INACTIVATION OF BACTERIOPHAGE T4r BY SPECIFIC ANTISERUM

Thesis by

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ABSTRACT

After a brief discussion of antigens, haptens, and antibodies, and a review of the literature on the phage antiphage reaction, a general theory of the inactivation of phage by antiserum is developed. The discovery by Jerne and Skovsted (38) that the rate of inactivation of phage T4r by specific antiserum is a thousand times faster in a medium of low ionic strength than in 0.1 M salt solution has made possible the study of the kinetics of the reaction at extremely low concentrations of antiserum. The use of low concentrations of antiserum has allowed the investigation of the reaction in conditions of phage excess at such low concentrations of phage that aggregation of phage does not take place. From a study of the kinetics of inactivation at low concentrations of phage and antibody in a low ionic strength medium quantitative estimates were made of the number of sites on a phage particle to which antibody molecules may attach (700), the number of these sites which, when occupied, result in the inactivation of the phage particle (10-30), the number of specific inactivating antibody molecules in antiserum (10^{16}/ml), and the efficiency of killing of a phage particle per collision of antibody with the tip of the tail (0.8).

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I. INTRODUCTION

This thesis is a study of *in vitro* reactions between a virus and specific anti-virus serum.

Viruses which require bacterial cells for the reproduction of their kind are called bacteriophages or phages. The group of "T" phages attacking *Escherichia coli* have been studied very intensively. The current concepts of the structure of a phage particle and of the mechanism of its invasion of the host bacterium have to a large extent been derived from work with three related phages, named T2, T4, and T6 (1,2,3,4,5). The phage particle consists of a head and a tail in a tadpole-like arrangement. Both head and tail present a protein surface. The head contains the genetic material, desoxyribose nucleic acid (DNA). When a phage particle comes into contact with a host bacterium, the first step of invasion is a reversible electrostatic adsorption of the tip of the tail of the phage onto the surface of the bacterium. An irreversible second step follows in which the bacterial wall is penetrated, and the phage DNA passes from the phage head through the tail into the bacterium. After this injection, the phage membrane remains attached to the bacterial surface but plays no further part in the replication of the phage and lysis of the host cell.

Of all virus-host cell systems the phage-bacterium system is experimentally the simplest. Especially important is the fact that the number of viable phage particles in a sus-

pension can be counted easily.

When phage particles are injected into an animal such as a rabbit or a horse, after a period of time, antibodies appear in the blood which can react with the type of phage particles that were injected. This reaction may take place in the tissues of the animal or in vitro and results in the inactivation (or neutralization, as it is often called) of the phage particle. The inactivation of phage by antibody is a manifestation of an antigen-antibody reaction. The phage-antiphage system has properties that make it singularly well suited to a detailed study of the kinetics of such a reaction. Most of the work to be described will be concerned with the kinetics of the inactivation of phage.

First, in the way of introduction, a discussion will be presented of some general aspects of antigen-antibody reactions and of some unique advantages of the phage-antiphage system over other antigen-antibody systems. Secondly, a review will follow of previous work done with the phage-antiphage system. Thirdly, a discussion of previous theories to describe the kinetics of phage inactivation and the development of a more general theory will be presented. Finally, experimental work will be described, and an interpretation in terms of the theory will be attempted.

II. ANTIGENS, HAPTENS AND ANTIBODIES

The purpose of this section is to review some of the current ideas about the nature of antigens, haptens and antibodies as a basis for a discussion of the phage-antiphage reaction (6,7,8,9).

A. The nature of antigens and haptens.

Antigens may be defined as substances which are capable of inducing the formation of antibodies in animals, and which can combine specifically with their antibodies. Haptens are able to combine with antibodies, but are not capable of inducing their formation. Antigens are usually large molecules (minimum molecular weight about 15,000). Almost every known protein has been found to be antigenic provided that it is foreign to the circulation of the test animal. Many polysaccharides and some other groups of substances are also known to be antigenic.

