BACTERIOPHAGES

MARK H. ADAMS

WITH CHAPTERS BY

E. S. ANDERSON, Central Enteric Reference Laboratory,
Central Public Health Laboratory, London

J. S. GOTS, University of Pennsylvania, Philadelphia

F. JACOB, Pasteur Institute, Paris

E.-L. WOLLMAN, Pasteur Institute, Paris

ELECTRON MICROGRAPHS BY

E. KELLENBERGER, The University, Geneva

INTERSCIENCE PUBLISHERS

a division of John Wiley & Sons, Inc., New York • London • Sydney
CHAPTER VIII

ANTIGENIC PROPERTIES

Early studies on the antigenic properties of bacteriophages have been reviewed by d’Herelle (1926) and Burnet, Keogh, and Lush (1937). The latter reference gives an excellent discussion of the mechanism of the antigen-antibody reaction from the kinetic viewpoint and, in addition, contains much experimental material which has not been published elsewhere.

Bordet and Ciuca (1921) first demonstrated that injection of rabbits with phage lysates stimulates the production of phage-neutralizing antibody. Their antisera also contained agglutinins for the host bacterium. The injection of host bacteria, however, failed to stimulate the production of phage-neutralizing antibody. Otto and Winkler-(1922) were able to remove all bacterial agglutinins by absorption with host bacteria without affecting the antiphage properties of the serum. These findings demonstrated that phage is antigenically distinct from its host.

It was early recognized that antiphage sera had considerable specificity, inactivating the homologous and some heterologous strains of phage but not others. The fact that serological relationship was correlated with other properties of bacteriophages was not generally appreciated, however, until the taxonomic work of Burnet and Asheshov.

Since these early experiments it has been found that the activity of antiphage antibodies is not confined to neutralization. The addition of a sufficiently concentrated suspension of phage particles to homologous antiserum results in visible aggregation of the virus particles followed by settling of the clumps as a precipitate. By suitable ultrafiltration methods one can sep-
arate from phage lysates soluble substances possessing the antigenic specificity of the phage particles. When antiphage antibody is allowed to react with such substances it loses its ability to neutralize phage. Some, if not all, phage-antiphage systems fix complement. These and other properties of antiphage sera will now be discussed in more detail.

1. Preparation of Antiphage Sera

Rabbits are the most convenient animals to use for antibody production, and most of the serological work with phage has been done with rabbit sera. The phages themselves are non-toxic and nonpathogenic for animals and can usually be injected in large amounts without damage to the recipient. Crude phage lysates, however, may contain toxic bacterial substances, such as the endotoxins of the enteric bacteria and the much more potent exotoxins of the corynebacteria and clostridia. In such cases the toxins must first be removed by fractionation or inactivated. For many purposes it is desirable to purify the phages before using them for immunization, since adequate purification will result in the absence of host-cell antibodies from the resultant antisera (Kalmanson, Hershey, and Bronfenbrenner, 1942). If crude phage preparations are used it may be necessary to remove host-cell antibodies by absorption with bacterial suspensions. This is essential, for instance, in studies involving complement fixation.

Stimulation of antibody production requires the administration of adequate quantities of antigenic material. The daily injection of about $10^{10}$ virus particles (about 10 μg.) for five days will usually result in the presence of considerable neutralizing antibody in serum obtained one week after the last injection. Repetition of this course of immunization will further increase the antibody titer. The final result does not seem to depend greatly on the route (intravenous, intraperitoneal, or subcutaneous) by which antigen is administered. Use of the subcutaneous route, however, is least likely to lead to toxic or anaphylactic reactions. Since there are large differences in anti-
ANTIGENIC PROPERTIES

body response from one rabbit to another, it may be desirable to immunize at least three rabbits with the same phage preparation. Judged by the neutralization test, antisera prepared against certain phages, such as T2, T3, T4, T6, and T7, usually have very high homologous antibody titers in comparison with antisera against others, such as T1 and T5, which may be relatively weak. Such differences do not necessarily reflect corresponding differences in antibody concentration, since the rate of neutralization may easily depend on other factors as well. Actually, a very few determinations have been made of the absolute antibody content of antiphage sera.

The effect of adjuvants on phage antigenicity has been very little studied, probably because most phages are already excellent antigens. Wahl and Lewi (1939) obtained a marked improvement in the antigenicity of a low-titer B. subtilis phage by adsorption to alumina. Intraperitoneal injection of $3 \times 10^8$ particles of unadsorbed phage gave little or no antibody response. Injection of the same amount of absorbed phage resulted two weeks later in an antiserum giving 90 per cent inactivation at a dilution of $1/50$. The titer increased to $1/2000$ in a month. This technique may be useful to workers dealing with low-titer temperate phages.

