THE PROTEIN COATS OR "GHOSTS" OF COLI PHAGE T2

II. The Biological Functions*

By ROGER M. HERRIOTT and JAMES L. BARLOW‡,§

(From the Department of Biochemistry, The Johns Hopkins University, School of Hygiene and Public Health, Baltimore)

(Received for publication, May 27, 1957)

ABSTRACT

Phage coats or ghosts, composed entirely of protein, appear to be responsible for protecting the phage nucleic acid from degradation by factors in the surrounding medium; attachment of the virus to its susceptible host; and delivering the nucleic acid to the interior of the cell. In addition, the ghosts have a number of biological actions which resemble similar actions of the parent phage. Thus, they both "kill" cells, inhibit pentosenucleic acid formation, interfere with subsequent infection by other virus particles, block adaptive enzyme formation, induce or trigger lysis of the host, and cause a leakage of phosphorus-containing fragments from the cell. Results to date fail to demonstrate a direct involvement of the ghosts in the passage of genetic information to the progeny. Several of the above changes induced in the host cell following attachment of ghosts could be derived from an alteration in but a single metabolic reaction. The stoichiometry of the ghost–bacterial cell interaction is different from that of the parent phage. Experiments to distinguish between a variable response of the host cell to reaction at different sites and a state of heterogeneity in the ghost preparations suggest the former but they are not decisive.

It was reported earlier (1) that certain biological properties of intact T2 phage were also found in its coat or ghost freed of the nucleic acid. The present article describes in some detail the experiments and results involved in an extension of that report.

It will be shown that the phage properties of (*a*) host range specificity, (*b*) "killing" action, (*c*) inhibition of host PNA synthesis, (*d*) interference, (*e*) induction of lysis in the host cells, and (*f*) induced leakage of host phosphorus-containing fragments are also properties of the proteinous coat or "ghost." No genetic activity was detectable in the ghosts. These findings point to a

* Aided by a grant from the National Foundation for Infantile Paralysis.

‡ Supported in part by a grant from the United States Atomic Energy Commission to R. M. H.

§ Present address: Laboratory of Protein Chemistry, Division of Laboratories and Research, New York State Department of Health, Albany.

high degree of functional specialization by the chemically and morphologically distinct components of T2 phage.

An article (2) describing the preparation and assay of T2 ghosts preceded the present one in order to define and establish the units and chemical nature of the ghosts before entering into a discussion of their biological properties.

Materials and Methods

The materials and methods used in the present work were in general those described earlier (2). Any deviation will be noted in the protocols of the experiments.

TABLE I

Host Range Specificity

Adsorption of S^{35} T2 Phage and Ghosts to Various Strains of E. coli

Materials.—The strains of B/2 and B/4 were obtained through the kindness of Dr. T. F. Anderson of the University of Pennsylvania. The phage was labelled as described earlier (2) and the ghosts were prepared from it by osmotic shock.

Procedure.—The procedure was similar to that described for Table III of the previous article (2).

Multiplicity	Counts per min. in supernatant			Per cent adsorbed		
Strains of cells.....	B	B/2	B/4	B	B/2	B/4
2 ghosts.....	73	15	73	28	80	0	62
2 phage.....	70	8	70	12	89	0	83
8 ghosts.....	280	95	317	140	66	0	50
8 phage.....	280	29	312	40	70	0	86

EXPERIMENTAL

Host Range Specificity.—It is well known that there are strains of host cells that are resistant to particular phage strains while being susceptible to others. It has been shown (3) that in some of these instances the phage does not attach to the resistant cell. Studies were initiated to compare the host range of the ghosts to that of the phage, and the results of these experiments are shown in Table I.

B/2 cells are resistant to phage T2 but susceptible to other phages, and B/4 are resistant to T4 phage but susceptible to others. If the ghosts determine the host range of the phage, they should exhibit the same selectivity of hosts as the intact virus. This was found to be the case.

Sulfur ^{35}-labelled phage and ghosts were incubated with aliquots of B, B/2, and B/4 cells after which the cells were centrifuged and the radioactivity remaining in the supernatant fluid determined. It is abundantly clear from the results in Table I that T2 ghosts like the parent phage do not adsorb to B/2 organisms, yet they both adsorb to B and B/4 cells. Though this con-

stitutes a small number of cases, it suggests that the host range specificity of a phage is determined by the specificity built into its protein coat—probably at the tip of the tail where contact with the cell wall is made (4).

The results showing that 15 to 20 per cent of the label is not adsorbed to host cells with radioactive S^{35}-labelled phage and ghosts, confirm the earlier experiments of Hershey (5, 6) and the recent ones of French and Siminovitch (7). However, using an electron microscope Bonifas and Kellenberger (8) have examined a comparable supernatant fluid following centrifugation and report that no ghosts could be found though as little as 0.1 per cent of the initial number could be detected if present. This means that some interpretation other than the non-adsorption of some ghosts must be found. Perhaps there is a partial breakdown of the labelled ghosts following adsorption. Since there is no such partial breakdown following phage infection, it must be concluded that either the breakdown observed in ghosts is confined to the "non-killers" (see section on "Killing" action of ghosts) or that there may be an important difference in the fate of the ghost when acting alone, from that when acting in conjunction with the phage DNA.

The suggestion of Bonifas and Kellenberger that the non-adsorbed isotope might represent labelled material detached from the coat at the time of osmotic shock has been removed by Hershey (6) who found only *ca.* 2 to 3 per cent of the phage sulfur in the supernatant fluid following removal of the ghosts from a "shockate" by sedimentation.

The "Killing" Action of Ghosts.—

It has been known for some time that the property of virulent phages of preventing their host cells from forming colonies is not dependent on the replicating capacity of the phage. Thus, ultraviolet light (9) or x-ray (10) inactivated phage "kills" the host cells. However, it is not possible to decide from these experiments whether the killing resulted from injection of the viral nucleic acid into the host or from an effect of the protein ghost, or both. Experiments using ghosts freed of nucleic acid leave no doubt that the host cells are rendered incapable of forming colonies (killed) by the protein ghost.

Fig. 1 illustrates how the number of viable cells (colony-forming units) decreases with increasing number of ghosts. A comparable experiment using the parent phage is included for comparison. As is well known for T2 phage the curve follows the theoretical "one-hit" or exponential curve (11). The linear nature of the ghost curve with its passage through the origin confirms the observations of Bonifas and Kellenberger (8) and constitutes strong evidence that the killing by ghosts is due to a single particle.

The smaller slope of the ghost curve compared to the phage suggests that only a fraction of the ghosts are effective killers. This situation has also been observed by others (8, 7, 6). The e^{-1} value, the number of ghost particles

equalling one inactivating dose, is found by interpolating at 0.37 survival. This dose is an average multiplicity of 1 per cell for phage, but between 2 and 3 per cell for ghosts. Interpretation of this finding will be delayed until the mechanism of action is considered.