Haptens are lower molecular weight substances. These may be attached to a protein carrier to form a "conjugated" hapten which is then antigenic. Much of the work yielding information about the specificity and nature of the antibody combining site on an antigen has been done with haptens of known chemical structure attached by chemical union to protein carriers.

Several lines of evidence indicate that the specificity of antigens is due to the chemical structure of haptenic sur-

face groups. This has certainly been shown to be the case with the conjugated haptens where the determinant group is known. Studies of these haptenic determinants indicate that antibodies react with small areas of the antigenic surface rather than with the antigen particle as a whole. Also, the fact that the ratio of antibody molecules to antigen particles in specific precipitates formed by mixing antigen and antibody in vitro under conditions of excess antibody is often high, supports the idea that the number of determinant sites on an antigen can be high. Natural protein antigens may be regarded, then, as mosaics of haptenic determinants where the determinants are combinations and arrangements of amino acid residues.

B. The nature of antibodies.

After an animal has been injected with an antigen, the serum of this animal acquires the new property of reacting specifically with the antigen injected. This new property is due to the production of antibodies. In most cases circulating antibody has the same chemical properties and composition as normal serum globulin, but, in addition, it is capable of reacting with the type of antigen injected. Antibodies, therefore, are globulin molecules having special sites adapted to react specifically with complementary determinant groups on the surface of the inducing antigen.

The antibodies of rabbit and human serum and some of the antibodies of horse serum have molecular weights of about 160,000. Analysis of ultracentrifugal and diffusion data

indicate that antibodies are elongate in shape with dimensions of the order of 27 x 44 x 230 Å. While the number of combining sites on antibody molecules is still disputed by some workers, there is much evidence in favor of the existence of two combining sites. There also exists so called "incomplete" antibody which may have only one effective combining site.

The affinity between the antigen and antibody combining sites has been ascribed to van der Waals forces, Coulomb forces and hydrogen bonding rather than to chemical bonding by primary valence forces. The strength of the bonding by these forces and the specificity of the combination results from the complementarity of the antigen and antibody combining sites. In order to achieve a certain minimum combining strength, the steric fitting of the two reactive groups need not be as close should coulomb forces predominate, as when the shorter range forces predominate. The antibody combining site may be thought of as a cavity in the surface of the antibody which more or less closely fits around the determinant group on the antigen. The closer the fit or the greater the predominance of the coulomb forces, the stronger the bonding. Antibodies that show a greater combining strength than others (of identical "specificity") are said to be more "avid."

The high degree of specificity characteristic of immunological reactions implies neither absolute specificity nor homogeneity among antibody molecules. Antibodies are found to cross react with antigens related to the antigen that

induced this formation, presumably because of similar chemical configurations of the determinant groups. Antibodies in different sera, and even in one specimen of serum, are also found to have varying degrees of affinity for the same antigen. Both of these facts are easily reconciled with the picture just presented of the antigen and antibody combining sites. Cross reactivity and combining power depend on the closeness of fit between the combining sites.

Purification of antibodies from crude serum may be accomplished in a number of ways. The procedures are either based on non-specific physical methods designed to separate the antibody containing components of immune serum, or on specific removal of antibody with antigen and subsequent separation from the antigen. The non-specific methods are usually designed to remove the gamma globulin fraction of serum by selective salting out or by means of electrophoresis. The limit of purity by these methods depends on what fraction of the gamma globulin is antibody. The specific methods consist of forming a precipitate of the antigen and antibody, dissociating the complex and separating the antibody. Dissociation has been accomplished by raising or lowering the pH, by raising the salt concentration, by heating, and by using inhibiting haptens. Unfortunately, with the phage-antiphage system dissociation of the precipitated complex has never been achieved.