Phages retain antigenicity, in some instances without appreciable loss, following careful inactivation of their infectivity by heat, formaldehyde, ultraviolet irradiation (Muckenfuss, 1928; Schultz, Quigley, and Bullock, 1929; Nagano and Takeuti, 1951; Pollard and Setlow, 1953; Miller and Goebel, 1954); phenol, photodynamic action of methylene blue (Burnet, Keogh, and Lush, 1937); osmotic shock, and sonic vibration (F. Lanni and Lanni, 1953; Nagano and Oda, 1954). Phage which has been "overneutralized" by homologous antibody is, however, nonantigenic in guinea pigs, even after treatment with papain (Kalmanson and Bronfenbrenner, 1943; Nagano and Takeuti, 1951).

The antigenicity of premature lysates of a staphylococcal phage has been studied by Rountree (1952); of purified T2
"doughnuts" by Lanni and Lanni (1953); of proflavine lysates of T4, and of T4- and T3-infected bacteria by Barry (1954); and of infected and lysogenic B. megaterium by Miller and Goebel (1954). The antigenicity of ultrafiltrable phage-related materials is discussed below.

2. Multiplicity of Phage Antigens

Until a few years ago it was believed, simply because there was no contradictory evidence, that each strain of phage possessed a single kind of antigen. Accordingly, all of the specific effects of antiphage serum were interpreted in terms of the reaction of a single antibody (neutralizing antibody) with the virus particles. In an extensive study, Lanni and Lanni (1953) (see also Rountree, 1952; De Mars, Luria, Fisher, and Lenthal, 1953) presented clear evidence for the existence of at least two distinct antigens in phage T2. One of the antigens, which appears to be localized in the phage tail, reacts with neutralizing antibody. The primary measurable effect of this reaction is neutralization of infectivity; under appropriate conditions virus aggregation and complement fixation can also be demonstrated. The second antigen, which appears to be localized in the phage head, participates in specific aggregation and complement fixation, but does not react measurably with neutralizing antibody. The two antigens are confined to the surface structures (protein) of the virus; thus far, the internal contents, liberated by osmotic shock, have given no sign of serological activity (see also Hershey and Chase, 1952; Hershey, 1955).

The available evidence indicates that staphylococcal phage 3A (Rountree, 1952) and phage T5 (Fodor and Adams, 1955) likewise possess two distinct antigens, only one of which reacts with neutralizing antibody. Evidence bearing on the serological heterogeneity of the phage tail will be discussed in later sections.

The failure until recently to recognize the antigenic complexity of phage attaches considerable doubt to many of the structural interpretations given to early serological observations.
3. Neutralization of Phage Infectivity

When a phage preparation is mixed with homologous antiserum there is a progressive decrease in the number of plaque-forming particles. The inactivation process can be interrupted at any time by diluting the phage-antibody mixture below the antibody concentration at which collisions between the reactants occur at a significant rate. Following this dilution there is, in general, no detectable reversal of the inactivation process; the titer of surviving phage does not increase. Apparently, dissociation of the phage-antibody complex does not occur at a measurable rate (Burnet, Keogh, and Lush, 1937; Hershey, 1943; see, however, Andrewes and Elford, 1933b; Jerne and Avegno, 1956). Attempts to facilitate dissociation by the addition of formalin-inactivated phage to bind the antibody were unsuccessful (Hershey, 1943). The addition of soluble phage antigen, however, appeared to have a slight reactivating effect (Burnet, 1933b). Although in most instances antibody does not appear to dissociate from the phage particles spontaneously, there is evidence that it can be removed by certain methods. Kalmanson and Bronfenbrenner (1943) found that neutralized phage could be completely reactivated by digestion of the antibody with papain providing the antibody had not been too concentrated or the serum treatment too prolonged. Apparently, if a sufficiently large number of antibody molecules had reacted with the phage particle, the inactivation became irreversible. Anderson and Doermann (1952b) succeeded in obtaining more than a 30-fold increase in titer of a neutralized preparation of phage T3 by subjecting it to sonic vibration.

In the cited reactivation experiments with papain and sonic vibration the concentration of phage during preliminary treatment with antiserum was sufficiently high in most instances as to admit the possibility that part, at least, of the plaque-count decrease was due to specific aggregation. It is not certain, therefore, to what extent the observed reactivation can be ascribed to reversal of neutralization, as opposed to disaggregation of virus clumps. However, Hershey (1943) found that
papain can reactivate phage which has been treated with serum under conditions minimizing the possibility of specific aggregation. These successful attempts to reactivate neutralized phage particles indicate that antibody does not kill or destroy phage particles. Presumably, it interferes with some step in the interaction of the intact virus particle with the host cell. The nature of this interference will be discussed in a later section.

From the foregoing discussion, it is clear that neutralizing antibody acts on the phage particles themselves, rather than on the host cell. Pretreatment of host cells with phage antibodies has no effect on the infectious process. Delbrück (1945b) found that, following phage adsorption to the host cell, the infected bacteria are immune to antiphage serum throughout the latent period (see, however, Lieb, 1953; Gross, 1954a; Nagano and Mutai, 1954a). The resistance of infected bacteria to antiphage serum is of great importance in phage technology because it permits the inactivation of unadsorbed phage without affecting the multiplication of phage which is already adsorbed.

