Later Krueger and Mundell (10) demonstrated that staphylococci grown in an oxygenated medium and subsequently stored at 5°C. in Locke's solution would increase [phage] 500 per cent within 2 minutes after phage was added to the cells. It was shown first, that the reaction took place in the complete absence of bacterial growth and second, that the increase in titre did not depend upon any anomalous influence on the titration system.

The results with the activated staphylococci were interpreted in terms of the precursor theory but in the absence of decisive data no attempt was made to decide whether the precursor-phage reaction involved the hydrolytic cleavage of a complex protein pro-phage or whether it was concerned with the catalytic completion of a cellular synthesis. Whatever may be the case experimental data show that cells which have been brought to a resting state by storage in Locke's solution at 5°C. after having undergone a period of active metabolism contain some component which reacts rapidly with phage to form more phage. Live resting cells that have not undergone the preparative period of increased metabolic activity do not increase [phage] when brought in contact with phage solutions. The precursor theory is compatible with the following experimental facts:

1. The reaction between activated cells and phage is completed

within 2 minutes after mixing the reactants. This, of course, does not rule out the possibility that the mechanism proceeds as d'Herelle pictured it but it is hard to see how a living parasite could bore its way into the interior of the cell, synthesize its own substance, and reproduce within such a short time.

2. The serial dilution experiment together with the control experiments cited in this paper obviate the objection that the reaction reported may involve anomalous effects on the titration system. In the serial dilution experiment it has been found feasible to mix relatively high concentrations of manganese-treated bacteria with sufficient phage to cause lysis without growth. The manganous ion depresses the lytic threshold and permits the use of sufficient cells so that the phage formed by the reaction between the activated cells and the added phage furnishes a measurable increment in [phage]. The newly formed phage is set free by cellular lysis and the reaction may be carried on in series, diluting the phage each time and adding fresh aliquots of activated cells. In these experiments the time elapsing is not concerned with phage production which takes place within 2 minutes but rather with the lytic process.

3. Activated staphylococci can be deprived of their phage-augmenting capacity by heat treatment without causing cell death. After 20 minutes at 45°C. activated cells cease to enhance [phage] when added to phage; at 50°C. 2½ minutes are required to bring about the same result. During the application of heat for the periods noted there are no significant numbers of cell deaths. The rates of heat inactivation follow the curve of a monomolecular reaction and show a very high temperature coefficient. The critical thermal increment is 90,000 and is of the order of magnitude characteristic of protein denaturation reactions in general. It is probable, therefore, that the phage-augmenting fraction of the activated cells either is a protein or that it contains a protein. However, another interpretation is not excluded for it is conceivable that as the cells are heated some entirely unrelated protein is denatured and that its denaturation alters cellular permeability preventing the access of phage to the cell.

4. The quantitative relationships outlined above are compatible with the precursor theory providing two assumptions are made: (A) That each activated cell contains a certain amount of precursor and

(B) That the phage formed remains attached to the cells. Under the conditions of our experiments only traces of phage can be demonstrated in the suspending fluid.

Starting with a small amount of phage the addition of a moderate number of activated staphylococci produces a measurable increase in [phage]. As [bacteria]<sub>0</sub> is increased the total phage formed rises, finally attaining a maximal limiting value beyond which the addition of more cells ceases to have an effect. With higher values of [phage]<sub>0</sub> the same general result is forthcoming except that the limiting plateau is shifted to the region of higher initial bacterial concentrations. With very high [phage]<sub>0</sub> the smaller concentrations of activated cells produce no detectable increase in [phage] because the phage formed is negligible compared to the phage added.

The reaction occurring when phage is added to activated bacteria cells represents only one phase of the complex phenomenon of bacteriophagy. It shows that a bacterial cell prepared by a period of active metabolism is capable of reacting very rapidly with phage to form more phage; it does not tell how the newly formed phage is released to react with more bacteria. Strong evidence for d'Herelle's concept that cellular lysis is an essential part of phage formation has been advanced recently by Ellis and Delbrück (17). Working with a strain of *B. coli* and a *coli* phage they found that the curve of phage increase was not strictly logarithmic with time but consisted of a series of plateaus connected by rather sharp slopes. According to their interpretation phage is produced within the cell and is set free by the lytic destruction of the organism; the plateaus of the phage production curve cover the phase of adsorption and phage production within the cell, the steep portions coincide with the setting-free of intracellular phage by lysis. For determining [phage] in their experiments they used the plaque count method, a procedure which gives a measure of the number of infected centers but does not estimate the total phage (*i.e.*, a single phage-containing bacterium will produce one plaque whether it contains 1 or 100 activity units). The curve of phage increase is therefore the resultant of three related but not identical reactions, namely, the adsorption of phage, the intracellular production of phage, and the lytic destruction of the infected cell. It expresses the rate of infection of a bacterial popula-