C. Antigen-antibody reactions.

1. Agglutination, precipitation, and complement fixation.

The reaction between antigen and antibody may manifest itself in many ways. When the antigen is particulate and microscopic in size as in the case of erythrocytes or bacteria, the interaction with antibody can result in a visible clumping of cells called agglutination. Combination of antibody with a submicroscopic particulate antigen such as phage or with a soluble antigen such as ovalbumin may result in precipitation. The precipitation reaction usually occurs only in a limited range of antigen and antibody concentrations and may be strongly influenced by salt concentration, pH, etc., of the medium. When fresh specific antiserum is added to bacteria or erythrocytes, the cells may be observed to undergo lysis or dissolve, while aged or heated serum may cause only agglutination. The heat labile factor called complement is responsible together with the antibody for the phenomenon of lysis. Many antigen-antibody reactions use up or "fix" complement irrespective of whether a special effect such as lysis or agglutination is produced. Complement fixation in an antigen-antibody mixture may be detected by testing for the presence of remaining complement by means of an erythrocyte-antierythrocyte indicator system. Detection of complement fixation is a sensitive method of inferring that a union of antigen and antibody has taken place. In some instances the characteristics of the antigen-

antibody system may be such that complement fixation is the only clue that a reaction has taken place.

2. Detection of primary union between antigen and antibody.

a. Toxin-antitoxin and enzyme-antienzyme systems.

In addition to complement fixation there are other examples of special effects with various antigen-antibody systems which make it possible to determine whether or not a reaction has taken place. Among these are the reactions of antigens which have special biological activity. They will be represented in this discussion by toxin-antitoxin, enzyme-antienzyme and phage-antiphage systems. These have the common feature of detecting the primary union of antigen and antibody. Agglutination and precipitation require secondary unions. Combination of toxin and antitoxin may be detected as a loss of toxicity for a test animal. The method of assay of toxin in animals has obviously many drawbacks. There are some cases where the combination of antienzyme with enzyme is reflected in loss of enzyme activity. The quantity of enzyme can be determined by measuring the rate of reaction with its substrate. Kinetic study of the union of enzyme and antienzyme is possible, but rather limited in application because of the limitations imposed by the method of assay of the antigen.

b. Phage-antiphage system.

The phage-antiphage system has unique advantages for the study of the kinetics of an antigen-antibody reaction. While it is impossible to detect experimentally a single toxin molecule or a single enzyme molecule, it is possible to detect a single phage particle by its ability to form a plaque. The assay of phage by counting plaques is quick, easy, precise and is about a billion times more sensitive a method of detecting antigen than the next most sensitive system. It is possible to detect a concentration of about 10^9/ml toxin molecules by the most sensitive skin reaction. The combination of antiphage with phage may result in the inactivation of the phage. The manifestation of the phage neutralization reaction is not limited to narrow concentration limits, but may be studied over wide ranges of concentration of both phage and antiserum. Kinetic studies are easily carried out because the reaction can be quickly stopped by dilution without reducing the sensitivity of the assay of antigen by the diminished concentration of the antigen. The rate of reaction between phage and antiphage can be slowed down enough by working at low concentrations to make possible measurement of the rate of reaction. This cannot be done in the toxin-antitoxin or the enzyme-antienzyme systems, because the less sensitive methods of assay impose a limit on the degree of dilution.

On the other hand phage appears to have some disadvantages. It is large and more complex than smaller antigens.

While a population of phage particles may be remarkably uniform antigenically--practically all particles being identical--a phage particle is known to possess at least two antigens. Whether the antigenic sites on the tail of the phage which are responsible for the neutralization reaction are more heterogeneous than the antigenic sites on an ovalbumin molecule, for example, is not known, but they may be quite homogeneous. Another uncertainty with the phage-antiphage system is the relationship between the attachment of antibody, and the effect of the attachment on the infectivity of the phage. Before the first steps of the phage infection cycle were known, it was a great puzzle to phage workers why phage adsorbed to bacteria was completely unaffected by strong neutralizing serum, yet was still capable of reacting with it. Now it is known that the antigenic phage coat is dispensable after injection of the phage DNA into the bacterium. Other recent knowledge has also added to the picture of how antibody molecules cause the inactivation of phage. One of the main themes of this thesis is concerned with the number of sites on a phage particle at which an attaching antibody molecule will inactivate the phage.

