BACTERIOPHAGE FORMATION WITHOUT BACTERIAL GROWTH

I. Formation of Staphylococcus Phage in the Presence of Bacteria Inhibited by Penicillin

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The bacteria-bacteriophage system lends itself to a study of virus-host relationships better than other systems. The experimental conditions may be carefully controlled and experiments performed rapidly. In addition, the results represent a statistically significant number of individual host-virus systems.

The first step in such an analysis would be the separation of phage production from the growth of the host cells. Krueger and Fong (1), Gratia (2), Northrop (3), and Ellis and Spizizen (4) have reported small increases in phage without bacterial growth. The possibility of the multiplication of a small number of cells, however, was not ruled out. Since a small increase in cells can give rise to fairly large increases in phage, it is imperative to measure the growth of the bacteria at frequent intervals by an accurate method.

Using igepol, a detergent, various pH, lysozyme, and ultraviolet irradiation, Northrop found that as soon as the growth of *B. megatherium* or *Staphylococcus muscae* was inhibited, the growth of the corresponding phage was prevented (5).

Evidence will be presented in this paper to show that bacteriophage can increase in the presence of bacteria whose growth has been inhibited by penicillin. In the second paper of this series experiments will be reported which indicate that penicillin prevents the utilization of a certain substance by bacteria which is essential for phage production (6). The third paper shows that adenosinetriphosphate is important in the formation of phage (7).

RESULTS

The addition of more than 10 γ of penicillin per ml. completely prevents the multiplication of *Staphylococcus muscae* (Table I). The addition of penicillin even in very high concentrations does not inhibit phage production (Table II). Fig. 1 gives the results of an experiment in which the cell concentration and phage concentration were determined at intervals in a culture containing 20 γ of penicillin per ml. The cell count was determined by a photoelectric colorimeter. The method is accurate to $\pm$ 2 per cent (Table IV). The curve in

Fig. 1 shows that there were no significant changes in the cell concentration and that a change in concentration of ± 3 per cent could have been detected. The plaque count on the other hand rose from 10^4 to 3×10^8. This experiment was repeated many times with the same result.

Effect of Initial Cell Concentration on Phage Production.—Under normal conditions more phage is produced by the inoculation of a system containing less than the maximum number of cells capable of growing in the media, than by the

TABLE I

Effect of Penicillin Concentration on Bacterial Growth

Each tube contained 5.0 ml. of broth and 0.5 ml. of Locke's solution with various penicillin concentrations. Growth measured at 4 hours. Two readings taken for each determination.

Tube No.	Penicillin concentration	Initial cell count	Final cell count
	γ/ml.		
1	2	1.0–1.1×10^8	2.1–2.1×10^8
2	5	0.9×10^7–1.1×10^8	1.5–1.6×10^8
3	10	1.0–1.0×10^8	1.0–1.1×10^8
4	20	1.0–1.1×10^8	1.0–1.0×10^8

TABLE II

Phage Formation at Various Penicillin Concentrations

Each tube contained 5.6 ml. of broth, 0.7 ml. of broth containing 2.1×10^5 phage plaque counts per ml., and 0.7 ml. of broth containing various penicillin concentrations. Initial bacterial count was 3.0×10^8 cells per ml. Samples taken at 8 hours when all tubes were completely lysed.

Sample	Penicillin concentration	Initial phage plaque counts per ml.	Final phage plaque counts per ml.
	γ per ml.		
1	20	2.1×10^5	9.4×10^8
2	100	2.1×10^5	1×10^9
3	500	2.1×10^5	8.1×10^7

inoculation of a culture which already contains the maximum number of cells (8). In the presence of penicillin, however, the opposite result is obtained. More phage is produced in the concentrated suspension than in the dilute cell suspension (Table III). The addition of penicillin therefore increases phage production in the concentrated suspension but decreases it in the dilute suspension. The increase in the concentrated suspension is due to the fact that the penicillin prevents the cells from using up a compound which is essential for phage production. This observation will be discussed in the following paper.

The decrease in phage production in dilute suspension is probably due to the

fact that the normal culture grows rapidly so that many more bacterial cells are present at the time of lysis than in the penicillin culture.