Although complement is not required for specific neutralization, it can modify the neutralization of T1 and T2 (Hershey and Bronfenbrenner, 1952). Complement and properdin together, but not separately, inactivate T2 in the absence of specific antibody (Van Vunakis, Barlow, and Levine, 1956).

4. Properties of Incompletely Neutralized Phage Preparations

Following the treatment of phage preparations with antiserum, the surviving infectious particles are not normal. Andrews and Elford (1933b) reported that the survivors of antibody treatment produced smaller plaques than normal. They interpreted this as indicating a delay in the initiation of the infectious process. The surviving particles also failed to pass through bacteriological filters and collodion membranes which were freely permeable to the same phage particles prior to serum treatment. This might be ascribed to increase of particle size or change of surface charge due to an antibody coating,
or to aggregation of phage particles. Frequency distribution curves for plaque sizes of phage Cl6 before and after treatment with antiserum are given by Burnet, Keogh, and Lush (1937). The distribution for the incompletely neutralized preparation was bimodal with one maximum at less than \( \frac{1}{3} \) the peak plaque diameter of the unimodal curve for normal phage particles. Surprisingly, Delbrück (1945b) failed to find any difference between normal T2 phage and serum-survivors at the 1 per cent level with respect to adsorption, latent period, or burst size (cf. Adams and Wassermann, 1956). The reason for the discrepant observations with these closely related phages is not obvious.

Gough and Burnet (1934) extracted from suspensions of the host bacterium a phage-inhibiting agent (PIA) which was able to inactivate phage particles in much the same manner as antiphage sera. They regarded the extract as a solution of the bacterial receptors for phage adsorption. Burnet and Freeman (1937) investigated the effect of PIA on phage preparations which had been inactivated to the extent of 90 per cent with dilute antiphage sera. The survivors of serum inactivation proved to be very resistant to the inhibiting action of PIA whereas the untreated phage population was 99 per cent inactivated by PIA. This is further evidence that phage antibody can modify the properties of phage particles without inactivating them. It also suggests that the isolated PIA is not identical to the receptor substance on the bacterial surface, since the survivors of the serum action are still able to infect the host cell although they are resistant to the action of PIA.

Another very striking property of partially neutralized phage preparations was demonstrated by Hershey, Kalman, and Bronfenbrenner (1944) using a variant of phage T2. The variant adsorbed very poorly to bacteria in a salt-free medium and had a relative efficiency of plating of \( 10^{-4} \). In a medium containing 0.1 \( N \) NaCl the phage adsorbed well and the efficiency of plating was about half of that under optimal conditions. The phage was treated with very dilute antiphage
BACTERIOPHAGES

serum (dilution $10^{-5}$) to a survival of about 0.2. The plaque count in a salt-free medium of the survivors of serum treatment was increased 1,000-fold as compared with that of the untreated phage preparation. Treatment with a small amount of antiserum had the same effect on phage infectivity as an increase in salt concentration. It is probable that these effects of salt and of antiserum on phage infectivity are due to effects on electrostatic charge (see Cann and Clark, 1955), although this explanation was discarded by the authors. This problem will be discussed further in the chapter on phage adsorption.

It is not certain at present which of the several antibodies in antiphage serum is responsible for the effects described above. A peculiar antibody which stabilizes the activated state of a tryptophan-requiring strain of T4 has recently been described (Jerne, 1956).

5. Complement Fixation

Early results (d'Herelle, 1926) left considerable doubt as to the ability of phage-antiphage systems to fix complement. These doubts have been dispelled in recent years by studies showing not only that such systems do, in fact, fix complement but also that complement fixation offers a valuable quantitative tool for the study of those phage antigens and phage-related materials that do not react with neutralizing antibody. An example is the work of Lanni and Lanni (1953) in their demonstration of a serological relationship between T2 doughnuts and T2 phage and their use of antidoughnut sera for studying the structure of the infective virus particle. The scope of the procedure is not restricted, however, to such antigens.

Other recent studies illustrate further the broad applicability of complement fixation to problems of phage structure and growth. In confirmation of the discovery of Hershey and Chase (1952) with T2, Lanni (1954) showed that the bulk of the complement-fixing antigens of infecting particles of T5 remained outside the infected host cells. Previous work had suggested instead that the complement-fixing antigens of T5 (Rountree,
1951b) and of staphylococcal phage 3A (Rountree, 1952) disappeared during the early stages of infection. Both workers observed that newly synthesized complement-fixing antigens appeared and increased intracellularly a few minutes before mature virus. Miller and Goebel (1954) injected lysogenic cells of *B. megaterium* into rabbits and failed to demonstrate the production of phage-specific, complement-fixing, or neutralizing antibodies. Since nucleic acids have not, in general, been found antigenic, this negative result, which extended an earlier failure by F. Lanni (cited by Lwoff, 1953), accords with the current conception that prophage consists of DNA. Fodor and Adams (1955) used complement fixation in analyzing the antigenic relationship of T5, the related phage PB, and T5 × PB hybrids. Details of a photometric complement titration are given by Lanni (1954).

