PHAGE FORMATION IN STAPHYLOCOCCUS MUSCAE CULTURES

II. The Release of the Virus from the Bacterial Cell

By WINSTON H. PRICE

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

(Received for publication, May 28, 1948)

The mechanism of the release of bacteriophage from its host has been one of the most controversial subjects in the study of bacterial viruses.

D'Herelle's experiments led him to conclude that phage was released on the lysis of the host, these "bursts" occurring at definite intervals. The picture given by d'Herelle (1) indicated a lag period of 30 minutes after the addition of the virus to the bacteria, after which a 6- to 60-fold increase in phage occurred in a quarter of an hour. The titer then remained approximately constant for another 30 minutes until another sudden increase occurred. Doerr and Grüninger (2), using a *coli* phage, found a tenfold increase each quarter of an hour until the limiting concentration of phage was approached.

Other workers, however, reported that the virus could reach very high titers without lysis of susceptible bacteria (3–9). The well known lysogenic strain of *megatherium* which continues to form phage and to multiply is further evidence that the virus may be released from the bacterial cell without lysis of the host (10).

In a very extensive study Krueger and Northrop (11), working with a phage for *S. aureus*, reported that phage was released continuously from the bacteria and did not cause lysis of the cell until a critical ratio of phage to bacteria was reached. This conclusion was confirmed by Clifton and Marrow (12) and by Lin (13).

Ellis and Delbrück (14), working with a *coli* phage and using plaque counts to determine phage, found that the bacterial virus was liberated in steps and considered that the steps corresponded to the lysis of the bacteria with the subsequent release of phage, thus confirming d'Herelle's original observations.

In discussing the earlier results of Krueger and Northrop (11), Ellis and Delbrück (14) stated that the method of assay used by Krueger and Northrop might have obscured the step-formation of phage. They also pointed out that in the early formation of phage, the lysis of the cells would be obscured since the lysed cells would represent only a small fraction of the total bacterial cells present. The lysed cells would not be detected until the cells undergoing lysis represented a large fraction of the total bacteria. Krueger and coworkers (15) have reexamined the staphylococcus system previously studied by Krueger and

Northrop (11) in which the virus seemed to be formed continuously. Krueger and coworkers found that this phage system also released the bacterial virus in steps.

It had been previously reported from this laboratory (16) that in Fildes' synthetic medium containing a small amount of hydrolyzed casein, *Staphylococcus muscae* phage may be released without cellular lysis. If the concentration of the casein is increased, the cells are lysed. Recently Maurer and Woolley (17) working with the *E. coli* system have demonstrated that the addition of apple pectin to a synthetic medium results in the release of phage without cellular lysis. It thus appears that by varying the composition of the medium, the bacterial virus may be released without lysis of the host.

A more detailed study of the release of the bacterial virus will be reported in this paper. It will be shown that (*a*) in veal infusion medium the increase in plaque count occurs in a step-wise manner and is correlated with the lysis of the cell; (*b*) in Fildes' synthetic medium (18) containing hydrolyzed casein, the phage is also released in steps although no visible lysis occurs. This last observation was confirmed by turbidity measurements, hanging drop slides of the infected bacteria, and finally by staining the bacteria and observing them under the microscope.

RESULTS

The Release of Phage in Veal-Infusion Medium.—Fig. 1 shows the release of phage in veal infusion medium from multiple infected cells. It can be seen that the phage titer remains constant for 30 minutes and then begins to increase at which time the cells begin to lyse; practically all the phage is released after the cells begin to lyse. It will be noted that the cells do not begin to lyse until 40 minutes. This is in fairly good agreement with the time of the minimum latent period. The relationship between the time of lysis in a multiple infected cell and the beginning of increase in phage titer in the *S. muscae* system is therefore similar under the above conditions to the *E. coli* B system previously studied by Delbrück (19). One-step growth curves carried out in veal infusion medium with single infected cells give average phage yields per cell in fairly good agreement with the average yield of virus particles from multiple infected cells. The minimum latent period is also the same as multiple infected cells; that is, 30 minutes. These observations are in agreement with the experiments reported by Delbrück for the *coli* system (19). It should also be observed that under these conditions cells infected with phage do not multiply. This result is in agreement with the studies of Cohen and Anderson (20) on the *coli* system.