III. THE LITERATURE OF THE PHAGE-ANTIPHAGE REACTION IN REVIEW

A. Antigenicity of phage.

1. Introduction

The antigenicity of phage was first established in 1921 by Bordet and Ciuca (10). They showed that serum from a rabbit repeatedly injected with a lysate would inhibit lysis of the host in liquid culture. Serum from a rabbit injected with the host organism only, had no effect on the activity of the lytic principle. At this time the nature of the lytic principle was unknown,and theories about its being a ferment or a toxin were as widespread as the idea of its being a virus. For the next five years or so, the studies of the reaction between the lytic agent and its antiserum were rather confused and contradictory for several reasons. First, many different phage-host systems of questionable homogeneity of phage type were used. Second, the plaque counting assay of phage was not yet widely employed and the assay methods for lytic activity of untreated and treated lysates were subject to great variation among different workers. Third, while it was recognized that crude lysates contained bacterial antigens and that antiserum produced by injection of crude lysates would contain antibacterial antibodies, the complicating effects of this fact were not generally appreciated. For a review of this early work see Schultz, et al. (11).

2. Stability of the phage antigen

Data on the stability of the phage antigen were early

summarized and rechecked by Muckenfuss in 1929 (12). He reported that phage retained its antigenicity after mild heat inactivation, but that boiled and autoclaved phage was not antigenic. Phage inactivated by formalin or antiserum as well as phage adsorbed to dead bacteria was antigenic. Burnet et al. (13), reported that phage inactivated by phenol, and phage inactivated photodynamically with methylene blue also retained its antigenicity. Evidently, the infectivity of phage is less stable than its antigenicity. This is in agreement with the idea that the phage adsorption site for bacteria is much larger than an antigenic determinant group and consists of a pattern of small groups perhaps of similar size to the antigenic group. A change, then, in only one or two of these small groups may alter the pattern sufficiently to disturb seriously the infectivity of the phage.

Incidentally, the report by Muckenfuss (12) indicates that the antigens provoking the formation of neutralizing antibody are not blocked by the adsorption of the phage to dead bacteria.

3. Distinction of phage antigens from bacterial antigens.

A number of early reports appeared ascribing opsonic, precipitating and complement fixing activity to phage and antiphage that almost certainly must have been due to the contaminating bacterial antigens and their respective antibodies. This complication was first properly recognized by Weiss and Arnold in 1924 (14). Their first attempt to obtain

the lytic principle free of bacterial antigen was not really successful. It was based on differential diffusion of phage and bacterial debris into a layer of 2% agar. (They probably would have gotten the same result by simply diluting the lysate.) Nevertheless, they also made the more important observation that the agglutinating and complement fixing activity of a serum specific for a phage active on a typhoid bacterium disappeared entirely when it was repeatedly absorbed with typhoid cells, while it still retained its antilytic activity against phage. Later attempts by the same workers to free lysates of bacterial antigen were more successful. They found it possible to selectively precipitate the bacterial antigens from a lysate by salting out with 14% sodium sulfate (15) or by using an appropriate concentration of an alcohol extract of heart muscle (16). They were also able to destroy the bacterial antigens with trypsin (17) without destroying the phage. With these purified phage preparations, they obtained sera having antilytic activity, but apparently having none of the other properties usually ascribed to antiphage serum at that time. The titers of their phage stocks were probably too low for precipitation or detectable complement fixation. They concluded (incorrectly, as we now know) that the phage-antiphage reaction manifested itself only as a neutralization of phage activity.