6. Specific Aggregation

Schlesinger (1933a) observed the aggregation of phage WLL particles by antiphage serum using the dark field microscope. Burnet (1933c) demonstrated that phage C16 gave a visible precipitate when mixed with antiserum. Photographs of the aggregated phage particles, taken by Barnard with the ultraviolet microscope, are included in Burnet's paper. A visible precipitate was formed when antiserum was mixed with phage C16 at all concentrations above about $2 \times 10^6$ particles per ml. For smaller virus particles a considerably higher concentration is needed to give a visible precipitate, since the amount of precipitate formed is dependent on the total mass of antigen added. The relationship between the size of an antigenic particle and the concentration of particles needed to give a visible precipitate has been discussed by Merrill (1936). This author also discussed the relationships between particle size and the minimal concentration required for complement fixation and for the stimulation of antibody production. These relationships should be of considerable value to virologists. Ignorance of them has been responsible for much wasted effort.
The phage-antibody system could be readily investigated by the techniques of the quantitative precipitation reaction, but the only attempt at such a study was made by Hershey, Kalmanson, and Bronfenbrenner (1943a) using coliphage T2. The authors concluded that the nitrogen of phage lysates which was specifically precipitable by phage antibodies corresponded to less than $10^{-13}$ mg. N per lytic unit. Chemical analyses of purified T2 phage preparations by various investigators agree with a figure for the nitrogen content of the lytic unit which is close to $10^{-13}$ mg. (Chapter VII). The amount of antibody nitrogen capable of precipitating with phage T2 was found to be 0.6 mg. per ml. for a serum with a $K$ value of about 360 minutes$^{-1}$. It would be of interest to know the ratio between antibody content and $K$ value for various other antiphage sera.

With sera of low neutralizing antibody content, it is possible to follow specific aggregation of phage by observing the consequent reduction in plaque count (Lanni and Lanni, 1953; Fodor and Adams, 1955).

7. Agglutination of Phage-Coated Bacteria

Individual cells of susceptible bacterial strains are able to adsorb a considerable number of phage particles. Delbrück (1940a) found that cells of *E. coli* became saturated after adsorption of from 20 to 250 phage particles depending on the physiological condition of the cells. Burnet (1933a) investigated the serological properties of such phage-coated cells using formalin-killed bacteria to avoid lysis. The formalinized bacteria were suspended in a high-titer phage stock for an hour, then centrifuged and washed to remove unadsorbed phage. These phage-coated bacteria were less susceptible than uncoated bacteria to agglutination by antibacterial sera, but were readily agglutinated by antiphage serum which had no effect on uncoated bacteria. The agglutination was phage-specific. Absorption of the serum with phage-coated bacteria depressed both the agglutinating and the phage-neutralizing activities. The result is interesting since it suggests that antigens capable
of binding neutralizing antibody exist elsewhere than at the tip of the phage tail. More work is needed, however, before this interpretation is accepted.

Burnet also performed cross-absorption experiments using serologically related phages. Absorption with bacteria coated with homologous phage reduced the neutralization titer for homologous and heterologous phages alike, whereas bacteria coated with heterologous phage absorbed antibody against this phage but had little effect on the titer against homologous phage. This type of experiment permits one to make an antigenic analysis of a series of related phages. Antiphage sera can also be absorbed with phage concentrates, the precipitated phage particles being removed by low-speed centrifugation (Hershey, Kalmanson, and Bronfenbrenner, 1943a, 1943b; Hershey, 1946a). An advantage of the use of phage-coated bacteria is that the adsorption of the phage particles to the bacteria serves to concentrate and purify them in one step. It should be kept in mind, however, that those phage antigens which are located at or near the site of adsorption to host cells may not be accessible to antibody.

8. Specific Soluble Substances

The first evidence for a soluble substance having the antigenic specificity of phage particles was obtained by Asheshov (1925). He filtered a phage preparation through a collodion membrane of a pore size small enough to retain the phage particles. The immunization of rabbits with the phage-free filtrate resulted in the production of specific phage-neutralizing antibody. Burnet (1933b) prepared sterile ultrafiltrates of high-titer stocks of coliphage C16 using Gradocol membranes of a suitable permeability to retain all the phage particles. The ultrafiltrates immunized rabbits with the production of neutralizing antibody for phage C16. The ability of the ultrafiltrate factor to react with antibody was most conveniently detected by mixing it with antiphage C16 serum and observing a decrease or blocking
of the phage-neutralizing activity of the serum. The antibody-blocking effect showed the serological specificity of phage C16. The ultrafiltrate factor was not adsorbed by phage-sensitive bacteria and had no bacteriolytic activity. It was relatively heat resistant, requiring treatment at 90 °C for 30 minutes for inactivation. On this basis Burnet suggested that it might be a polysaccharide.