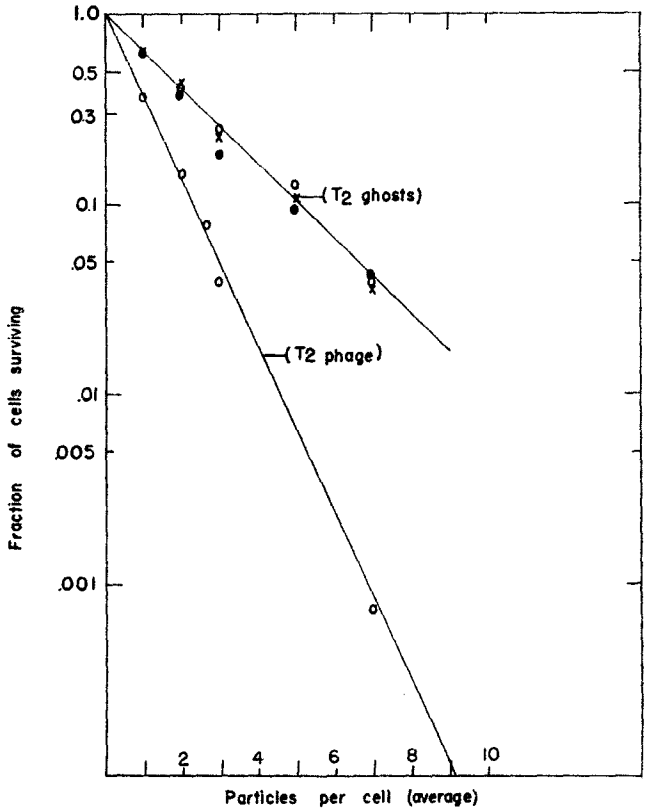

FIG. 1. Killing of nutrient broth-grown *E. coli* B by ghosts. Open circles on the ghost curve were obtained with ghosts prepared by osmotic shock. The crosses represent ghosts prepared by crenation with sucrose, and the closed circles represent ghosts prepared by the action of 2×10^{-3} M pyrophosphate at pH 8.9.

Although there are reports (8, 7) of considerable variation in the proportion of killers in ghost preparations, our preparations have been relatively constant with one or two possible exceptions. Perhaps the variation observed by others is due to variations in divalent cations, known to depress ghost action (12). The report (7) of Weidel's obtaining greater than 90 per cent killers is most interesting.

In an attempt to determine whether the low proportion of killers had its

origin in the method of preparation, ghosts were prepared by three quite different methods—osmotic shock using sodium sulfate or glycerine, crenation with concentrated sucrose solution (13), and liberation of the nucleic acid by dilute pyrophosphate (14). All preparations showed the same proportion of killers (about 45 per cent) as seen in Fig. 1. Purified ghosts, *i.e.* separated from the nucleic acid, had the same killing efficiency as the crude preparations. In addition, ghosts formed from phage produced in nutrient broth-grown cells were not different from their counterpart obtained from synthetic medium-prepared phage. In similar experiments, Hershey (6) found about the same fraction of killers as reported here.

It is of some interest that Siminovitch (15) has suggested that ghosts of the temperate phage λ do not kill the host cell even though they adsorb. As he points out, this may have important implications regarding the differences in the course of infection of the two types of phages.

Experimental Procedure (Fig. 1)

Coli B grown in nutrient broth to 2 to 3 × 10^8/ml. in 10 ml. quantities was mixed with 1 ml. quantities of T2 phage or ghosts of the appropriate concentration to yield the indicated multiplicity. These tubes were shaken at 37°C. for 5 minutes after which they were diluted and plated for colony-forming units. Appropriate controls were included. The methods of preparing ghosts have been described (2).

Inhibition of Growth by Non-Killing Ghosts.—

Experiments earlier in this paper suggest that half or more of the ghosts in a preparation do not kill or lyse susceptible cells to which they adsorb. It was of some interest to determine whether the non-killing ghosts are deficient in other functions. It was expected that any effect on the metabolic systems of the host would produce a diminished growth rate so that a correlation was made of the turbidity changes with time of cultures at a low multiplicity of ghosts. A low multiplicity (0.5) was chosen in order to observe as nearly as was practicable the effect of singly "infected" cells and to prevent equivocal results at higher multiplicities due to lysis which, of course, would offset a corresponding growth.

The experimental results are shown in Fig. 2 from which it may be concluded that non-killer ghosts inhibit cell multiplication and, therefore, confirm the results of French and Siminovitch (7). On the basis of a random distribution of ghosts among the cells it was expected that at a multiplicity of 0.5, the cell growth would be depressed to 60 per cent of normal, if all the ghosts were effective inhibitors of growth and would follow the same curve as a system infected with phage at this multiplicity. If, however, only the killer ghosts inhibited growth, the turbidity rise would lie between the two curves. The experimental observations of both the phage and ghosts fell on

the same curve which has a slope of 58 per cent of the control. It is difficult to see how a composite of normal and recovering cells in any proportion could account for this result unless it is assumed that under the particular experimental conditions growth of normal uninfected cells is depressed by just the necessary amount. It is tentatively concluded, therefore, that non-killing ghosts inhibit cell growth.

French and Siminovitch (7) have reported that some ghost-treated cells recover in synthetic medium after about 80 to 90 minutes. This extended delay may be due to the nature of the medium and/or to the magnesium ion.

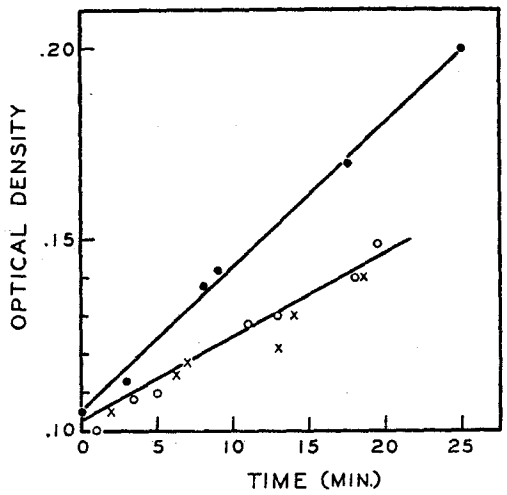

FIG. 2. Evidence of inhibition of growth of *E. coli* B by non-killer ghosts. Closed circles are from the uninfected control. The open circles represent ghosts at an average multiplicity of 0.5. The crosses represent a phage control at an average multiplicity of 0.5. The ordinate is optical density units read from a Coleman Jr. spectrophotometer at 650 mμ.

In the present studies the inhibited but viable cells began to multiply soon after dilution into fresh nutrient broth as shown by a logarithmic rise in colony count, yet no evidence of toxic or inhibitory material was detectable in ghost lysates of cells.

It was expected from the above results that growth curves as represented by turbidity measurements of cultures having varying multiplicities of ghosts should turn upwards after an initial delay or drop due to inhibition or lysis. This was not observed in Fig. 2 of the previous paper (2) and it was indicated that the residual phage in the ghost preparation could produce sufficient phage *in situ* to infect most of the cells after 20 to 30 minutes. To examine the effect of ghosts without the complicating effect of phage the latter was

reduced by differential centrifugation and by short exposures to ultraviolet light. Such preparations, when tested as described earlier (2), produced the curves shown in Fig. 3. It is clear that in the absence of phage the growth of cells is observed, but the systems are too complex to attempt to separate normal and recovering cell growth.

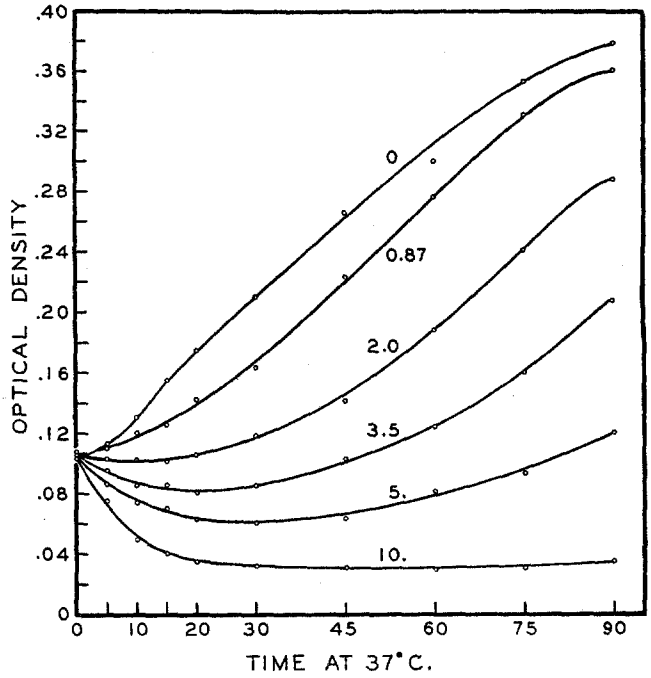

FIG. 3. Effect of time on the change in cell concentration at various multiplicities of ghosts. The phage concentration in this purified ghost preparation was reduced to below 0.01 per cent. The number on each curve is the average multiplicity of ghosts.

