JOURNAL OF BACTERIOLOGY, Sept., 1965
Vol. 90, No. 3

Printed in U.S.A.

Assay for the Killing Properties of T2 Bacteriophage and Their "Ghosts"[1]

DONNA H. DUCKWORTH[2] AND MAURICE J. BESSMAN
McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland

Received for publication 14 May 1965

ABSTRACT

DUCKWORTH, DONNA H. (Johns Hopkins University, Baltimore, Md.), AND MAURICE J. BESSMAN. Assay for the killing properties of T2 bacteriophage and their "ghosts." J. Bacteriol. **90**:724-728. 1965.—A procedure for the assay of bacteriophage and their "ghosts" which is based on their ability to kill cells is described. The method is derived from the well-known ability of phage and ghosts to prevent the induction of β-galactosidase. Conditions are described whereby a direct relationship is found between the decrease in β-galactosidase and the number of phage or ghosts present during the induction period. The number of phage measured by this method was found to be identical with the number of plaque-forming units found in a fresh lysate. The method has been used to follow the fate of ghosts under several conditions and to measure killer (but nonviable) particles in various preparations of phage.

Despite the rapid advances during the past few years in describing the biochemistry of the bacteriophage-infected cell, the primary lethal event in the infection process is still obscure. Since the empty protein capsule, or ghost (Anderson, 1950; Herriott, 1951), from which the deoxyribonucleic acid (DNA) has been extruded is also effective in inhibiting normal metabolic processes in the bacterial cell, it seemed advantageous to use ghosts instead of whole phage particles to study the killing process, thereby avoiding additional complications attendant with the injection of DNA and subsequent multiplication of bacteriophage.

To study this problem on a quantitative basis, it was necessary to develop a reliable assay for measuring ghosts based on some feature of their ability to alter the normal metabolism of the host. Methods which relate the number of ghosts to the lysis of bacterial cultures (Herriott and Barlow, 1957*a*) or to surviving colony formers are not applicable, because cells may be killed but not lyse (Lehman and Herriott, 1958), and "killed" bacteria have been observed to recover the ability to form colonies under certain conditions (French and Siminovitch, 1955). [We have also observed recovery of "killed" bacteria as evidenced by a resumption of growth and increase in colony formers. However, the recovery was observed only in synthetic medium (glucose-salts). In nutrient broth and nutrient broth mixed with glucose-salts, no recovery occurred.]

This report describes a simple and accurate method for the assay of ghost particles and whole phage which is based on their ability to prevent the formation of adaptive enzymes (Monod and Wollman, 1947; Benzer, 1953; French and Siminovitch, 1955; Sher and Mallette, 1954; Levin and Burton, 1961).

MATERIALS AND METHODS

$T2r^+$ is a wild type isolated in our laboratory in 1960 from a single plaque. *Escherichia coli* B is a wild type grown from a single colony originally isolated in 1958. Phage ghosts were prepared and purified by the procedure of Herriott and Barlow (1957*a*). Isopropyl β-D-thiogalactopyranoside (IPTG) and *o*-nitrophenyl β-D-galactopyranoside (ONPG) were purchased from Mann Research Laboratories, New York, N.Y.

Bacterial titers were read from a standard curve relating turbidity in a Klett colorimeter (no. 66 filter) to viable count. Bacteriophage were titered on nutrient agar plates by use of the agar layer technique (Adams, 1950). β-Galactosidase was assayed according to the procedure of Lederberg (1950), modified as described below. Cells were grown at 37 C by shake culture in M-9 medium (Herriott and Barlow, 1952).

RESULTS

Proportionality of induced β-galactosidase to cell number. Since the success of this method is based on the relationship between cell number and enzyme activity, it was necessary to define a

[1] Contribution No. 446 of the McCollum-Pratt Institute.

[2] Predoctoral fellow of the National Institutes of Health.