The Release of Phage in Synthetic Medium

One-Step Growth Curve.—From Fig. 2 it can be seen that there is a minimum latent period of 30 minutes in synthetic medium. This is the same as in the

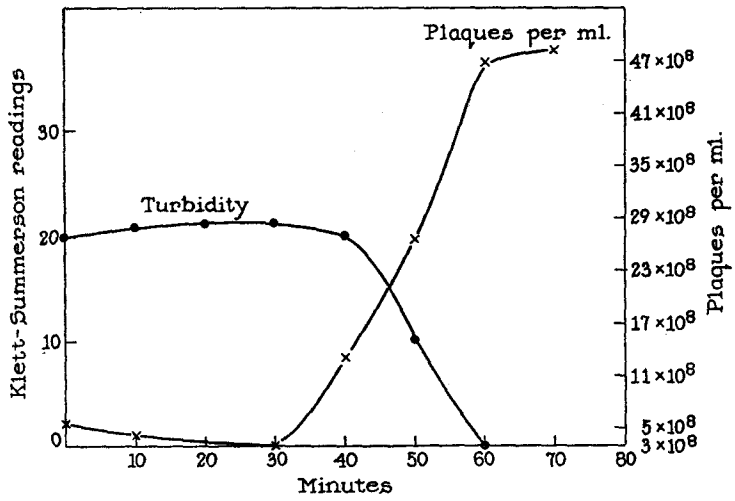

FIG. 1. Multiple infection in veal infusion medium. The bacteria were prepared as described under Methods. The bacteria were then added to 10.0 ml. of veal infusion medium and incubated 1 hour. After 1 hour the count was 1.1×10^8 cells per ml. Phage was added to give a final concentration of 5.2×10^8 particles per ml. The measurements were started. This experiment was run simultaneously with the experiment shown in Fig. 3 using the same bacterial and phage preparations.

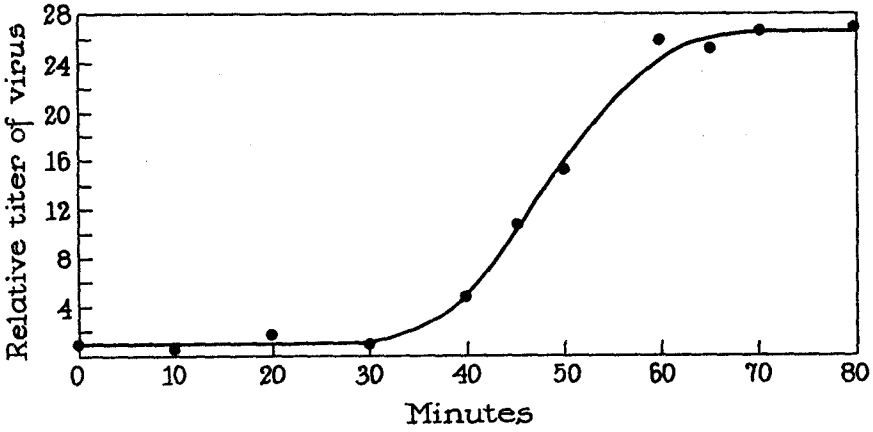

FIG. 2. One-step growth curve in synthetic medium. The cells were prepared as described under Methods. The synthetic medium, containing 10 mg. of hydrolyzed casein, was inoculated with 1×10^7 cells per ml. and allowed to incubate 3 hours. At this time the cell count was 5.1×10^7 cells per ml. 0.1 ml. of stock phage solution was added to give a final titer of 3.1×10^7 particles per ml. The tube was shaken for 18 minutes, and then diluted 1:2000 in synthetic medium containing 10 mg. of hydrolyzed casein. Centrifugation of the sample showed that 45 per cent of the virus was adsorbed. Samples were then taken from the diluted tube for phage assay.

veal infusion medium, although the cells grow more slowly in the synthetic medium. This observation is in agreement with the results obtained in the *coli* system (19).