Experimental Procedure (Fig. 2)

To 10 ml. aliquots of *coli* B grown in nutrient broth to an O.D. of 0.115 ≈ 3.8 × 10^8/ml., was added 1 ml. of dilutions of 1.9 × 10^9/ml. ghosts or phage which was shaken at 37°C. The turbidity was read in a Coleman Jr. spectrophotometer at 650 mμ immediately after mixing and at intervals thereafter. Controls of free growing cells were also included.

Experimental Procedure (Fig. 3)

As described for Fig. 2 of the earlier paper (2) except that the ghosts were freed of phage by fractional sedimentation (16) or by short exposures to ultraviolet light (20 seconds at 80 cm. to 1 to 2 mm. layer of saline solution). This treatment had no detectable effect on the ghost lytic action.

Lysis by Ghosts.—

Lysis by ghosts of growing cultures of *coli* B was the property which first stimulated this study of the specific biological functions of the ghosts. Since lysis was essentially complete in a short time and was correlated with the number or concentration of ghosts added, an assay procedure was developed in which the extent of lysis was evaluated from turbidity measurements (2). Useful as this method is, it does not permit a direct evaluation of the exact number of ghosts needed for lysis of a cell. If each ghost is able to trigger the lytic mechanism, then the decrease in number of unlysed cells should be logarithmic with increasing multiplicities of ghosts. The equation:

$$N/N_0 = e^{-\alpha m}$$

describes such a case, in which N/N_0 is the fraction of surviving unlysed cells and m represents the average multiplicity used. α is a constant which indicates the proportion of effective ghosts in the preparation. If the combined action of more than one ghost is necessary for lysis, the curve will start with a small slope and then curve downwards.

In nutrient broth a turbidimetric analysis of the lytic system was complicated by the growth of free cells. Freshly grown log phase cells washed and resuspended in buffered saline responded to ghosts as shown in Fig. 4. The linear nature of the results indicates that a single ghost triggers the lytic process. However, the value of α is about 0.35, suggesting that only 35 per cent of the ghosts are effective. In the presence of 0.001 M cyanide α equalled 0.45. It may be noted here that the correlations reported in a previous paper (2) between changes in turbidity and loss of cells showed that "lysis" was essentially an all-or-none process. It has been assumed that in saline that relationship still holds.

Lysis by ghosts has many features in common with lysis by high multiplicities of phage noted by Krueger and Northrop (17) for staphylococcus phage and described by Delbrück (18) for the *coli* systems as "lysis from without" (LFW). Lysis is also induced prematurely when the energy-generating systems of the cells multiply infected with wild type phage are uncoupled by the addition of cyanide (19), iodoacetate (20), or dinitrophenol (21) (see (20) for a discussion). In all these instances lysis was initiated within a few minutes in marked contrast to lysis following infection.

The triggering of cell lysis by ghosts and LFW by phage are inhibited by divalent cations (22, 12, 23), thereby explaining why the process in synthetic medium is incomplete or requires many more ghosts.

A few additional experiments will be summarized briefly because of their relationship to the problem of lysis. Attempts to lyse infected cells with as many as 100 ghosts per cell were unsuccessful. Ghosts induce lysis in cells suspended in physiological saline so that exogenous nutrilites are not essential to the reaction.

Some years ago Anderson (24) reported a "lysin" obtained by irradiating T2 phage with ultraviolet light. Since the lysin failed to sediment in an hour at 30,000 R.P.M., it was suggested that the agent was an enzyme. It was active on cells irradiated with ultraviolet light. Ralston *et al.* (25) have more recently observed a lytic factor in lysates of *Staphylococcus aureus* infected with P14

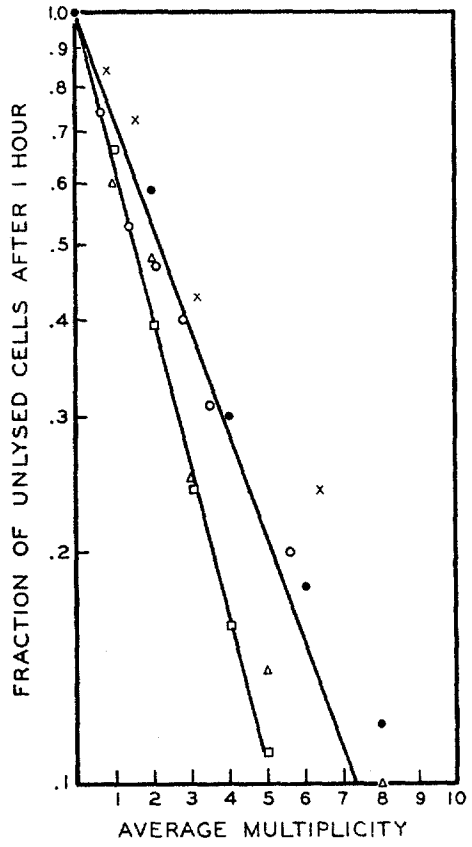

FIG. 4. Lysis of *coli* B by ghosts in buffered saline. The left-hand curve was obtained when 0.001 M NaCN was added to the buffered saline.

phage. This agent also fails to sediment at 35,000 × *g* in an hour and lyses heat-killed cells or live cells when acting with the phage. It appears that this agent is quite different from T2 ghosts.

Bronfenbrenner (26) has suggested an interesting mechanism for lysis of bacteria, and it is quite possible that in ghost lysis or even phage LFW that the cell's metabolism is so disrupted that there follows an enzymatic breakdown of large molecules with a corresponding increase in osmotic equivalents. Cells undergoing lysis by ghosts usually become spherical prior to bursting

indicating that they may be experiencing a weakening in the cell wall structure, an increase in internal pressure, or both.

Little is known about the mechanism of lysis by ghosts or LFW by phage, but any hypothesis will necessarily have to be fitted to many facts, some of which have been enumerated above. Perhaps the most interesting are the complete blocking of LFW by increasing the magnesium ion concentration to 0.01 molar (12, 23), the complete resistance of infected cells to ghost lysis, and the initiation of lysis by interrupting the energy metabolism of cells infected with wild type phage (20, 21).

Experimental Procedure (Fig. 4)

Coli B were grown in nutrient broth to 1×10^8/ml. after which they were chilled, centrifuged, washed once with saline, and then resuspended in one-third to one-half its original volume of saline buffered with M/50 phosphate pH 7.2. After warming for 5 minutes at 37°C., 1 ml. of saline solution of freshly prepared ghosts was added to 10 ml. of the cells and the turbidity read in a Coleman Jr. spectrophotometer at 650 mμ after 30 and 60 minutes' shaking. The fraction of unlysed cells after 60 minutes was calculated after first subtracting a blank of 0.01 representing the turbidity of completely lysed cells. Lysis in nutrient broth to which was added sodium cyanide was studied in a comparable series. The final concentration of sodium cyanide was 0.001 M.