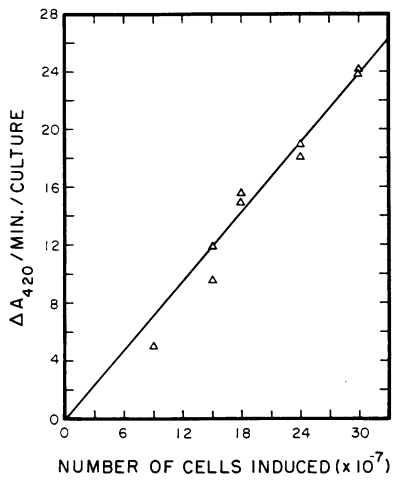

FIG. 1. *Influence of cell number on the amount of β-galactosidase activity induced. Cells were induced with IPTG under standard conditions described in the text. Initial rates of β-galactosidase activity were measured in a Klett colorimeter with ONPG as substrate.*

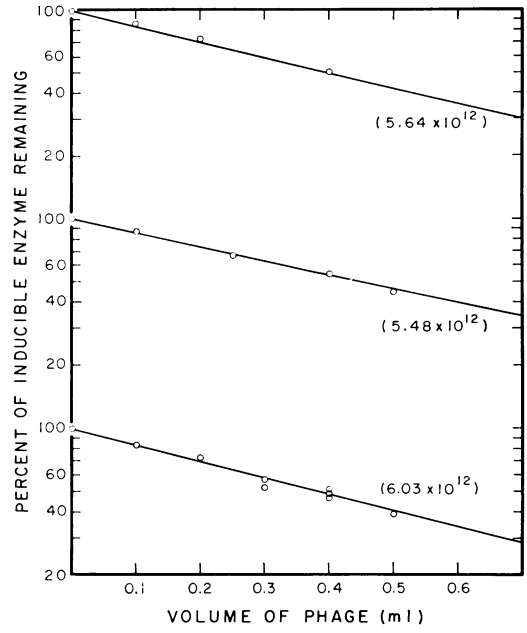

FIG. 2. *Assay of bacteriophage by inhibition of β-galactosidase induction. A phage preparation which contained 3 × 10¹² plaque-forming units per milliliter was assayed according to the procedure described in the text. The numbers of bacteria per incubation mixture in the top, middle, and bottom curves, respectively, were 3.3 × 10⁸, 3.5 × 10⁸, and 3.3 × 10⁸. Values in parentheses represent the number of phage per milliliter calculated from the 37% survival value of each curve. The volume of phage represents a 1:1,000 dilution.*

set of conditions which gave a reproducible dependence of β-galactosidase activity on bacterial cell titer. The following set of conditions satisfied this requirement. (i) A saturating concentration of a gratuitous inducer (Monod and Cohen, 1952) was used. (ii) Bacterial cells were harvested in log phase and shifted immediately prior to use from a glucose-containing medium to a medium devoid of a carbon source. (iii) Induction was allowed to proceed for 12 to 20 min.

Cells were grown in M-9 to a density of 3 × 10^8 per milliliter in a shake culture; they were then chilled, centrifuged, and suspended in M-9 minus glucose to their original density. Samples (10 ml) were transferred to 50-ml Erlenmeyer flasks and warmed to 37 C. Then 0.1 ml of freshly prepared 0.05 M IPTG (inducer) was added, and the flasks were shaken for 12 min. The cultures were then chilled and centrifuged, and the cells were resuspended in 5 ml of 0.02 M sodium phosphate buffer (*p*H 7.5). Ten drops of toluene were added, and the suspensions were left at 37 C for 30 min, with intermittent vigorous shaking. The solution was clarified by centrifugation, the toluene was removed by aspiration, and cell debris was suspended in the supernatant fluid. An 0.8-ml sample was then taken for enzyme assay. To this was added 0.2 ml of 0.005 M *o*-nitrophenyl β-D-galactoside, and rates were measured in a recording spectrophotometer or colorimeter at 420 mμ. The relationship between cell number and enzyme activity under these conditions is demonstrated in Fig. 1.