Phage Release from Multiple Infected Cells.—Multiple infected cells in synthetic medium appear to release their phage in a step-wise manner before lysis of the cell. In the experiment shown in Fig. 3, there is a 20 minute interval between the lysis of the cell and the peak in phage formation. Plating out on agar showed that all the cells were infected after 30 minutes.

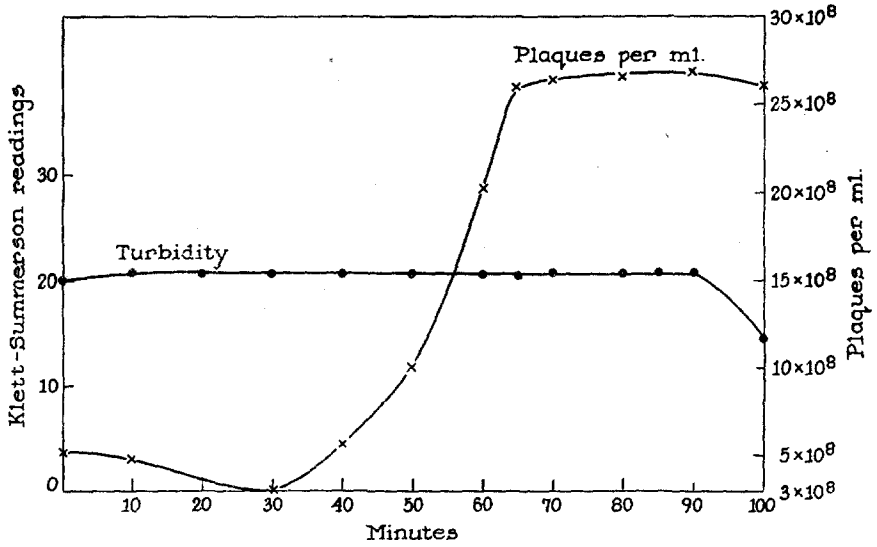

FIG. 3. Multiple infection in synthetic medium. The bacteria were prepared as described under Methods. The bacteria were then added to the synthetic medium. After incubation for 1 hour the cell count was 1.4×10^8 cells per ml. Phage was added to give a final concentration of 5.2×10^8 particles per ml. The measurements were then started. A control tube containing the same initial bacteria but no virus contained 3.2×10^8 cells per ml. at the end of 2 hours.

It may be argued that part of the cell is ruptured, but that the part remaining still gives the full turbidity reading. In order to check this point bacteria were removed from the tube and methylene blue smears made of the infected bacteria at various times before and after the phage had been released in a step. Many slides containing many fields of infected bacteria were examined. Such slides, under oil immersion, showed that the bacteria had a normal morphology and no evidence of cellular rupture was seen even though all the virus was released. In order to account for the phage formed, all the cells would have to be ruptured, as pointed out below, and if this had occurred, such smears should have disclosed the ruptured cells. Finally, hanging drop slides showed the lysis of

the cells to occur at about the same time as the drop in turbidity and not at the time the step was completed.

In other experiments the difference in time between reaching the "step" and onset of lysis varied from 10 to 30 minutes. The time at which the "step" was reached varied from 60 to 82 minutes in different experiments. The minimum latent period also varied in some experiments appearing at 45 minutes instead of 30 minutes.