Inhibition of PNA Syntheses.—

A number of years ago Cohen (27) reported that T2 phage inhibits PNA synthesis in *E. coli* B, and Manson (28) has confirmed this with P^{32} studies. Hershey's data (5), however, suggest that the inhibition is not complete for in his experiments a rapid turnover of about 2 per cent of the PNA was observed immediately after infection. Since this general inhibition is one of the characteristic properties of at least the T-even phages, it was looked for in ghost-"infected" cells. To overcome the necessity of correcting for RNA synthesized by cells having no ghosts a multiplicity of 4 was used. This multiplicity left only 2 to 3 per cent of the cells free of ghosts; it killed over 80 per cent of the cells, and lysed about 15 per cent. Over 80 per cent of the cells remained intact as judged by turbidity and they synthesized some protein and deoxyribosenucleic acid (DNA) but no net synthesis of PNA could be detected in a 45 minute period (29, 30).

Using uptake of inorganic P^{32} phosphate into the PNA fraction as the measure of PNA synthesis, it may be seen in Fig. 5 that on the average twice as many ghosts as phage were necessary for a given inhibition. A logarithmic fall-off was obtained. By itself this might not be highly significant for there are many unproven assumptions involved in such an experiment. Nevertheless, with other properties responding in a similar manner this result takes on somewhat more significance and suggests that a single ghost blocks the forma-

tion of RNA. The similar observation that only one in every 2 to 3 ghosts is responsible for the killing action suggests that only the killer ghosts carry the other properties, but other alternatives will be discussed later.

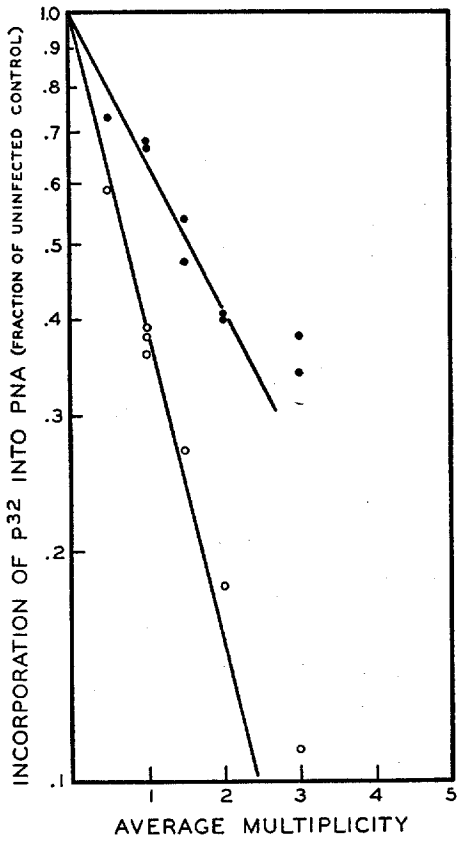

FIG. 5. Inhibition of PNA synthesis by ghosts. Solid circles were obtained with crude and purified ghosts. The open circles were obtained with T2 phage and they serve as a control.

The tendency of the lower points in both curves in Fig. 5 to fall above the straight lines suggests that there may be a small incorporation of P^{32} phosphate into PNA and that this assumes a more prominent place as the inhibition of PNA synthesis is more extensive. This is supported by the results noted above of Hershey and Chase (5). Lehman (30) observed considerably more incorporation in ghost-inhibited cells than in phage-infected cells (5) which agrees with the greater deviation noted in Fig. 5.

The ghost curves were not extended to higher multiplicities because of the complications in interpretation due to lysis.

Experimental Procedure (Fig. 5)

Fifty ml. aliquots of *E. coli* B in nutrient broth were grown to 3 × 10^8/ml. after which 1 ml. quantities of ghosts or phage were added followed by 1 microcurie of P^{32} as inorganic phosphate. The suspensions were then agitated for 20 minutes at 37°C. after which the turbidities were checked and the suspensions chilled, centrifuged, washed with cold 5 per cent TCA, and extracted in N/1 KOH according to the Schmidt-Thannhauser procedure (31), and after acid precipitation the radioactivity of the soluble fraction was assayed with a Geiger counter which had been standardized. Four separate experiments were performed.

Inhibition of Adaptive Enzyme Formation.—

It is known (32, 33) that phage infection blocks the formation of adaptive enzymes and interrupts adaptation if it is under way. Sher and Mallette (34) found that the adaptive formation of lysine decarboxylase was blocked by T2 ghosts. French and Siminovitch (7) reported similar results for β-galactosidase. The latter workers have presented evidence that suggests that the inhibition of adaptive enzymes is a property of non-killer as well as killer ghosts.

Leakage of Phosphorus-Containing Components.—

A number of investigators (35–37) have observed that phage-infected cells lose certain cellular constituents soon after infection is initiated. Prater (36), Puck and Lee (38), and Lehman (30) obtained similar findings following the interaction of phage ghosts and *coli*. In our earlier paper (2) in which the evidence bearing on the correlation of turbidity changes with lysis of cells was considered, it became clear that cellular phosphorus appeared in the supernatant before there was true lysis of cells. Additional data from tracer studies will be offered in the succeeding papers (39), but it seems certain that leakage of cellular phosphorus must be included among the effects produced by ghosts. It is not entirely clear whether this leakage is due in part or entirely to an effect on cell wall permeability, to a breakdown of nucleic acids, or to some other response.

Cytological Changes.—

The infection of *coli* by T2 phage produces characteristic cytological changes which have been examined in some detail by others (40–41). An independent study (8) covering the action of ghosts has already appeared and our results[1] are essentially in agreement with those so that only the conclusions will be noted here.

The cytological changes observed either in unstained cells under phase

[1] The writers are indebted to Dr. Katherine Schaeffer for her counsel and examination of the specimens.

microscope or in cells stained with Giemsa after acid treatment showed that ghosts produce a change in cells which is distinguishable from that seen in phage-infected or normal cells. For the first 5 minutes the cells infected with phage or ghosts were very similar, after which cells with ghosts showed an accumulation of the opaque material in the ends of the cells like polar bodies leaving a vacuole or clear area in the center; whereas in phage infection there were marginal vacuoles. Normal cells under the phase microscope remain uniformly opaque.

Interference.—

In common with many viral (42) and rickettsial agents (43, 44) the *coli* T viruses possess the property of initiating in their host a mechanism (45) against subsequent infection by other virus particles. Hershey (46) has pointed out that there are probably two kinds of interference—one, mutual exclusion in which a cell produces only one of the viruses even though the cell is infected essentially simultaneously with both. The other type of interference has been analyzed by Dulbecco (47). In it a period of metabolism (3 to 5 minutes) after the primary infection prevents the bulk of superinfecting particles from contributing genetic markers to the progeny.

It has been known that ultraviolet light (45) or x-ray (10) inactivated phage T2 interferes with active phage infection, but in these instances as with active phage it is not possible to assign the interference function to a particular viral component for both protein and nucleic acid were present. An answer to this puzzle was obtained using ghosts freed of nucleic acid.

Fig. 6 contains a semilogarithmic plot of the results of a set of interference experiments in which the fraction of secondarily infecting particles which produce progeny is plotted against the average multiplicity of ghosts used in primary infection. Once again the linear nature of the curve suggests that a single ghost induced the changes leading to interference as had been observed for phage. Others (8, 48, 7) have also observed interference by ghosts. French and Siminovitch (7) report that even non-killer ghosts interfere. The present studies do not support their conclusion (see Fig. 6), but it is worth noting that the present studies were carried out with nutrient broth–grown cells, whereas their work was performed with synthetic medium cells. Lysis of host cells by the ghosts cannot account for the interference since a microscopic count of the cells during the hour after adsorption of the virus showed many more normal appearing cells than the number which formed plaques.