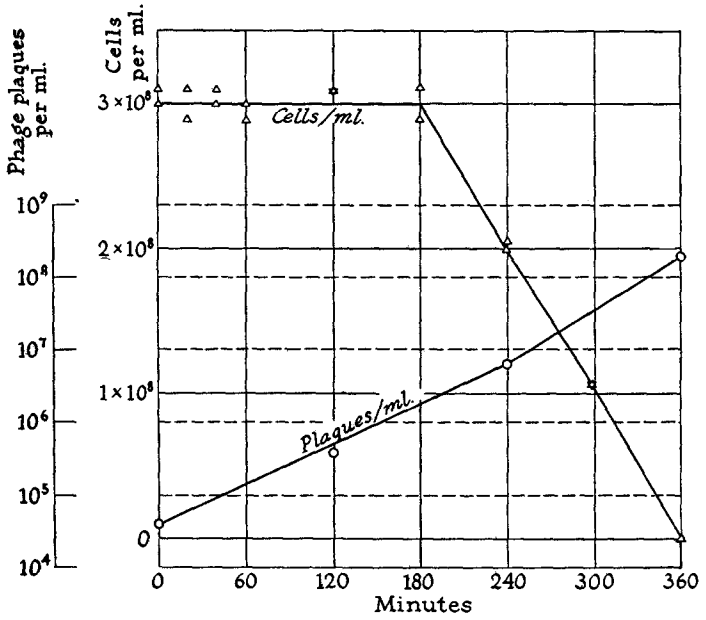

FIG. 1. Lysis of bacteria with formation of bacteriophage in the presence of penicillin. Sample contained 5.6 ml. of broth, 0.7 ml. of broth containing 140γ of penicillin, and 0.7 ml. of phage solution. Two readings taken for each cell determination.

TABLE III

Effect of Initial Cell Concentration on Phage Produced from Normal and Penicillin-Treated Bacteria

All tubes contained 5.6 ml. of broth, 0.7 ml. of phage solution, and 0.7 ml. of 0.85 per cent NaCl with and without penicillin. Samples taken at end of 6 hours.

Tube No.	Penicillin concentration	Initial cell count	Maximum cell count	Initial phage plaque count per ml.	Final phage plaque count per ml.
	γ/ml.				
1	—	8×10^8	1×10^9	4×10^4	3×10^6
2	—	5×10^7	1×10^8	4×10^4	3×10^8
3	20	8×10^8	8×10^8	4×10^4	4.8×10^9
4	20	5×10^7	5×10^7	4×10^4	2×10^8

From Table III it can also be seen that normal bacteria produce, under our method of assay, 3 plaques per bacterial cell, while the penicillin-treated organisms produce 1 plaque per cell. The difference between these two figures

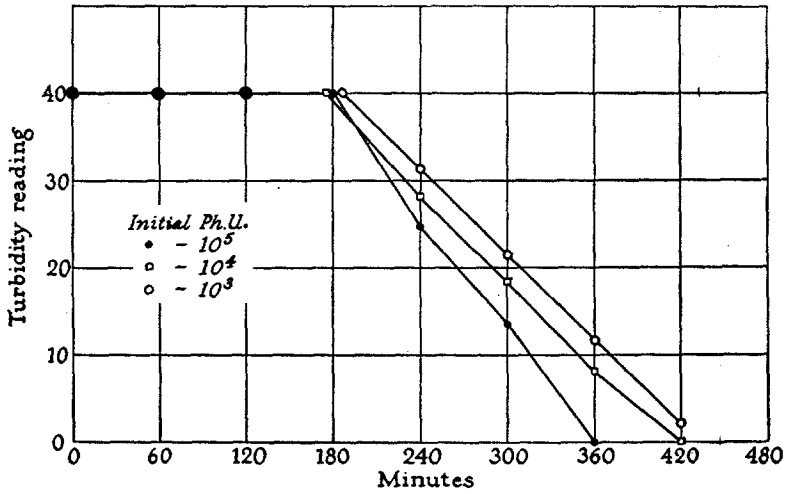

FIG. 2. Bacterial lysis at various initial phage concentrations. Each tube contained 5.6 ml. of broth, 0.7 ml. of broth containing 140γ of penicillin, and 0.7 ml. of phage solution.