DeMars, Luria, Fisher, and Levinthal (1953) demonstrated the presence of similar specific soluble substances in bacteria infected with coliphages T2, T4, or T5. The substances were liberated from the infected bacteria during the latent period by premature lysis, and were separated from phage particles and bacteria by ultrafiltration through collodion filters. The quantity of ultrafiltrable specific antigen increased during the latent period. What is probably the same type of substance was liberated from bacteria infected with phage T2 or T6, in which normal phage development had been prevented by the addition of proflavine (see also DeMars, 1955; Barry, 1954). The ultrafiltrable fraction had the same serological specificity as the infecting phage. At equivalent antibody-blocking activity the ultrafiltrable fraction from T2 lysates had roughly the same complement-fixing activity as intact virus (Lanni and Lanni, 1953).

Crude T2 lysates contain a second noninfectious antibody-blocking component, which appears to be intermediate in size between the ultrafiltrable component and the infectious virus particle (Lanni and Lanni, 1953; DeMars, 1955). The latter reference should be consulted for details of the measurement of antibody-blocking agents and for a description of their intracellular development. Actually, the antibody-blocking measurement may be regarded as an abbreviated absorption experiment, in which the absorbing agent and its attached antibody are allowed to remain in the system during subsequent measurement of residual neutralizing activity.

Lanni (1954) has found that at least one-half of the total phage-specific complement-fixing activity of T5 lysates is
ANTIGENIC PROPERTIES

associated with a noninfectious fraction which sediments more slowly than the virus.

Efforts to isolate phage antigens by degradation of the virus particle have met with partial success (Lanni and Lanni, 1953; DeMars, 1955; see also Hershey, 1955).

9. Kinetics of Neutralization

It had been recognized since the work of Prausnitz (1922) that the neutralization of phage activity by antiserum was a relatively slow process, and that a small proportion of the phage population resisted inactivation by antibody. There seems to have been no further attempt to study the kinetics of phage neutralization until the work of Andrewes and Elford (1933a). These authors systematically varied the concentrations of antiserum and of phage in the reaction mixture, and determined the decrease in the number of infectious phage particles as a function of time. The “percentage law” is a summary of their experimental results: “over a very wide range a given dilution of serum neutralized in a given time an approximately constant percentage of phage, however much phage there was present.” This relationship held from concentrations of a few hundred phage particles per ml. up to at least $10^8$ per ml., and for serum concentrations from $10^{-4}$ to undiluted. Although this relationship between phage and antibody is taken for granted by phage workers today, it was very startling at the time of its discovery, and its implications are still not understood or appreciated by many immunologists and virologists.

Burnet, Keogh, and Lush (1937) presented kinetic measurements for the inactivation of various phages by antiserum. In most cases the proportion of active virus decreased logarithmically with time after a slight initial lag. The rate of inactivation was directly proportional to the concentration of antiserum. The inactivation was assumed to be the result of a collision between a phage particle and an antibody molecule. Since the reaction is usually carried out in the presence of a large excess of antibody, phage neutralization does not decrease
the concentration of antibody perceptibly. Therefore, the kinetics is that of a first-order reaction, represented by the equation:

\[-\frac{dP}{dt} = \frac{KP}{D}\]

Integration gives the very useful result:

\[K = \frac{2.3D}{\log_{10} \left(\frac{P_0}{P}\right)}\]

in which \(K\) is the velocity constant with the dimension \(\text{minute}^{-1}\), \(D\) is the dilution of serum (100 for serum diluted 1/100, etc.), \(t\) is the time in minutes, \(P_0\) is the phage assay at zero time, and \(P\) is the phage assay at time \(t\).

Burnet found that the kinetics of neutralization of phage C16 agreed satisfactorily with this equation at serum concentrations from \(\frac{1}{20}\) to \(\frac{1}{30,000}\). Phage neutralization was logarithmic to an inactivation of more than 99 per cent, but then the rate slowed, the survivors appearing to be more resistant. Most of the phages studied by Burnet behaved in this manner, including a streptococcal phage and a staphylococcal phage as well as various immunological types of enterophages. However, he noted that the neutralization of coliphages in serological group 3 deviated markedly from a first-order reaction, and that a relatively large proportion of the phage population was resistant to inactivation by even high concentrations of antiserum. Delbrück (1945b) found that the neutralization of T2 and T7 obeyed the equations above, but that neutralization of T1 did not. The rate of inactivation of T1 decreased progressively throughout the course of the reaction. Similar anomalous behavior has been observed with phages related to T5 (Adams, 1952), with the M phages of \(B.\ megaterium\) (Friedman and Cowles, 1953), and with D20, a relative of T1 (Adams and Wade, 1955).