Effect of bacteriophage on induction of β-galactosidase. The number of phage particles present in a given preparation may be estimated by measuring their ability to prevent the induction of β-galactosidase. Cells were grown as in the previous experiment. When the cells reached a density of approximately 3 × 10^8 per milliliter, they were centrifuged and then suspended in M-9 minus glucose at about 3 × 10^8 per milliliter; 10-ml samples of the suspension were pipetted into 50-ml Erlenmeyer flasks and warmed to 37 C. Various samples of phage were added, and after 10 min (for adsorption) the inducer was added and the remainder of the assay was conducted as in the previous experiment.

A control flask containing no added phage was arbitrarily set at 100% induction. The decrease in inducibility of β-galactosidase with increasing numbers of phage was delineated graphically on semilog paper by plotting the volume of phage suspension added versus the percentage of inducible bacteria remaining. Typical results are depicted in Fig. 2. The slope of the graph is propor-

tional to the concentration of phage, and the number of killing phage may be calculated from the expression: per cent inducible bacteria remaining = $\ln e^{-m} \times 100$, where m = the number of phage divided by the number of bacteria in the culture. At 37% survival, $m = 1$, so that the number of phage at this point on the graph is equal to the number of bacteria in the culture. Multiple determinations on the same phage preparation, on the same or successive days agreed to within 5% of the mean.

It is important to include a control containing no phage with each assay to establish the value for 100% enzyme induction, since this can vary from day to day by as much as 100%. This variation in absolute amount of inducible enzyme in no way effects the precision of the assay. In Fig. 2, the results of typical assays are depicted. This preparation was assayed three times over a 1-week period, and results agreed to within 4% of the mean value.

Effect of bacteriophage ghosts on induction of β-galactosidase. As far as their effects on inducibility of β-galactosidase is concerned, phage ghosts behave similarly to whole phage particles. Inactivation curves identical to those seen with whole phage were obtained (Fig. 3). The procedure for ghosts was identical to the assay for bacteriophage, although the precision was less, varying between 10 and 20% of the mean. Dilutions used routinely contained between 10^9 and 10^{10} ghosts or phage per milliliter. There is no reason, however, why the sensitivity could not be increased at least 10-fold with the proper volume adjustments. In either assay, an alternate procedure may be used to lyse the cells. Instead of adding toluene, a high multiplicity of phage

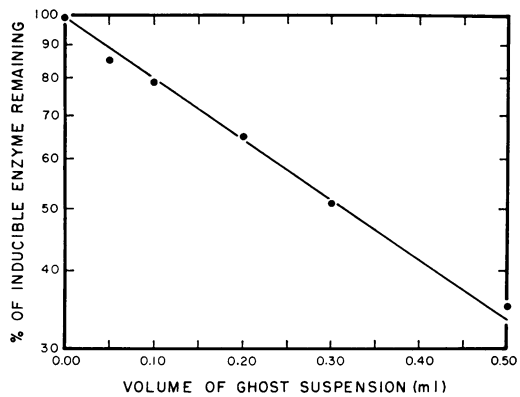

FIG. 3. *Assay of bacteriophage ghosts by inhibition of β-galactosidase induction. The number of bacteria in each incubation was 3.25 × 10^8 per milliliter. The volume of ghosts added represents a 1:100 dilution.*

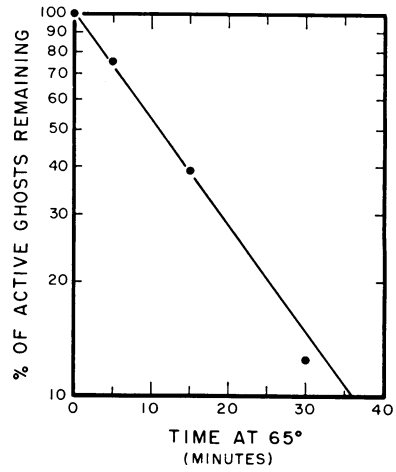

FIG. 4. *Heat inactivation of ghosts. A suspension of ghosts in M-9 medium was incubated at 65 C. At the indicated times, samples were chilled and assayed for their ability to prevent the induction of β-galactosidase in the standard procedure.*