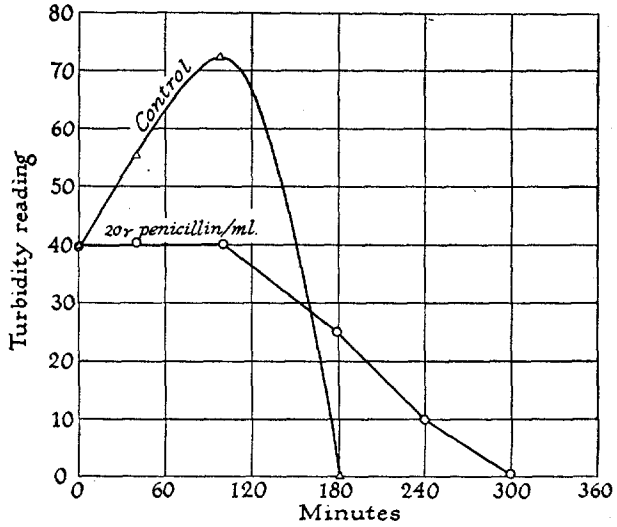

FIG. 3. Comparison of lysis of normal bacteria and penicillin-treated bacteria. Each tube contained 5.6 ml. of broth + 0.7 ml. of broth with or without penicillin and 0.7 ml. of broth containing 10^5 phage units per ml. Both samples showed approximately 10^9 phage units per ml. at complete lysis.

could conceivably be due to the fact that the penicillin is continually killing the cells and that there are really fewer viable cells in the penicillin culture than can

be determined by the turbidity method, which only measures total bacteria and does not distinguish between living and dead cells. This effect of penicillin on bacteria is well known.

It could be argued that in the presence of penicillin there is a continual slow multiplication of bacteria which is too low a concentration to be detected. This does not seem likely, since there does not appear to be any inhibition of phage formation in penicillin concentrations ranging from 20 to 100 γ (Table II). If the phage formation was due to undetected growth, one would expect that there would be a difference in phage formation under such conditions. The addition of 500 γ of penicillin results in less phage formation than adding 100 γ of penicillin. This is probably due to the fact that such high concentrations of penicillin kill the bacteria.

It should also be noted that not only is cellular multiplication inhibited, but that the cells do not grow at all, for any increase in the size of the bacteria could be detected with the colorimeter.

Effect of Phage Concentration on Lysis Time.—The rate of lysis of the penicillin-treated bacteria depends on the concentration of phage (Fig. 2). A 100-fold dilution of phage results in approximately a 60 minute increase in lysis time. This result agrees quite well with the observation of Krueger and Northrop (9) for normal bacteria.

The rate of lysis is slower in the presence of penicillin than in the normal culture (Fig. 3). The reason for this phenomenon is not known at the present time.

DISCUSSION

Penicillin prevents the multiplication of the *Staphylococcus muscae* but does not prevent the formation of the staphylococcus phage. This finding is important as it is the first step in an analysis of phage production. In systems where phage formation depends on bacterial growth a biochemical analysis is practically impossible, since all observations would be greatly complicated by variations in the growth of the host.

Cohen and Anderson (10) have reported that *E. coli* stop multiplying as soon as they are infected with T_2 phage. This observation is in agreement with the results presented in this paper that phage can be formed in the presence of non-multiplying bacteria. It should be pointed out, however, that inhibition of multiplication by the T_2 phage cannot be a universal bacteria-bacteriophage effect, for strains of *megatherium* are known which produce phage but continue to multiply (11).

SUMMARY

Bacteriophage will increase 100,000 times in *Staphylococcus muscae* cultures whose multiplication has been completely inhibited by penicillin.

I am indebted to Dr. John H. Northrop for advice during these experiments. I also wish to thank Dr. Richard E. Shope for a culture of the bacteria and virus used in this work.

Experimental Methods

Preparation of Standard Suspensions of Staphylococcus muscae.—18 to 24 hour bacterial cultures grown on veal infusion agar slants were washed off with sterile broth and incubated 2 to 3 hours at 37° unless otherwise stated.