4. Specificity of the phage antigen and cross reactivity.

The specificity of phage antigens as tested by neutrali-

zation with antiphage was recognized very early by a number of workers (11), and there was also general agreement that the specificity of the phage was independent of the host in which it was grown (13). This latter finding was later confirmed by Kalmanson and Bronfenbrenner (18) who found that coliphage T2 was inactivated at the same rate whether it had been grown on E. coli, B. dysenteriae Shiga, or B. dysenteriae Flexner.

The difference in specificity of two phages showing cross inactivation by their respective antisera was shown in an ingenious way by Burnet (19). Using two phages of the coli-dysentery group, he showed that formalin-killed bacteria coated with these phages could be agglutinated by antiphage serum from which the antibacterial antibodies had first been removed by absorption with bacteria. This showed that phage-coated bacteria were still able to react with antiphage antibody and provided a method of concentrating enough phage antigen in an easily precipitable form to effect the absorption of antiphage from serum. Using phage-coated bacteria, he exhaustively cross-absorbed the two antisera of the two related phages. He found that while homologous absorption took out all neutralizing activity for both homologous and heterologous phage, heterologous absorption removed all activity for itself, but left practically full activity against the homologous phage. This experiment demonstrated, incidentally, that phage adsorbed to bacteria is still capable of combining with neutralizing antibody. The antigens capable of combining

with neutralizing antibody are therefore not blocked by adsorption of the phage to bacteria.

5. Phage classification by serology.

The use of serological specificity for classification of phages was first reported by Watanabe (20) in 1923. He classified a group of shiga bacteriophages by the range of bacterial hosts they attack, and by serology, and found a general correlation between the groupings. There was also an indication in his work of a correlation of plaque type with serological type. The serological classification of phages was later suggested by other workers (12). In 1933 Burnet (21) classified some of the coli-dysentery bacteriophages by this method. His findings were similar to Watanabe's in that he observed correlation of the serological type with the host range classification. While all the phages in a serological group were found to belong to the same major host range group (with one exception), one major host range group was subdivided into several serological groups. One exception was found where two phages belonging to different host range groups definitely cross reacted serologically. He also reported that all phages of a serological group produced plaques of the same morphological type and roughly of the same size. Burnet considered particle size to be the primary basis for a gross classification of phages and serological type and host range as secondary characteristics. In a later review (13), however, Burnet concluded that the best basis for a classification of phages was provided by comparative

serological work. The advantage of a serological classification in comparison with a host range classification was effectively settled by Delbrück (22). He pointed out that for the coli "T" phages cross-resistance tests bring together T1 and T5, and T3, T4 and T7 which are serologically and morphologically diverse groups, and separate T2, T4 and T6 which are closely related serologically and are identical morphologically. Furthermore, while mutations of the T phages affecting a serological character have never been observed, abundant mutations of phages affecting host range can be obtained without a concomitant change in serological character. It may be concluded, therefore, that a serological classification yields the better classification for establishing mutual relationships between viruses. (cf. Adams, 23,24.)

6. Heterogeneity of phage.

a. Heterogeneity in resistance to neutralization by antibody.

The question of whether phage particles in a lysate differ in their resistance to antibody was very early a concern of the investigators of the phage-antiphage reaction. Andrewes and Elford (25) gathered some information on this question by fractionating a phage preparation in several ways and looking for different rates of inactivation of the different fractions. The 10% of a phage population which were inactivated last by serum were found to be inactivated slower than the first 90%, but the progeny of these phages, when subcultured, were inactivated at the same rate as the original

phage preparation. The serum resistance of this fraction was, therefore, not heritable and the question of whether the phage preparation was originally heterogeneous in this respect or whether heterogeneity developed during the course of the inactivation by serum remained unanswered. They found no difference in the rate of inactivation between unfractionated phage and the fractions surviving heat inactivation or the fraction which is poorly adsorbable to the susceptible host, nor was there any difference between fresh and old preparations of phage. The evidence seemed to indicate that the phage was rather homogeneous with respect to the rate of inactivation by antiserum. Many studies since then have shown that the time course of inactivation under conditions of antibody excess is logarithmic, often down to less than 0.1% survivors. This evidence indicates that 99.9% or more of a phage population react in exactly the same way toward antiserum. The antigenic homogeneity of a carefully prepared phage stock is, therefore, as good as that of any antigen prepared by chemical methods.

b. Composition of lysates.