(greater than 100 phage per bacterium) may be used. This has the advantage of reducing the blanks, because lysis with phage produces clear solutions, whereas treatment with toluene produces turbid suspensions. For most purposes, either technique may be used and both give the same results, although the former procedure is much more economical of phage.

Heat inactivation of ghosts. An application of this technique is illustrated in Fig. 4 where the inactivation of phage ghosts by heat is demonstrated. Each point on the curve was determined with the assay described in this paper. The curve indicates that the property in ghost preparations capable of preventing β-galactosidase induction in inducible cells is inactivated with first-order kinetics at 65 C and has a half-life of about 11 min.

DISCUSSION

The technique described in this paper provides a convenient means for measuring phage ghosts based on their ability to prevent the induction of β-galactosidase in inducible cells. This method was used to follow the fate of the ghosts during their preparation by the procedure of Herriott and Barlow (1957*a*). It was found that, in the first step, the osmotic shock used to disrupt the phage particle into coat protein and nucleic acid inactivates 99.9% of the plaque-forming capacity of the preparation and 30 to 50% of its ability to prevent enzyme induction. The addition of gelatin (Hershey, 1955) to the shocking medium did not prevent this loss. Thus, the structure(s) in the

phage coat responsible for the prevention of enzyme induction can be damaged by osmotic shock. During the remaining steps in the purification procedure, a further 25% was lost, but this can be accounted for entirely in the supernatant fractions of the differential centrifugations. In the steps following the osmotic shock, the specific activity of the particles remained constant; that is, the number of effective units per milligram of protein did not change.

Except for special instances in which the electron microscope has been used to count phage membranes directly (Bonifas and Kellenberger, 1955), the general procedure for quantitating a given ghost preparation has been to assume that its titer is numerically equal to the titer of the phage (plaque-forming units) from which it was prepared. An important feature of the assay described in this paper is that it is not based on this assumption. It is possible, for example, to produce more active ghosts than the number of plaque-forming units originally present in a given preparation. This can come about if the particular preparation contains a mixture of viable and nonviable phage. If ghosts are produced from both types, their number can exceed the viable phage originally present. This is not a hypothetical situation but occurs frequently in preparations of phage that have been stored under various conditions. We have observed that the number of phage present in a fresh lysate measured by the conventional plaque count (Adams, 1950) is the same as the number of phage measured by our assay. In one preparation, the respective titers were 1.9×10^{11} and 1.7×10^{11}. However, when the phage were then purified according to the procedure of Herriott and Barlow (1952), the titer by our assay was twice the titer obtained by plaque count. In two different purified preparations, the results were 3.0×10^{12} and 4.4×10^{12} plaque-forming units, as opposed to 6.0×10^{12} and 8.4×10^{12} β-galactosidase-inhibiting particles, respectively. Thus, during the purification procedure, there is a greater loss of plaque-forming units than β-galactosidase-inhibiting units. This might be expected, since it is probable that there are more ways to damage the complex apparatus needed for reproduction of a phage particle than there are to damage the structure(s) responsible for enzyme inhibition.

It is anticipated that, in addition to its usefulness in titering ghost preparations and intact phage, this procedure will be applicable to other problems which have been difficult to approach. For example, it should be possible to follow the synthesis of enzyme-inhibiting units during the course of phage maturation by using this assay on premature lysates of the host bacterium. It should also be useful in any instance in which it is important to know the total number of particles capable of inactivating bacteria. Such information is essential for interpretations of experiments in which the multiplicity of infection is crucial, or in experiments on the induction of early enzymes, where a large number of nonviable but killer ghosts would reduce the total population of productive bacteria.