When phage was mixed with ghosts and this mixture added to nutrient broth–grown cells, prevention of the development of infective centers also occurred as may be seen in Fig. 7. Here the fraction of the infected units observed is plotted against the average multiplicity of ghosts. It is again clear that the ghosts prevent the development of infective centers even though they

are applied simultaneously. When the mixture of phage and ghosts was adsorbed to washed starved cells (49) and then placed in nutrient, a much higher fraction of infective centers was found indicating considerably less interference. This last picture is complicated by a low (30 per cent) recovery of infective centers in the absence of ghosts—a situation reported earlier by Ben-

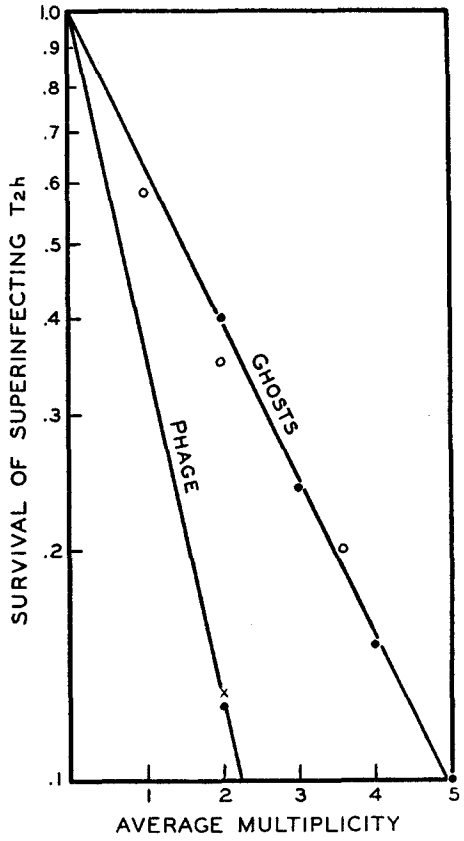

FIG. 6. Ghost interference with delayed superinfection.

zer (49). This combined with lysis at high multiplicities for which a correction has been indicated in Fig. 7, and it is readily seen that any conclusion about this system is very tenuous.

Freshly shocked or purified ghosts also interfered with delayed superinfection by T1 and T4 phages in essentially the same way as noted for T2, thus confirming the observations of French and Siminovitch (7).

The present work throws very little light on the mechanism of the interference phenomenon. It appears to be metabolic in nature, and it is clear that

ghosts induce metabolic changes. Interference by ghosts is apparently not due to a breakdown of the superinfecting viral nucleic acid (48). Whether the mechanisms of ghost and phage interference are the same has not been revealed, but it seems established that in actively growing cells ghosts induce interference.

"Lysis Inhibition".—

The property which distinguishes the wild type "r^+" strain of the T-even phages from the rapid lysis "r" strain is the capacity of the former to inhibit

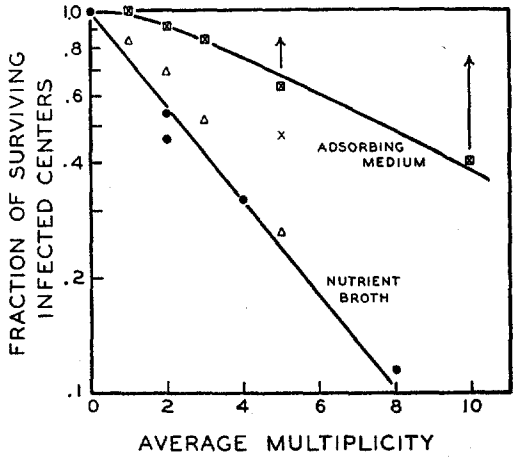

FIG. 7. Ghost interference with simultaneous infection. The closed circles and open triangles represent experiments in which the mixture of phage and ghosts was added to cells in nutrient broth at 37°C. The squares were obtained by first adsorbing the mixture of phage and ghosts to washed starved cells suspended in adsorbing medium after which nutrient was added. The arrows indicate the position of the points if correction is made for lysis.

or delay lysis of infected cells (50). To determine whether this property resides in the protein or is a function of the whole virus, cells were singly infected with T2h virus at a multiplicity (P/B) of 0.1 and 10 minutes later T2 ghosts or phage were added so the multiplicity was 2.5. The lysis time in these experiments was detected by determining the release of newly formed phage. To prevent readsorption of the released phage the cells were diluted extensively in warm nutrient broth after which samples were withdrawn every few minutes and plated for infectious centers on B/2 cells. Use of B/2 cells permitted detection of the lysis of T2h-infected cells without the necessity of correcting for the T2 from the control in which T2 phage was the secondary inhibiting agent.

The results in Fig. 8 show that the T2 ghosts delayed lysis of T2h-infected cells by not more than 2 to 3 minutes, whereas T2 phage delayed lysis by 9 minutes.

In every experiment the ghosts delayed lysis by a few minutes so that it cannot be said that they have no inhibitory power, but in no case did the inhibition exceed a third of that exhibited by whole phage. The stronger inhibitory power of whole phage is quite clear.

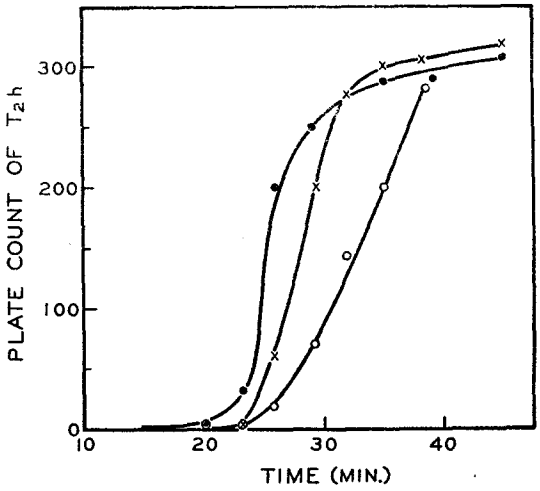

FIG. 8. "Lysis inhibition" by T2 ghosts. Closed circles are from a control in which no secondary agent was added. Crosses are from the experiment in which T2 ghosts were the secondary agent. The open circles were obtained when T2 phage was the secondary agent.

Experimental Procedure (Fig. 8)

Materials

The following solutions were prepared in nutrient broth:
I = 4×10^7/ml. T2h
II = 1×10^9/ml. T2
III = 1×10^9/ml. T2 ghosts prepared by osmotic shock
C = *coli* B grown up to 2×10^8/ml.

Procedure

To three tubes containing 1 ml. of cells (C) was added 0.5 ml. of T2h(I). These were shaken 10 minutes at 37°C. after which 0.5 ml. of nutrient broth was added to the first tube; 0.5 ml. of T2(II) was added to the second; 0.5 ml. of T2 ghosts(III) was added to the third. The tubes were shaken for another 5 minutes after which they were diluted 4×10^5-fold in warm nutrient broth and 10 ml. of the final dilu-

tion shaken at 37°C. At 3 or 4 minute intervals starting at 20 minutes after mixing the cells with T2h phage, 0.5 ml. aliquots were mixed with 10 ml. of diluting agar and B/2 cells, and 2 ml. pipetted onto nutrient agar plates.

Recombination.—

It seemed important to determine whether any discernible relationship existed between the ghost and the formation of recombinants. Cross-reactivation (intracellular restoration of an inactivated phage by a related active one)

TABLE II

Cross-Reactivation with Ghosts

E. coli B grown to 1×10^8/ml. in nutrient broth were chilled, centrifuged, and washed once with cold adsorbing medium (5), and then resuspended in one-quarter of the original volume of adsorbing medium. To 0.5 ml. aliquots of this suspension of cells were added 0.5 ml. aliquots of primary phage, T2h or T4, mixed with the cross-reactivating component to give the multiplicity noted in the table. In the systems containing T4, 5 μg. per ml. of sterile *l*-tryptophan was added to promote adsorption of the phage. These systems, after agitation at 37°C. for 5 minutes, were diluted with 9 ml. of warm nutrient broth, incubated 10 minutes, then diluted and plated on the indicated organism. Ultraviolet-treated phage contained 0.2 per cent residual active phage.