The presence in lysates of bacterial antigens has already been discussed. The problem of the presence of phage antigens other than plaque-forming phage will now be considered.

In 1933 Burnet (26) reported the effect of a specific soluble substance (SSS) from bacteriophage. A phage-free ultrafiltrate of a lysate was shown to contain specific phage antigen by its ability to block the inactivating effect of

antiphage serum. The test is analogous to hapten inhibition of a precipitin reaction. When present in large amounts, this substance was found to stop the neutralization of phage. The SSS was heat labile at 65° C, was not adsorbed by host bacteria, and was able to induce the formation of phage neutralizing antibodies when injected into a rabbit. Recent work by DeMars et al. (27) also showed the presence of a substance in a phage ultrafiltrate which had serum blocking power.

Luria et al. (28) compared titers of phage preparations by plaque count with the titers by electron microscope counts. They concluded that there was good agreement between the titers by the two methods, indicating that the plaque counting technique is an almost ideal method of titration and is a valid measurement of the number of phage particles in a phage preparation. A lysate does not contain a large fraction of inactive particles which do not show up as plaque formers, but which might combine with antiphage antibodies.

c. Antigenic structure of the phage particle.

DeMars et al. (27) reported the isolation of non-infectious particles without tails (doughnuts) from lysates made in the presence of proflavine. These particles fixed complement with antiphage serum, but reacted poorly with phage neutralizing antibody. This was the first clue that phage may contain more than one antigen. A detailed investigation of the antigenic structure of T2 was undertaken by Lanni and Lanni (3). They measured both the complement fixing activity (CFA) and

the serum blocking power (SBP) of different phage and doughnut preparations purified by differential centrifugation, relative to a standard phage preparation. It turned out that phage had a fixed ratio of CFA/SBP while the doughnut preparations had very little SBP, making their CFA/SBP ratio very large. Furthermore, treatment of the doughnut preparations with intact susceptible host cells greatly reduced the SBP but reduced the CFA very little. In this way it was made evident that the doughnut preparations contained at least two physically separable antigenic components. One of these components fixed complement, but did not react with neutralizing antibodies. The other attached to bacteria and had both CFA and SBP. The major component without SBP was identified with doughnuts, and the other was identified with tailed particles. A strong antiphage serum exhaustively absorbed with a purified doughnut preparation lost 90-100% of its CFA and precipitating activity for doughnuts, retained 94-98% of its neutralizing activity and 75% CFA for phage and was still able to precipitate phage. Finally, antidoughnut serum was shown in electron micrographs to cause head to head aggregation of whole phages, while antiphage serum absorbed with doughnuts caused aggregation of phage by their tails. From all this evidence, it may be concluded that phage has at least two antigens, one associated with the head and one with the tail. The reaction of tail antigen with its homologous antibody fixes complement and is responsible for neutralization of the phage. The reaction of the head antigen and its homologous

antibody also fixes complement, but is not responsible for phage neutralization except by means of aggregations.

B. The effect of antiserum on phage.

1. Manifestations of the phage-antiphage reaction.

a. Complement fixation.

Soon after the discovery of the antigenicity of phage, attempts were made to find out whether the phage-antiphage system fixed complement and whether complement influenced the rate of inactivation. It is difficult to decide from the old reports whether complement fixation was actually observed because of the complications of contaminating bacterial antigens and their corresponding antibodies. Where precautions were taken to avoid these complications (15) no complement fixation was found, indicating that the phage quantities used were too low to fix a detectable amount of complement. Recent work by Lanni and Lanni (3) describing complement fixation by phage and antiphage has already been discussed.