In several experiments the result shown in Fig. 4 was obtained. The step was finished at 72 minutes and the virus count then remained constant until

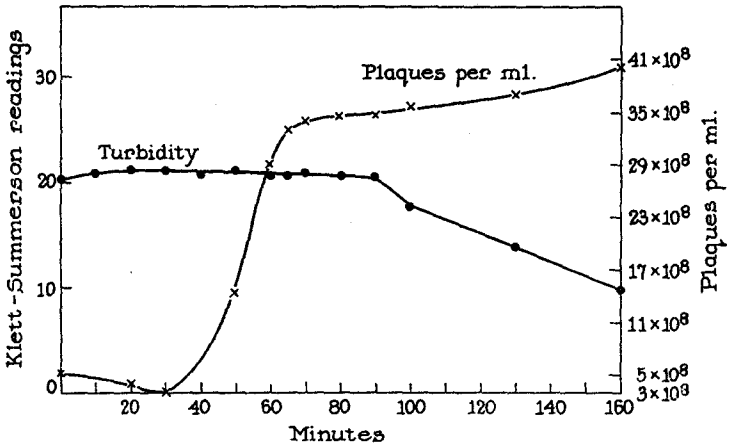

FIG. 4. Multiple infection in synthetic medium. The bacteria were prepared as described under Methods. 7.8×10^7 bacterial cells per ml. were added to the synthetic medium and allowed to incubate for 1 hour at which time the count was 1.0×10^8 cells per ml. Virus particles were then added to give a final concentration of 5.0×10^8 particles per ml. The measurements were then started.

90 minutes at which time lysis started. Phage counts during this lysis period showed a very slow rise in titer with lysis of the cell. This slow increase in phage titer is what one would expect since the plaque count method only determines infective centers. Some of the phage released in the step formation, which occurred in 72 minutes, would be adsorbed on bacteria. These bacteria would only give rise to one plaque even though they may contain two or more phages. As the cells were lysed from "without," however, the bacteria would release the adsorbed particles and then increase the titer.

These experiments seem to indicate two facts: (1) that phage may be released without any observable lysis of the cell and (2) that the step formation of phage need not necessarily be due to the lysis of the cell, since under the above condition of non-lysis, there is still a step formation of phage. It does not appear that this system is a lysis-inhibitor system such as that recently re-

ported by Doermann (21) for the following reasons: (*a*) phage release is correlated with cell lysis in the veal infusion medium, (*b*) there is no observable lysis corresponding to the release of phage as there is in Doermann's *coli* system, and (*c*) there are none of the other characteristics of Doermann's system. It does not appear likely that the phage is formed by a few lysed cells since a one-step growth curve in single infected cells carried out at the same time as the experiment in Fig. 3 showed that the average yield of virus particles liberated per cell was 22. To reach a titer of 2.5×10^9 virus particles per ml. as observed in the experiment shown in Fig. 3, there would have to be lysis of 1×10^8 bacteria per ml. if the phage produced is due to lysing cells. As the method of bacterial determination is accurate to 3 per cent (22), this could not have occurred, since there were only 1.0×10^8 bacterial cells to start with and no cellular lysis was observed until after the maximum virus titer was reached. It does not appear that lysis is obscured by multiplying cells since in this case the cells, when infected by phage, do not multiply and one is able to begin and end with the same number of cells and still reach the maximum phage titer. It should also be noted that the phage is released in this system and not contained within the cell. If the phage was not released, the plaque count method would not show any increase in titer, since this method only gives the titer of infective centers, as pointed out earlier.