The value of \(K\), the velocity constant, is a characteristic of a particular lot of serum and may vary from one rabbit to another or from one bleeding to another from the same animal. The \(K\) value is a convenient measure of the neutralizing potency of
ANTIGENIC PROPERTIES

a serum, a high $K$ value indicating a high titer. Once the $K$ value of a sample has been determined, it can be substituted in the equation and used to calculate the dilution, $D$, of this serum required to inactivate any desired fraction of the phage in any given time. The range of inactivation over which the equation is applicable must be determined by experiment. For most purposes an inactivation of 90 to 99 per cent is adequate and within the range of first-order kinetics.

Any phage-antibody system which follows first-order kinetics will necessarily obey the percentage law of Andrewes and Elford. The law is more general, however, since it applies also to phages in serological group 3, which do not follow first-order kinetics.

Hershey, Kalmanson, and Bronfenbrenner (1943a) found that the inactivation of phage T2 by antiserum followed first-order kinetics, the $K$ value being independent of $D$, $t$, and $P_0$ up to a concentration of about $10^9$ phage particles per ml. Above this concentration, aggregation of phage particles by antiserum resulted in an increased $K$ value.

Kalmanson, Hershey, and Bronfenbrenner (1942) studied some of the variables influencing the rate of neutralization of phage T2 by antibody. A particularly careful study of the reaction using highly diluted antiserum confirmed the initial lag in the neutralization that had been noted by Burnet. Analysis of the convexity of the inactivation curve suggested that between 2 and 3 antibody molecules must react with each phage particle on the average for neutralization to occur. Neutralization of T2 by antibody thus appeared to be a multi-hit process. However, it is now known (Sagik, 1954) that a large fraction of the phage particles in certain stocks of T2 are unable to form plaques when plated directly, but become infective during treatment with antiserum. The inhibited particles may also be activated in other ways, for example, by heating or by lowering the salt concentration. Activated stocks showed no sign of deviation from first-order neutralization kinetics (Cann and Clark, 1954). Since allowance must be made for genetic differences in the lines of T2 employed by
different workers and for differences in antisera, these findings do not necessarily explain all examples of multi-hit neutralization kinetics (see Park, 1956).

The temperature coefficient for the velocity constant $K$ was determined by Burnet, Keogh, and Lush (1937) for phage Cl6. The $K$ value at 0, 37, and 45° C. was 45, 930, and 1350 minutes$^{-1}$, respectively, the $Q_{10}$ thus being about 2.1. Similar studies were made by Kalmanson, Hershey, and Bronfenbrenner (1942) with the serologically related phage T2. The $K$ value was found to be 0.05 seconds$^{-1}$ at 0° C. and 0.7 seconds$^{-1}$ at 37° C., corresponding to a $Q_{10}$ of about 2. The Arrhenius constant was determined by Hershey (1941) to be about 11,000 cal. per mole. The meaning of these estimates has been placed in doubt by the finding of Cann and Clark (1954) that the neutralization of activated T2 (see above) shows a $Q_{10}$ of only 1.4, suggesting that the process is limited solely by diffusion. However, their measurements, unlike earlier ones, were made using media of low salt concentration.

Essentially all serological work with viruses has been done with antiserum diluted either in physiological saline or in broth containing added salt because such diluents are conventional in serology. Recently Jerne (1952) and Jerne and Skovsted (1953) reported that physiological diluents actually inhibited phage neutralization, monovalent cations above $10^{-2}$ M and divalent cations above $10^{-3}$ M being inhibitory. Optimum rates of inactivation were obtained in $10^{-3}$ M NaCl containing a few $\mu$g. per ml. of certain proteins which appeared to act as cofactors. Highly diluted normal serum, egg white, and crystalline lysozyme were effective as cofactors. An anti-T4 serum with a $K$ value of 600 minute$^{-1}$ in broth gave under optimal conditions a $K$ value of 100,000 or more. The kinetics were first order to a phage survival of $10^{-3}$ to $10^{-4}$. It has long been recognized that salt concentration has a marked effect on reactions involving colloidal particles because of surface charges, but no one had anticipated such an astonishing effect on the rate of the antigen-antibody reaction. Additional
data and discussion of these effects are given by Cann and Clark (1954, 1955).

Hershey (1941) discussed the absolute rate of the phage-antiphage reaction, giving theoretical equations from which the maximum rate could be calculated. The calculations and some of the discussion in this paper are incorrect because of erroneous concepts of the nature and size of phage T2 current at that time. However, recalculation using Hershey’s methods and more modern data indicates that the actual inactivation rates observed by Jerne are of the same order of magnitude as the maximum rate attainable assuming that every collision resulted in antibody attachment. Control of the ionic environment and the use of cofactors in Jerne’s experiments resulted in increasing the number of effective collisions between antibody molecules and susceptible sites on the phage particle to nearly the theoretical limit.

A theoretical analysis of the neutralization of T4 is given by Mandell (1955).