There is general agreement in the older literature that the addition of complement or the destruction of complement by heating does not affect the rate of phage inactivation. In 1947, however, Hershey and Bronfenbrenner (29) published an interesting note on this subject. They reported that in the case of T1, the addition of complement caused the neutralization to stop at the 10% survival level. When T1 was exposed to antibody alone and complement was added soon, inactivation occurred down to 10% survivors. When inactivation was first allowed to proceed to lower survival levels,

the addition of complement resulted in reactivation back to the 10% level. This was also the case for T2, but with quantitative differences. Unfortunately, experimental details were not included in this abstract, and no subsequent report was made. It is, therefore, fruitless to try to interpret these findings.

b. Precipitation of phage by antiserum.

A true precipitin reaction between phage and antiphage was first reported by Burnet in 1933 (19). He showed that phage coated bacteria could be specifically agglutinated by antiphage serum. He then reported (30) that phage alone could be precipitated by antiphage serum if a high enough titer of phage was used. At least 2×10^9 phage/ml were required for visible precipitation.

The aggregation of phage by antiserum is obviously a complicating factor that must be taken into account when measuring the rate of inactivation of phage. The problem of aggregation has been discussed by Hershey (31,32). The rate of inactivation under conditions of serum excess is detectably increased by aggregation effects when the concentration of phage exceeds 10^9/ml. Measurements of depleting power of phage are likely to be even more greatly influenced because of the longer times of reaction usually involved. Lanni and Lanni (3) present evidence that in the presence of high titer antihead serum, aggregation effects can be detected in one hour with phage concentrations slightly below 10^7/ml. They have estimated from kinetic theory that the frequency of

binary collisions between phage particles at a concentration of 10^7/ml is about one collision per phage per minute.

c. Neutralization

1) Properties of serum treated phage.

a) Serum survivors.

Andrewes and Elford (33) reported that the plaques of serum survivors at low levels of survival are smaller than normal in the case of some phages, but not in the case of others. A study of phage C36,which showed this effect, revealed that while adsorption of this phage to host bacteria was normal, there was a delay of about two hours before multiplication could be detected. Burnet et al. (13) plotted the frequency distribution of plaque sizes of phage C16 after reacting with antiserum for different lengths of time. At about the 1% survival level, the frequency distribution of sizes changed from a unimodal to a bimodal distribution, the second hump representing the appearance of pinpoint plaques. It is difficult to interpret the result of very long exposure to very dilute serum on the ability of a phage to produce plaques because of the complications of aggregation. It should be possible to account for the reduction in plaque size of serum survivors either by a delay in adsorption of the phage or by a delay in injection. More recent work by Delbrück (34), however, reveals that T2 serum survivors at the 0.26% survival level adsorb to bacteria as quickly as untreated phage and show no delay in multiplication. In the light of these results the reason for the reduced plaque size of serum sur-

vivors remains obscure. It may be related to the presence of antibody combining with the phage in a reversible manner (see Section 3 below).

b) Serum inactivated phage.

The mechanism by which antiserum causes phage to lose its ability to multiply and to form plaques has not been thoroughly investigated. Serum inactivated phage may still be able to adsorb to bacteria. This was shown by Burnet (13) by the use of the agglutination of phage-coated bacteria with antiphage serum. He showed that serum inactivated phage was still capable of causing the agglutination of bacteria in the presence of antiphage serum. Adsorption to bacteria of serum inactivated T3 and T4 labeled with radioactive sulfur was also demonstrated by G. Sato (1951, 1955 personal communication). At least some of the serum inactivated phage particles could still adsorb. Evidently, a step in the phage growth cycle after adsorption is interfered with by antiserum, probably injection. Suggestive evidence that serum inactivated phage does not inject DNA was reported by Delbrück (34), who showed that serum inactivated T2 does not interfere with the growth of untreated T2 or T1.