DISCUSSION

The experiments reported in this paper indicate that the medium in which the experiment is carried out determines whether phage is released by lysis of the host cell or without visible lysis. In veal infusion medium the release of phage in *S. muscae* systems is correlated with the lysis of the host. Under these conditions, the release of the virus is very similar to that reported for the *E. coli* system (19). In Fildes' synthetic medium containing hydrolyzed casein, the results indicate that phage is released in a step-wise manner before visible lysis of the cell occurs. Under these latter conditions the disintegration of the cell may be due to "lysis from without." A comparable situation may exist in the *E. coli* system, since the release of phage is very similar in the two systems in a rich medium. Due to the greater capacity of *E. coli* to synthesize metabolites, it may not be possible to demonstrate phage release without lysis by the method used in this paper since the substance(s) involved in the lysis phenomenon may be synthesized by this host. Recently, however, Maurer and Woolley (17) found that the addition of apple pectin to a synthetic medium resulted in the release of *E. coli* phage without cellular lysis. This observation, together with the fact that phage may be formed in *E. coli* cells rendered non-viable by mustard gas (24), makes it possible to determine whether the virus is released in a step-wise manner in the *E. coli* system without cellular lysis.

The situation reported in this paper, in which the response of the host to a virus infection depends on the medium, may have its counterpart in the effect of various nutrient deficiencies on animal virus infections. Thus Elvehjem and coworkers (25) have shown that thiamine-deficient mice injected with Western equine encephalomyelitis do not show the typical pathological signs although the virus multiplied very well and killed the host. Other similar cases have also been reported (26).

It is interesting to speculate why the virus stops multiplying in the synthetic system before the cells begin to lyse. One interpretation could be that there is a substance in the cell essential for virus multiplication, which the virus-infected cell cannot synthesize, and virus formation stops when this compound is depleted. This hypothesis of limiting substrate could account for the great variation in virus yields per cell which has been observed by Delbrück (27) in the *coli* system, since cells could have varying amounts of the compound. In the *E. coli* system, furthermore, it seems probable that the yield per cell is also influenced by lysis, since Doermann (21) has shown that in lysis inhibition, the phage yield per cell is increased. If the virus synthesis does stop because of a depletion of some substance in the cell, attempting to prolong the increase in phage by adding various bacterial fractions at the time the "step" normally stops in the synthetic medium would be of interest. Another way of approaching the problem would be an analysis of the cell before and after the phage is formed. If the cells are centrifuged out from such systems, the phage inactivated with acid, and new phage and cells added to the medium, there is a good increase in phage. This result indicates that the virus does not stop multiplying because of some deficiency in the medium.

Another point of interest raised by these experiments is the explanation of the minimum latent period in phage formation. If one assumed lysis of the cell, the explanation of the minimum latent period was easy to understand. However, when the virus is released without lysis, the mechanism is difficult to visualize in view of Doermann's results that the phage has increased in the cell before it begins to be released. Why the minimum latent period has such a great time constancy under conditions of non-lysis must await future investigation.

The writer was assisted in this work by Mr. Mortimer Litovchick.

SUMMARY

1. The release of *S. muscae* phage in veal infusion medium is correlated with lysis of the host.

2. The release of the bacterial virus in Fildes' synthetic medium occurs in a step-wise manner before observable lysis of the cells occurs. This result has been confirmed by both turbidimetric readings and direct microscopic examination of the infected cells.

Methods

Bacteria were grown on agar slants for 20 hours as described previously (22). The bacterial cells were washed off with water, centrifuged, washed once with water, and then suspended in water before being added to the various systems. Phage and bacteria were determined as described previously (22). The phage used in this work was prepared in the synthetic medium described below to which was added 20 mg. of hydrolyzed casein. After complete lysis of the cells, the bacterial debris was centrifuged out at 5000 R.P.M. for 5 minutes. Such centrifuged lysates had a titer of approximately 4 to 9 $\times$ 10^9 plaques per ml. All phage dilutions were made in sterile distilled water.