10. Anomalous Neutralization Behavior

A fact which puzzled Andrewes and Elford (1933a) was that neutralization of their phages proceeded rapidly until 90 to 99 per cent of the phage was inactivated and then slowed down rather abruptly, the residual phage activity appearing to be very resistant to neutralization. They demonstrated that this could not be due to exhaustion of antibody and hence must be due to inhomogeneity of the phage population with respect to susceptibility to inactivation. That this inhomogeneity was not genetic in origin was demonstrated by subculturing plaques of phage particles which had survived treatment with antiserum. The descendants proved to be normally susceptible to neutralization by antiserum.

The possibility that the inhomogeneity was caused by the action of antiserum itself, combining with the phage particles in such a way as to make them resistant to neutralization, was next considered. This possibility was discarded because the
resistant survivors of the action of \( \frac{1}{100} \) serum were inactivated by \( \frac{1}{10} \) serum at the same rate as untreated phage particles; however, the description of this experiment leaves much to be desired. Attempts to correlate resistance to antiserum with other properties such as heat resistance, ability to adsorb to host cells, age of phage stock, and plating efficiency on different bacterial hosts were unsuccessful.

Delbrück (1945b), dealing with the anomalous neutralization behavior of T\( \text{I} \), suggested that the inhomogeneity might be inherent in the virus population or, more likely, that it might develop during the course of the reaction as a result of attachment of antibody at noncritical sites on the phage. Although many samples of anti-T\( \text{I} \) serum are like that described by Delbrück in failing to follow first-order kinetics, Hershey (personal communication) found that anti-T\( \text{I} \) sera from two rabbits gave excellent first-order inactivation curves. Similarly, Fodor and Adams (1955) found only one antiserum, of some dozen prepared against various strains of the T5 serological group, which gave good first-order neutralization curves. It is not clear, therefore, whether the observed neutralization anomalies reflect an inherent heterogeneity of phage preparations, to which not all rabbits respond, or a heterogeneity which is acquired during interaction with certain samples of antiserum. Nor is it clear that the responsible serum factor is necessarily specific antibody. According to Hershey and Bronfenbrenner (1952) anomalous neutralization behavior may be an uncontrolled effect of complement. Recent studies (Van Vunakis, Barlow, and Levine, 1956) show that serum properdin, together with complement, have nonspecific effects which could confuse the study of specific neutralization. Unfortunately, nonspecific serum effects have not received explicit attention in most studies of anomalous neutralization behavior.

11. Host Dependence of Serum-Survivor Assay

It has been known for many years (d’Herelle, 1926) that different assay hosts may give different estimates of the fraction of
unneutralized phage in a given phage-antiserum mixture. Kalmanson and Bronfenbrenner (1942) analyzed this host effect with a strain of T2 that plated on certain strains of Shiga and Flexner dysentery bacteria with very nearly the same efficiency as on *E. coli*. The host-range properties and susceptibility to neutralization by antiserum were independent of the bacterial strain used as host for production of phage stocks. When the kinetics of neutralization were studied by plating replicate samples of serum-phage mixtures on each of the three host strains, it was found that the rate of neutralization was decidedly greater when measured with the Shiga and Flexner hosts than when measured with *E. coli* (see also Friedman, 1954; Tanami and Miyajima, 1956). This was interpreted to mean that a certain fraction of the initial phage population lost its ability to produce plaques on the dysentery strains while retaining its infectivity for *E. coli*. Subculture of these “monovalent” survivors on *E. coli* resulted in a “polyvalent” phage stock with full infectivity for all three host species. Reaction with antiserum had seemingly produced a phenotypic modification of host range. Bronfenbrenner had previously been able to produce the same kind of modification of host range in this phage by mild heat treatment.

The authors suggested (see also Tanami and Miyajima, 1956) that their experiments provide evidence for a serological heterogeneity inherent in individual phage particles. In the present conceptual framework, this heterogeneity would be a feature of the phage tail. It is not necessary, however, to postulate heterogeneity, either of antibody-attachment or of host-attachment sites, in order to explain the results. The differential host effects could easily be due to minor differences in the phage receptor sites on the several bacterial hosts, such that there would be differences in bonding strength between phage and the different hosts. In the random reaction of antibody molecules with uniform antigen sites on the phage particles, the virus surface could be so altered that certain of the particles would adsorb abortively, or even be unable to adsorb, to the
BACTERIOPHAGES

dysentery host cells, whereas stronger bonds with E. coli would still allow productive interaction. Both of these interpretations attribute the observed effects to antibody-induced phenotypic modification of host range, as opposed to selection from a genetically uniform, phenotypically heterogeneous population (see Lanni and Lanni, 1956). Direct evidence that “monovalent” survivors have indeed reacted with antibody in a significant fashion is contained in a brief note by Lanni and Lanni (1957).

12. Inheritance of Antigenic Specificity

The perpetuation of serological character of phages during continued subculture indicates, of course, that antigenic structure is genetically stable. On the other hand, the existence of phage strains which show partial serological cross-reaction implies the possibility of mutations affecting serological behavior.