Primary phage Cross-reactivating phage or ghosts Plating organism	T2h T2 B/2	T4 T4r47 K12 (λ)
Composition of system*	Titers per ml.	
0.1 P.P.‡ alone	1.1×10^7	2.4×10^7
0.1 P.P. + 2 C.R.§ phage	1.1×10^7	2.0×10^7
0.1 U.V.P.P.‖ alone	2.6×10^5	3.0×10^5
0.1 U.V.P.P. + 2 C.R. phage	1.5×10^6	6.3×10^6
0.1 U.V.P.P. + 2 C.R. ghosts	2.1×10^5	2.9×10^5

* The figures indicate the average multiplicities.
‡ Primary phage.
§ Cross-reactivating phage.
‖ Ultraviolet-treated phage.

is a type of recombination which lends itself to the present problem. Wild-type T2 ghosts were mixed with ultraviolet light–inactivated T2h phage in a ratio of 20:1 and added to washed and starved log phase *E. coli* B suspended in Hershey's adsorbing medium (5). The multiplicity of T2 ghosts was 2. These were then plated on B/2. As seen in Table II, the ghosts failed to cross-reactivate; whereas, whole phage in place of the ghosts resulted in good cross-reactivation.

In similar experiments, ultraviolet-inactivated T4 was not reactivated by ghosts of the T4rII47, nor was the opposite cross successful; whereas in the

controls using phage in place of the ghosts, reactivation was easily demonstrable by plating on the K12 *coli* lysogenized with λ phage (51, 52). These results are seen in Table II.

While the above results represent only a few experiments and, therefore, do not settle the point, they suggest that the protein component of the phage does not enter into the formation of recombinants. This result was not unexpected in the light of the accumulating evidence that phage DNA carries the genetic properties.

The Mechanism of Ghost Action.—

Throughout this paper the different responses of the cell to ghosts have been emphasized and have even been indicated as biological functions of the ghost. This may be misleading for several of the different responses could be manifestations of but a single change in the cell's metabolic or physiologic make-up. That there are at least two independent functions of ghosts is suggested by the difference in the cell's response to killer and non-killer ghosts. Both inhibit growth but the former destroy the cell's capacity to form progeny, whereas the latter do not. Perhaps the non-killer ghosts inhibit growth by reducing protein synthesis (7).

It has been found that on the average about one in every two ghosts kills a cell, interferes with superinfecting phage, and inhibits PNA synthesis. If these are not independent responses, they probably occur in the same cells. This would suggest that perhaps the killer ghost acts by inhibiting PNA synthesis and this change leads to exclusion of superinfection and to a block in cell division. Interpretation of these relationships is complicated, however, by changes in the cell's permeability following ghost action (36, 30, 38) and by changes in the chromatin-staining structures, the consequences of which are probably vital. Yet, the effects produced by ghosts are reasonably specific and do not produce a general breakdown for no change was observed[2] in the oxygen uptake, respiration, or phosphorus uptake and esterification (29, 30).

It is reasonably clear from kinetic studies that the lethal action is brought on by a single ghost and not by the cumulative action of several non-killer ghosts for the latter would have produced a "shouldered" curve near the origin instead of a straight line through it.

Use of the terms killer and non-killer ghosts appears to acknowledge as fact a state of heterogeneity in ghost preparations, but as the next section will show there are other equally suitable and likely explanations which assume only a single type of ghost.

[2] French (53, 7) has reported a drop in these properties following adsorption of ghosts and, therefore, differs with the observations of this laboratory (30). These results will be discussed in greater detail in the forthcoming paper (39).

Heterogeneity in the Ghosts or Cell Receptor Sites

Kinetic evidence has accumulated which suggests that a single ghost produces changes in many properties of the host cell. Thus, the linear drop from the origin of a semilogarithmic plot of surviving normal cells against an increasing mean multiplicity of ghosts indicates that the change is induced by a single ghost (11). In such a plot if every ghost is effective, the slope of the curve should be 0.434. A plot of surviving cells against increasing multiplicities of phage T2 has this slope. However, with ghosts a slope of 0.2 is obtained as may be seen in Figs. 1 and 4, suggesting that perhaps the stoichiometry of the ghost action is greater than one to one and, hence, that a fraction of the ghosts is ineffective.

The ratio of observed slope to 0.434 is a measure of the proportion of effective ghosts. Despite the use of such apparently meaningful terms as "effective" or killer ghosts the analyses thus far do not permit a decision on which reactant in the ghost-cell system is heterogeneous for it could arise from any of the following possibilities or a combination of them:

(*a*) *Failure of a Fraction of the Ghosts to Adsorb.*—Although as much as 15 to 20 per cent of the isotope of S^{35}-labelled ghosts has been found in the supernatant following adsorption to sensitive cells (8, 6, 2), a decisive interpretation of these findings is not possible (see section on "Host range specificity" for discussion). Even if the non-adsorbing 15 to 20 per cent should prove to be different ghosts, this is insufficient to account for the 40 to 50 per cent level of heterogeneity of most preparations.

(*b*) *The Heterogeneity Is in the Parent Phage.*—Despite the essentially theoretical distribution of phage on host cells as judged by its infectivity or killing action it is possible that phage is heterogeneous only in respect to its ghosts. However, isolates from single phage plaques did not yield ghosts exhibiting a proportion of killers to total ghosts that was different from the bulk of the phage. Some variation in the proportion of killers in ghost preparations has been reported from other laboratories (8, 7), but it is known that the composition of the medium, particularly the divalent cations (22, 12), influences ghost action, and it is not clear whether this played any part in the variation. Considerable variation in neutralization of T2 by antiserum as judged by infectivity in different hosts has been observed by Tanami and Miyajima (54). These results were independent of the host in which the phage was prepared which, as the authors point out, shows that the phage is hereditarily stable. It does not exclude the possibility of heterogeneity in the phage. However, Tanami and Miyajima (54) prefer a "polyphasic" neutralization of a complex site of attachment to account for their results. The magnitude of the variation is similar to that observed with ghosts. Since the experiments are not decisive, further consideration must be given to this possibility.

(*c*) *The Ghosts Are Heterogeneous.*—Using fundamentally different methods of preparation (2), ghosts essentially indistinguishable in properties were produced so that the heterogeneity must be independent of the method of formation. The possibility remains that the release of nucleic acid by any method may injure a fraction of the ghosts from even homogeneous phage. Attempts to fractionate ghost preparations have thus far proven unsuccessful.

(*d*) *The Host Cells Are Heterogeneous.*—This is incompatible with a logarithmic decrease in normal cells as the average multiplicity of phage or ghosts increases. If the host cells were heterogeneous, then with the destruction of susceptibles there should be an increase in the proportion of resistants. This was not observed.

(*e*) *The Receptor Sites of the Host Are Different.*—Cells are known to have over a hundred receptor sites (55) and since most of the collisions between cells and phage T2 lead to infection (55) each site must be equal in this respect. It seems possible, however, that these sites may not be equal with respect to their reaction with ghosts. It could be supposed, for example, that the location or nature of some receptor sites is such that ghosts of uniform properties elicit different physiological responses in the host cells depending on the site to which the ghost is attached.

From the preceding discussion only possibilities (*a*) and (*d*) appear to have been eliminated. There is indecisive evidence bearing on (*b*). Therefore, some effort was directed to a further consideration of (*b*), (*c*), and (*e*). If other conditions could be found in which phage exhibited less than full efficiency of action on cells, it might be considered as evidence of heterogeneity in the phage.