tion, which rate is dependent upon the speed of phage adsorption, the speed of phage formation, and the time required for the infected cell to burst. Our present data indicate that the first two of these reactions take place very quickly under the conditions outlined. Although it is not possible to apply these findings directly to the process of bacteriophagy taking place under normal conditions, they are not incompatible with the results of Ellis and Delbrück. They supply no evidence for the mechanism of phage transference from an infected cell to an uninfected one.

#### EXPERIMENTAL METHODS

1. Both the staphylococcus and anti-staphylococcus bacteriophage used are the ones described in previous papers (1). In preparing the staphylococcus suspensions for daily use the organisms were grown on nutrient agar made with infusion broth, 1 per cent neopeptone, and containing 2.5 per cent agar. The incubation period was 18 hours at 37° C. after which the growth was harvested in Locke's solution and was washed once in Locke's solution before using. The cell concentration was determined by the centrifuged sediment method (18). The broth used was standard beef infusion containing 1 per cent Difco neopeptone, 0.5 per cent sodium chloride, and was adjusted to pH 7.4.

2. Phage titres were determined by the activity method of Krueger (2). The activity unit is the smallest amount of phage which will cause lysis when added to a certain number of susceptible cells under standard conditions. Our standard phage contains  $1 \times 10^{10}$  activity units/ml. In practice three successive tenfold dilutions of each unknown were titrated.

3. For the preparation of activated cell suspension the washed staphylococci were suspended in broth in a concentration of  $5 \times 10^9$  bacteria/ml. The broth suspension was placed in a glass container arranged so that oxygen could be bubbled through the broth while the mixture was being shaken in a water bath adjusted to 36° C. After 1 hour of shaking the cell suspension was diluted with an equal volume of broth and the oxygen treatment was continued for another hour at 36° C. The cells were then centrifuged down and were resuspended in Locke's solution at pH 7.4. The suspension was kept at 5° C. for 2 hours before using.

4. In testing the serum prepared against nonactivated staphylococci and activated staphylococci 1 ml. of each serum dilution was mixed with 1 ml. of a suspension of activated organisms containing  $1 \times 10^9$  bacteria/ml. in Locke's solution at pH 7.4. The mixtures were kept at 5° C. for 1 hour after which 1 ml. of phage diluted in Locke's solution to  $1 \times 10^9$  P.U./ml. was added. The mixture was kept an additional 5 minutes at 5° C. and was then titrated. Controls included the same procedure carried out with normal rabbit serum and

tests for antiphage and bactericidal activity. None of the sera showed any significant capacity to inactivate phage or to kill staphylococci.

#### SUMMARY AND CONCLUSIONS

1. Staphylococci activated by rapid growth in the presence of excess  $O_2$  and subsequently brought to a resting state by storage in Locke's solution at  $5^\circ C$ . produce a significant rise in [phage] when added to phage-containing solutions.

2. For satisfactory activation the staphylococci require a period of active growth in the presence of oxygen. Activation proceeds best on the acid side of neutrality although variation in pH from 5 to 9 has relatively little effect. Activated cells retain their phage-augmenting property for from 4 to 24 hours, and this property may be destroyed by heating the cells at temperatures which do not kill them. The critical thermal increment for heat inactivation is 90,000 suggesting that the reaction involves protein denaturation.

3. The reaction between activated cells and phage has the following characteristics:

A. It is complete in 1 to 2 minutes after mixing the reactants.

B. The increase in phage does not depend upon bacterial growth nor does it involve any untoward effect on the titration system.

C. Serum prepared by injecting rabbits with normal live staphylococci or with activated staphylococci when mixed with activated cells before the addition of phage will prevent the customary increase in [phage].

4. The phage-producing reaction which follows the addition of activated cells to phage can be interpreted in terms of the precursor theory. It is likely that the precursor either is a protein or contains a protein as an essential component.

5. There is no way of deciding at present whether the reaction between phage and precursor represents the hydrolytic cleavage of a protein or whether it is the final step in a synthesis catalyzed by phage.

We wish to express our thanks to Robert Brown for helpful technical assistance.

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