Efforts to demonstrate serological mutation in phage, by examining either the survivors of prolonged serum action or mutants selected for nonserological markers, have generally been unconvincing (d’Herelle and Rakieten, 1934, 1935; Burnet and Freeman, 1937) or unsuccessful (Luria, 1945a; Hershey, 1946a, 1946b). Recent reports, however, indicate that some success may have been achieved in isolating serological mutants of a Xanthomonas pruni phage (Eisenstark, Goldberg, and Bernstein, 1955) and of T5 (Lanni and Lanni, 1956, 1957). Detailed verification of these briefly reported observations could make the antigenic structure of these phages amenable to ordinary genetic analysis.

Several workers, proceeding along a different line, have obtained valuable information by examining hybrids obtained from crosses of serologically related phages. Fodor and Adams (1955) have shown that certain T5 × PB hybrids resemble one or the other parent serologically, while others resemble both parents to some extent. By contrast, Streisinger (1956b) found that the genetic determinants of serological specificity in T2 and T4 are allelic and inseparable from the host-range determinants. Progeny from T2 × T4 crosses pos-
sessed the serological genotype of one or the other parent, but
never of both. Interestingly, the first-cycle T2 × T4 progeny
were found to fall into three phenotypic classes: the two parental
classes, and a class with mixed serological phenotype (Streisinger,
1956c). The quantitative distribution among these classes
was independent of genotype (Chapter XVI).

At the moment, it is not possible to give a unique structural
interpretation to the particles, obtained in either the T5 ×
PB or the T2 × T4 crosses, which resemble both parents serologi-
ically. It is tempting to draw the conclusion that they con-
tain two distinct tail antigens, corresponding to the distinct
parental antigens. They could, however, contain neither of
these but a third antigen, which cross-reacts with both anti-
bodies. In this respect, serological data obtained with cross
progeny are no less ambiguous than data obtained by cross-
absorption analysis of related phage strains. This ambiguity,
clearly stated for phage workers by Hershey and Bronfenbrenner
(1952) and Luria (1953a), is conveniently overlooked by most
serologists. But while the heterogeneity of the tail antigen
has proved elusive, there can be no question of the heterogeneity
of neutralizing antibody. The simple demonstration that ex-
haustive absorption of an antiserum with heterologous phage
removes part but not all of the antibody for the homologous
phage proves that the neutralizing antibody molecules in the
serum are not all alike. Unfortunately for structural interpre-
tations, it is well known that a single antigen can stimulate the
production of a variety of antibody molecules.

13. Mechanism of Phage Neutralization

We shall briefly review the observations that seem especially
relevant to the mechanism of neutralization by antibody.

1. Neutralized phage can be reactivated by treatment with
proteolytic enzymes or high frequency sound. Hence, antibody
molecules do not destroy the phage, but interfere mechanically
with infection by their presence at the phage surface.

2. Although a particle of phage T2 can accommodate a total
BACTERIOPHAGES

of about 4,600 antibody molecules (Hershey, Kalmanson, and Bronfenbrenner, 1943b, Hershey, Kimura, and Bronfenbrenner 1947), not more than two or three molecules, and possibly only one, actually participate in neutralization, regardless of how many attach.

3. Depending on the conditions of preparation, neutralized phage may absorb to host cells normally, poorly, or not at all (Burnet, Keogh, and Lush, 1937; Hershey and Bronfenbrenner, 1952; Nagano and Mutai, 1954b; Nagano and Oda, 1955). It may even adsorb to one host, which it proceeds to infect, but not to another, for which it has been neutralized (Lanni and Lanni, 1957). A normal particle which has already adsorbed to a host cell may or may not be resistant to neutralization.

4. The tip of the phage tail is the site of adsorption to the host (T. F. Anderson, 1952).

5. Following adsorption of a normal phage particle, the phage DNA enters the host cell, while the phage protein (ghost) remains outside and can be stripped off without interfering with phage reproduction (Hershey and Chase, 1952). Hence, infection consists in the injection of phage nucleic acid into the bacterium, this process being mediated by the phage tail in a way which is not yet clear. Neutralized phage that succeeds in adsorbing to a host cell does not inject its DNA (Nagano and Oda, 1955; Tolmach, 1957).

6. Neutralizing antibody is just one of several species of antiphage antibody. There is evidence that its action is confined to the phage tail, while other antibodies react mainly with the phage head (Lanni and Lanni, 1953).

These observations clearly indicate that the phage tail is critically important for neutralization. It would appear that attachment of one or a few antibody molecules at appropriate sites on the tail can render the virus noninfectious either by preventing adsorption, or by interfering with injection if the virus remains capable of adsorbing. Reaction of adsorbed virus with antibody, after injection of the DNA, would have no consequence for the infection. How antibody manages to prevent injection
without preventing adsorption is not known, but mechanisms are not difficult to imagine. Nor is it difficult to understand, in outline, how attachment of antibody at other sites on the phage might even, by altering the distribution of surface charges or in other ways, facilitate adsorption rather than inactivate the phage (Hershey, Kalmanson, and Bronfenbrenner, 1944; Sagik, 1954; Jerne, 1956).