A number of investigators (49, 56–58) have noted that with starved host cells in salts solution, or normal cells at 0°C., the fraction of cells infected or killed by T2 phage differs markedly from the expected. In general, they found from one-fifth to one-half the effect expected of phage at 37°C. in nutrient broth. Adams (58) gave careful consideration to the various possible explanations including heterogeneity of receptor sites on the host cell but reached no satisfactory conclusion. The results in Fig. 9 illustrate the quantitative nature of the deviation from expectation of some of the actions of T2 phage. These results make it clear that the altered environmental conditions have brought out a state of heterogeneity which is formally similar to that observed in ghosts, but as in the other instances it is not possible to determine which reactant is heterogeneous. These results are so similar to those obtained with ghosts as to suggest that the altered environmental conditions which lead to abortive infections (58) leave the cells with ghosts and that they respond therefore as though ghosts had been added. Should this prove to be a correct analysis it would eliminate from possible causes of the heterogeneity only the highly artificial methods of preparing ghosts.

There is a suggestion in the effect of cations on ghost action (12) to be reported in more detail later which throws some light on the present problem. It was observed that the number of cells protected by magnesium ion from the lytic and killing action of ghosts increased with the concentration of magnesium, but at any one concentration the number protected was independent of the multiplicity of ghosts. This points to a cation equilibrium with the cell rather than with the ghost.

Different experiments are needed to completely unravel this problem. At the moment, the cell's receptor sites are suspected of being responsible for the observed heterogeneity.

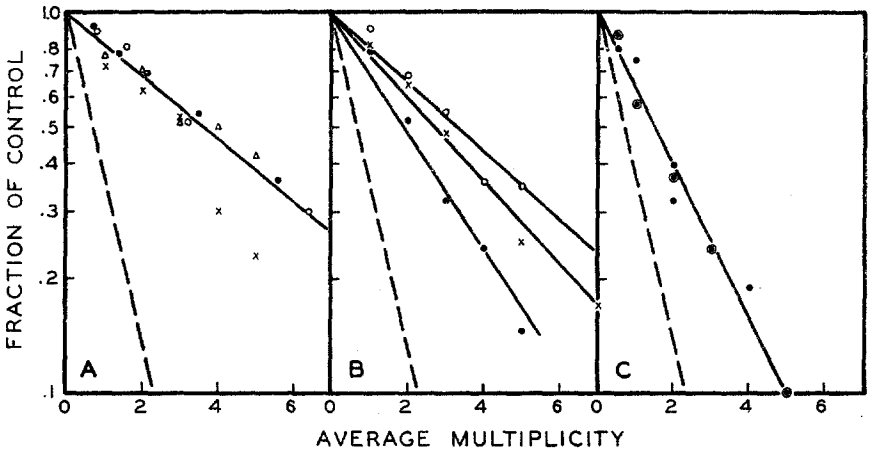

FIG. 9. The action of phage T2 on *E. coli* B under various conditions.
In all instances the broken line is the theoretical curve (slope = 0.434).
A. Lysis of cells in buffered saline at 37°C.
B. Lysis of cells in buffered saline + 0.001 M NaCN at 37°C.
C. Killing of cells at 0°C. in nutrient broth.

Experimental Procedure (Fig. 9)

A. Coli B were grown in nutrient broth to 1.5×10^8/ml. at 37°C. after which they were chilled, centrifuged, washed once with saline-$\frac{M}{50}$ phosphate buffer at pH 7.2, and resuspended in half the original volume of buffered saline. To a series of tubes containing 10 ml. of these cells was added 1 ml. of saline or T2 phage of varying quantities. These tubes were read in a Coleman Jr. spectrophotometer at 650 mμ immediately and after 60 minutes of agitation at 37°C. After subtracting a blank of 10 per cent for the residual turbidity after complete hydrolysis the fraction of residual cells was calculated directly from the turbidity changes and plotted against the average multiplicity.

B. The procedure was the same as in *A* except that the resuspending buffer-saline was made 0.001 molar with respect to sodium cyanide.

C. Coli B were grown in nutrient broth to a concentration of 3×10^8/ml. at 37°C. The suspension was then chilled rapidly in ice water and 1 ml. aliquots pipetted into a series of cold tubes. To each was added 0.5 ml. of cold nutrient broth or nutrient broth dilutions of T2 phage. These tubes were maintained at 0°C. for one-half hour after which 1.5 ml. of 1:100 anti-T2 sera was added and the tube incubated at 37°C. for 10 minutes. These suspensions were then diluted and the viable cell count determined from the colony formation on nutrient agar plates. Infective centers were also determined.

DISCUSSION

The particular aspects of the present work have been so thoroughly discussed in the separate sections of the paper that only certain broader issues will be considered here.

In only a few instances have the biological functions of any virus been assignable to particular chemical constituents. Thus, in vaccinia virus the hemagglutinin was found to be a phospholecithin-like compound (59) whereas the nature of the soluble antigen was nucleoprotein (60). The very important discoveries reported by Gierer and Schramm (64) and by Fraenkel-Conrat and Williams (61) establish that the nucleic acid (RNA) of tobacco mosaic virus is infective in the absence of its protein coat. The TMV protein apparently protects the nucleic acid from destructive insults which it would normally encounter in nature.

In the present study many biological properties of phage are also found in the isolated protein component. These properties are so similar in the two units that the conclusion is virtually inescapable that these functions of the phage are attributable to the protein component. However, Hershey's ingenious blendor experiment (5, 6) has provided some information which must be compatible with the present results if the over-all picture is to be clearly understood. Hershey found that within a few minutes after infection and without reducing the infectivity of the cells, 80 per cent of the phage protein (as measured by S^{35} content) could be stripped from the infected cells by stirring violently in a Waring blendor at low ionic strength. The consequences of this interesting work will be considered in some detail for it has been suggested that the protein may have no function other than to deliver the phage DNA to the host cell.

There are three possible interpretations which can be placed on the results of the blendor experiment:

Case I.—The 15 to 20 per cent residual or "non-strippable" protein represents the tip end of the tail of all the ghosts, and this is the part which modifies the cell's activity.

Case II.—The entire ghost has been removed from 80 per cent of the infected cells. The other 20 per cent carry intact ghosts.

Case III.—A small active unit, perhaps less than 1 per cent of the ghost,

is the functional unit and it remains in the cell after the 99 per cent is removed by the Waring blendor.

In case I it is necessary to attribute to the tip end of the tail some of the properties of the ghost. This presents no difficulty. In case II, however, it would be necessary to assume either that the action of the ghost involves initiation of changes in the cell that are self-sustaining after removal of the ghost, or that the effects produced by ghosts are in no way related to phage synthesis. If the last suggestion is correct, it might be possible to infect a protoplast directly with only the phage DNA.[3]

Case III is not essentially different from case I. It merely assigns the biological functions of the ghost to a lower order of unit and makes experimental confirmation more difficult.

Theoretically, cases I and II should be distinguishable by radioautographs of cells following adsorption of labelled ghosts and then treatment in the blendor. Regardless of which interpretation is correct, it will indeed be surprising if such properties of the ghost as the inhibition of PNA synthesis are fortuitous and utterly dissociated from phage synthesis.

The outstanding biological feature of the parent phage which is apparently lacking in the ghost is the replicating capacity. Anderson (62) was the first to point this out when he observed that osmotic shock destroyed the plaque-forming unit. In a later paper (63) it will be demonstrated that neither phage protein nor nucleotides containing hydroxymethylcytosine are formed in cells modified by ghosts. The present report also includes the finding that ghosts do not appear to participate in formation of recombinants. While these results are not decisive because they are negative, nevertheless they are in agreement with the concept that the genetic information is carried by the phage DNA.