Medium.—The veal infusion medium was prepared as described previously (22). The synthetic medium used was that of Fildes (18) which consisted of 6.0 ml. of amino acid mixture, 0.25 ml. of M/2 glucose, 0.25 ml. of M/500 ferrous ammonium sulfate, 0.10 ml. (100 γ) of nicotinic acid, 0.10 ml. (100 γ) of thiamine, 0.10 ml. of M/60 magnesium sulfate, 0.20 ml. of M/100 cystine, 0.20 ml. of M/100 methionine, 0.10 ml. of M/5 sodium nitrate, and 0.10 ml. of M/1000 tryptophane. To this medium was added 0.02 ml. of vitamin-free casein hydrolysate (equivalent to 2.0 mg. of casein) from General Biochemicals, Inc. To this solution was added the bacteria and phage. After all these additions, the solution was made up to 10.0 ml. with water.

Incubation of Reaction Mixtures.—All tubes were shaken at 36°C. as described previously (22).

One-Step Growth Curves.—The procedure of Delbrück and Luria (23) was followed.

REFERENCES

1. D'Herelle, F., The Bacteriophage and Its Behavior, translated by G. H. Smith, Baltimore, The Williams and Wilkins Co., 1926.
2. Doerr, R., and Grüninger, W., *Z. Hyg. u. Infectionskrankh.*, 1922, **97**, 209.
3. Doerr, R., and Berger, W., *Z. Hyg. u. Infectionskrankh.*, 1923, **97**, 422.
4. Otto, R., and Munter, H., *Z. Hyg. u. Infectionskrankh.*, 1923, **100**, 402.
5. Brutsaert, A., *Arch. méd. Belges*, 1924, **77**, 839.
6. da Costa Cruz, J., *Compt. rend. Soc. biol.*, 1924, **91**, 840.
7. Matsumoto, T., *Centr. Bakt., 1. Abt., Orig.*, 1924, **91**, 413.
8. Wahl, R., *Ann. Inst. Pasteur*, 1946, **72**, 73.
9. Krueger, A. P., and Fong, J., *J. Gen. Physiol.*, 1937, **21**, 137.
10. den Dooren de Jong, L. E., *Centr. Bakt., 1. Abt., Orig.*, 1931, **120**, 1–15.
11. Krueger, A. P., and Northrop, J. H., *J. Gen. Physiol.*, 1930, **14**, 223.
12. Clifton, C. E., and Marrow, G., *J. Immunol.*, 1936, **31**, 441.
13. Lin, F. C., *Proc. Soc. Exp. Biol. and Med.*, 1934, **32**, 488.
14. Ellis, E. L., and Delbrück, M., *J. Gen. Physiol.*, 1939, **22**, 365.
15. Krueger, A. P., Scribner, E. J., and Brown, B. B., *J. Gen. Physiol.*, 1946, **30**, 25.
16. Price, W. H., *J. Gen. Physiol.*, 1948, **31**, 233.
17. Maurer, F. D., and Woolley, D. W., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 379.
18. Fildes, P., and Richardson, G. M., *Brit. J. Exp. Path.*, 1937, **18**, 292.
19. Delbrück, M., *The Harvey Lectures*, 1945–46, **41**, 161.
20. Cohen, S. S., and Anderson, T. F., *J. Exp. Med.*, 1946, **84**, 511.

21. Doermann, A. H., *J. Bact.*, 1948, **55,** 257.
22. Price, W. H., *J. Gen. Physiol.*, 1947, **31,** 119.
23. Delbrück, M., and Luria, S. E., *Arch. Biochem.*, 1942, **1,** 111.
24. Herriott, R. M., and Price, W. H., *J. Gen. Physiol.*, **33,** 63.
25. Kearney, E. B., Pond, W. L., Plass, B. A., Maddy, K. H., Elvehjem, C. A., and Clark, P. F., *J. Infect. Dis.*, 1948, **82,** 177.
26. Kearney, E. B., Pond, W. L., Plass, B. A., Maddy, K. H., Elvehjem, C. A., and Clark, P. F., *J. Bact.*, 1948, **55,** 89.
27. Delbrück, M., *J. Immunol.*, 1945, **50,** 131.