Bacterial Concentration.—Bacterial concentrations were determined in a Klett-Summerson photoelectric colorimeter using 1.5 × 12 cm. sterile test tubes containing

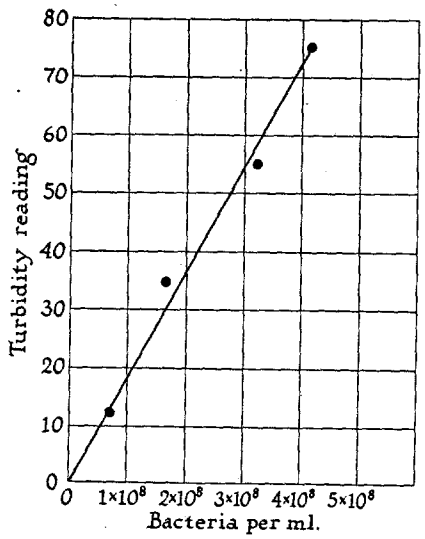

FIG. 4. Standard curve for determining bacterial concentrations.

5.0 cc. of the solution. The colorimeter was set for the reading by using 5.0 cc. of nutrient broth as the standard and reading against filter No. 54. Unknown values were read off a standard curve (Fig. 4). The curve was plotted from turbidity readings of standard suspensions in which the cell concentration was determined by microscopic count and checked by colony counts. Table IV shows five turbidity readings on the same sample. It can be seen that the accuracy is about ± 2 per cent for determining cell growth by this method.

Phage Assay.—Phage was determined by plaque counts according to Gratia (11). All dilutions were 1/10 and were made in broth. The final dilution prior to plating consisted of 3.5 ml. broth + 0.5 ml. phage sample + 1.0 cc. of 2.5 per cent agar. This mixture contained about 3×10^8 cells per ml. One ml. of the total mixture was pipetted into nutrient agar dishes and incubated about 18 hours at room temperature before counting the plaques.

Phage was also determined by the dilution method. In this method the pipette was rinsed in every dilution five times. All dilutions were 1/10 and made in broth.

The concentration of phage as determined by this method is expressed in phage units (Ph.U.). One phage unit, as determined by this method, is the smallest amount of phage solution that will cause lysis of 5 ml. of standard bacterial suspension containing 5×10^7 cells per ml. at 37° in 24 hours. The phage unit is the reciprocal of the maximum dilution which will cause lysis; *i.e.*, if 1×10^{-4}/ml. causes lysis [Ph.U.]/ml. = 10^4.

Preparation of Veal Infusion Broth.—To 500 gm. of ground veal is added 1000 ml. of H_2O and infused overnight in the ice box. The meat was strained next morning through fine cheese cloth and the filtrate made up to 1000 ml. with H_2O and heated in the steamer. Ten gm. of peptone and 5 gm. of NaCl was then added to every

TABLE IV

Accuracy of Turbidity Reading

Tube contained 5.0 ml. broth. Five separate readings taken, each time removing the tube from the colorimeter.

Reading	Value	Cells per ml. from Fig. 4
1	52	3.7×10^8
2	50	3.5×10^8
3	52	3.7×10^8
4	51	3.6×10^8
5	51.5	3.7×10^8
		3.64 ± 0.07*

* Average error of a single observation.

1000 ml. of broth. The pH was adjusted to 7.6 with N NaOH and the solution filtered through fine filter paper and autoclaved.

Penicillin.—Usually 20α of penicillin was added per ml. of reaction mixture to prevent multiplication. Several different samples of penicillin were used although most of the work was carried out with crystalline penicillin G sodium Squibb. The penicillin was incubated 90 minutes with the bacterial suspension before the addition of phage in order to ensure complete inhibition of bacterial growth. Ten α of penicillin per ml. gave complete inhibition of bacterial growth (Table I).

Phage.—All the experiments during this work were carried out with the staphylococcus phage described by Shope (12).

Reaction Mixtures.—All reaction mixtures were carried out in 2.0 × 15 cm. sterile test tubes, and were shaken rapidly without causing foam to appear. All experiments were carried out at 37°C.

Addendum.—After this paper was accepted for publication, Dr. A. P. Krueger informed us that his group at the University of California had independently observed that *Staphylococcus aureus* phage would multiply in the presence of bacteria under conditions where penicillin prevented any demonstrable multiplication of bacteria.

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