We should like to point out that the use of phage ghosts to study the lethal event(s) in the infection process does not imply that the killing process of ghosts and whole phage is identical. Indeed, it is clear that the whole phage and ghost are quite different in their overall effect on the host's metabolism (Herriott and Barlow, 1957*b*; French and Siminovitch, 1955; Lehman and Herriott, 1958; Bonifas and Kellenberger, 1955). However, the profound effect on bacterial metabolism attendant with ghost infection, which under suitable conditions leads to the ultimate death of the cell, and the rapidity with which these effects are manifested, suggest that a thorough investigation of the biochemical basis of ghost-induced cell damage will be instrumental in clarifying the sequence of events leading to phage-induced cell death.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant C-4088 from the National Cancer Institute.

We wish to thank Shirley Varricchio for her help in the preparation of this manuscript.

LITERATURE CITED

ADAMS, M. H. 1950. Bacterial viruses. Methods Med. Res. **2**:3–73.

ANDERSON, T. F. 1950. Destruction of bacterial viruses by osmotic shock. J. Appl. Phys. **21**:70.

BENZER, S., 1953. Induced synthesis of enzymes in bacteria analyzed at the cellular level. Biochim. Biophys. Acta **11**:383–393.

BONIFAS, V., AND E. KELLENBERGER. 1955. Etude de l'action des membranes du bacteriophage T_2 sur Escherichia coli. Biochim. Biophys. Acta **16**:330–338.

FRENCH, R. C., AND L. SIMINOVITCH. 1955. The action of T_2 bacteriophage ghosts on Escherichia coli B. Can. J. Microbiol. **1**:757–774.

HERRIOTT, R. M. 1951. Nucleic-acid-free T_2 virus "ghosts" with specific biological action. J. Bacteriol. **61**:752–754.

HERRIOTT, R. M., AND J. L. BARLOW. 1952. Preparation, purification and properties of E. coli virus T_2 . J. Gen. Physiol. **36**:17–28.

HERRIOTT, R. M., AND J. L. BARLOW. 1957*a*. The protein coats or "ghosts" of coliphage T_2 . I. Preparation, assay, and some chemical properties. J. Gen. Physiol. **40**:809–825.

HERRIOTT, R. M., AND J. L. BARLOW. 1957*b*. The protein coats or "ghosts" of coliphage T_2. II. The biological functions. J. Gen. Physiol. **41**:307–331.

HERSHEY, A. D. 1955. An upper limit to the protein content of the germinal substance of bacteriophage T2. Virology **1**:108–127.

LEDERBERG, J. 1950. The beta-D-galactosidase of Escherichia coli, strain K-12. J. Bacteriol. **60**:381–392.

LEHMAN, I. R., AND R. M. HERRIOTT. 1958. The protein coats or ghosts of coliphage T_2. III. Metabolic studies of Escherichia coli B infected with T_2 bacteriophage "ghosts." J. Gen. Physiol **41**:1067–1082.

LEVIN, A. P., AND K. BURTON. 1961. Inhibition of enzyme formation following infection of Escherichia coli with phage T_2r^+. J. Gen. Microbiol. **25**:307, 314.

MONOD, J., AND M. COHN. 1952. La biosynthèse induite des enzymes (adaption enzymatique). Advan. Enzymol. **13**:67–118.

MONOD, J., AND E. WOLLMAN. 1957. L'inhibition de la croissance et de l'adaption enzymatique chez les bactéries infectées par le bacteriophage. Ann. Inst. Pasteur **73**:937–956.

SHER, I. H., AND M. F. MALLETTE. 1954. The adaptive nature of the formation of lysine decarboxylase in Escherichia coli B. Arch. Biochem. Biophys. **52**:331–339.