The present studies with ghosts have the virtue of having been performed (or could have been) in the absence of phage nucleic acid. The reverse, the action of the phage nucleic acid in the complete absence of the phage protein would appear difficult to achieve and has not been reported at the present writing.[3]

The writers are indebted to Dr. I. R. Lehman, Dr. Helen Van Vunakis, and Dr. Sol H. Goodgal for their helpful discussions of the work and to Patsy Miller Perlman, Mary T. Dunn, and Bernard C. Jenkins for their technical assistance.

REFERENCES

1. Herriott, R. M., *J. Bact.*, 1951, **61**, 752.
2. Herriott, R. M., and Barlow, J. L., *J. Gen. Physiol.*, 1957, **40**, 809.

[3] Spizizen's interesting and important article (65) does not entirely clarify the situation.

3. Puck, T. T., Garen, A., and Kline, J., *J. Exp. Med.*, 1951, **93,** 65.
4. Anderson, T. F., *Cold Spring Harbor Symp. Quant. Biol.*, 1953, **18,** 197.
5. Hershey, A. D., and Chase, M., *J. Gen. Physiol.*, 1952, **36,** 39.
6. Hershey, A. D., *Virology*, 1955, **1,** 108.
7. French, R. C., and Siminovitch, L., *Canad. J. Microbiol.*, 1955, **1,** 757.
8. Bonifas, V., and Kellenberger, E., *Biochim. et Biophysic. Acta*, 1955, **16,** 330.
9. Luria, S. E., *Proc. Nat. Acad. Sc.*, 1947, **33,** 253.
10. Watson, J. D., *J. Bact.*, 1950, **60,** 697.
11. Lea, D. E., Actions of Radiations on Living Cells, New York, Macmillan Co., 1947.
12. Barlow, J. L., Thesis on The Effect of Divalent Cations on the Interaction of T2 Phage and T2 Ghosts with Host Cells, The Johns Hopkins School of Hygiene and Public Health, 1954. Available on loan from Welch Medical Library, Baltimore.
13. Anderson, T. F., Rappaport, C., and Muscatine, N. A., "Le Bacteriophage," Royaumont, *Ann. Inst. Pasteur*, 1952, **84,** 5.
14. Van Vunakis, H., see reference 2.
15. Siminovitch, L., "Le Bacteriophage," Royaumont, *Ann. Inst. Pasteur*, 1952, **84,** 265.
16. Lehman, I. R., and Herriott, R. M., data to be published.
17. Krueger, A. P., and Northrop, J. H., *J. Gen. Physiol.*, 1930, **14,** 223.
18. Delbrück, M., *J. Gen. Physiol.*, 1940, **23,** 643.
19. Doermann, A. H., *J. Gen. Physiol.*, 1952, **35,** 645.
20. Cohen, S. S., *Bact. Rev.*, 1949, **13,** 1.
21. Heagy, F. C., *J. Bact.*, 1950, **59,** 367.
22. Barlow, J. L., and Herriott, R. M., *Bact. Proc.*, 1954, 46.
23. Puck, T. T., The Dynamics of Virus and Rickettsial Infections, (F. W. Hartman, F. L. Horsfall, Jr., and J. G. Kidd, editors), Philadelphia, The Blakiston Co., 1954, 6.
24. Anderson, T. F., *J. Cell. and Comp. Physiol.*, 1945, **25,** 1.
25. Ralston, D. J., Baer, B. S., Lieberman, M., and Krueger, A. P., *Proc. Soc. Exp. Biol. and Med.*, 1955, **89,** 502.
26. Bronfenbrenner, J., *Science*, 1952, **116,** 517.
27. Cohen, S. S., *J. Biol. Chem.*, 1948, **174,** 295.
28. Manson, L., *J. Bact.*, 1953, **66,** 703.
29. Lehman, I. R., and Herriott, R. M., *Fed. Proc.*, 1954, **13,** 249.
30. Lehman, I. R., Doctoral Dissertation on Metabolic Studies of *Escherichia coli* B Infected with T2 Bacteriophage—Ghosts, The Johns Hopkins School of Hygiene and Public Health, 1954. Available on loan from the Welch Medical Library, Baltimore.
31. Schmidt, G., and Thannhauser, J. J., *J. Biol. Chem.*, 1954, **161,** 83.
32. Monod, J., and Wollman, E., *Ann Inst. Pasteur*, 1947, **73,** 937.
33. Benzer, S., *Biochim. et Biophysic. Acta*, 1953, **11,** 383.
34. Sher, I. H., and Mallette, M. F., *Arch. Biochem. and Biophysic.*, 1954, **52,** 331.
35. Murray, R. G. E., Gillen, D. H., and Heagy, F. C., *J. Bact.*, 1950, **59,** 603.

36. Prater, C. D., Dissertation presented to the Faculty of the Graduate School of the University of Pennsylvania, Philadelphia, 1951.
37. Price, W. H., *J. Gen. Physiol.*, 1952, **35,** 409.
38. Puck, T. T., and Lee, H. H., *J. Exp. Med.*, 1955, **101,** 151.
39. Lehman, I. R., and Herriott, R. M., data to be published.
40. Luria, S. E., and Human, M., *J. Bact.*, 1950, **59,** 551.
41. Mudd, S., Hillier, J., Beutner, E. H., and Hartman, P. E., *Biochim. et Biophysic. Acta*, 1953, **10,** 153.
42. Hoskins, M., *Am. J. Trop. Med.*, 1935, **15,** 675.
43. Breinl, F., *J. Infect. Dis.*, 1928, **42,** 48.
44. Price, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1953, **82,** 180.
45. Luria, S. E., and Delbrück, M., *Arch. Biochem.*, 1943, **1,** 207.
46. Hershey, A. D., and Bronfenbrenner, J., Viral and Rickettsial Infections of Man, (T. M. Rivers, editor), New York, J. B. Lippincott Co., 1952.
47. Dulbecco, R., *J. Bact.*, 1952, **63,** 209.
48. French, R. C., Graham, A. F., and Van Rooyen, C. E., *J. Bact.*, 1952, **64,** 597.
49. Benzer, S., *J. Bact.*, 1952, **63,** 59.
50. Doermann, A. H., *J. Bact.*, 1948, **55,** 257.
51. Lederberg, E. M., and Lederberg, J., *Genetics*, 1953, **38,** 51.
52. Benzer, S., *Proc. Nat. Acad. Sc.*, 1955, **41,** 344.
53. French, R. C., *J. Bact.*, 1954, **67,** 45.
54. Tanami, Y., and Miyajima, Y., *J. Bact.*, 1956, **72,** 721.
55. Schlesinger, M., *Z. Hyg. u. Infektionskrankh.*, 1932, **114,** 149.
56. Puck, T. T., *Cold Spring Harbor Symp. Quant. Biol.*, 1953, **18,** 149.
57. Gross, S. R., *J. Bact.*, 1954, **68,** 36.
58. Adams, M., Dynamics of Virus and Rickettsial Infections, (F. W. Hartman, F. L. Horsfall, Jr., and J. G. Kidd, editors), Philadelphia, The Blakiston Co., Inc., 1954, 86.
59. Burnet, F. M., and Stone, J. D., *Australian J. Exp. Biol. and Med. Sc.*, 1946, **24,** 1, 191, 197.
60. Shedlovsky, T., and Smadel, J. E., *J. Exp. Med.*, 1942, **75,** 165.
61. Fraenkel-Conrat, H., and Williams, R., A Symposium on The Chemical Basis of Heredity, (W. D. McElroy and H. B. Glass, editors), Baltimore, The Johns Hopkins Press, 1957.
62. Anderson, T. F., *J. Appl. Physics*, 1950, **21,** 70.
63. Herriott, R. M., data to be published.
64. Gierer, A., and Schramm, G., *Nature*, 1956, **177,** 702.
65. Spizizen, J., *Proc. Nat. Acad. Sc.*, 1957, **43,** 694.