2. Neutralization of adsorbed phage.

Delbrück (34) reported that when antiserum is added after the phage is adsorbed to its host, no infected cell fails to lyse, the latent period is not changed, and the burst size is normal.

3. Reversibility.

a. Effect of dilution.

There are many lines of evidence in the literature that the reaction between phage and neutralizing antibody is almost completely irreversible by dilution under all experimental conditions tested. Muckenfuss (12) reported no reactivation of serum inactivated phage after incubation for one day diluted in buffers of pH 4-10. Hershey (35) has summarized the evidence that the inactivation is not reversible by dilution. Specific precipitates do not dissolve in an excess of antigen which on initial addition would have prevented precipitation. Inactive phage greatly diluted with or without salt or detergent showed no activity after weeks in the refrigerator.

Recently, however, N. K. Jerne (1954, personal communication) discovered that phage inactivated by serum taken early during the course of immunization was reactivated by dilution. There is also evidence for the presence of a very small proportion of such reversible antibodies in serum taken from a hyperimmunized animal.

b. Reactivation with papain.

The reactivation of serum neutralized phage with papain was described by Kalmanson and Bronfenbrenner (36). They found that phage allowed to react with antiserum for one hour and inactivated to 10% survivors was 100% reactivable by treatment with papain. A duplicate mixture after one day in the refrigerator had 0.4% survivors and was 10% reactivable.

Another duplicate mixture after two days had no survivors and was 4% reactivable, and mixtures after four days were not reactivable at all. Another experiment involving different concentrations of serum and phage incubated for different times indicated that the degree of reactivability with papain depended on the amount of serum that had reacted with the phage rather than the length of time of union of phage and antibody. The inability of papain to effect reactivation of phages combined with large numbers of antibody molecules was attributed by the authors to the failure of papain to digest the antibody molecule completely.

c. Reactivation by sonic vibration.

Anderson and Doermann (37) gave evidence that T3 neutralized by antiserum could be reactivated by sonic vibration. A small volume containing 10^8 T3 particles per ml was allowed to react with 1:250 antiserum for six minutes and was then diluted 1/500 in growth medium and sonically vibrated. Before vibration there were 0.6% survivors. After 20 minutes of vibration there were 2.0% survivors, representing a reactivation of 1.4% of the original phage titer. The evidence that sonic vibration brought about reactivation by eluting antibody from the phage is incomplete. The reactivation may have been due to breaking up of clumps of survivors aggregated by serum, or it may have been due merely to reactivation by dilution of a small fraction of phages united with reversible antibody--the reactivation having been accelerated by

the increase in the temperature from the sonic vibration.

4. Cofactor requirement.

During an investigation of the rate of inactivation of coliphage T4r by specific antiserum in distilled water, Jerne and Skovsted (38) came across an interesting phenomenon. When a series of twofold dilutions of the antiserum was made in distilled water and the serum activity measured in 10 ml volume by adding about 3000 phage particles per ml and assaying for survivors after various times, there occurred a sudden inhibition of serum activity below a certain serum concentration. For example, with a dilution of 1:50,000 there were no survivors after one minute, while with a dilution of 1:100,000 there was no inactivation of phage after two hours. This inhibition could be removed by adding 1:5,000 normal serum or by carrying out the reaction in a liter volume instead of 10 ml. The interpretation offered for this phenomenon was the existence of a cofactor in serum which reversibly combined with antibody to form an active complex which then inactivated phage. Inhibition in small volumes was thought to be due to absorption of cofactor onto the glass wall. Analysis of the cofactor in normal serum revealed it to have chemical properties similar to lysozyme. Furthermore, lysozyme and lysozyme-containing materials exhibited cofactor activity.

C. Kinetics of phage inactivation

1. Survival curves in serum excess.

a. The percentage law.

Before 1933 the form of the survival curve of phage in the presence of antiserum was not known. Arnold and Weiss (39) presented superficially convincing evidence that the inactivation of phage followed the laws of definite proportion. The results obtained may have been substantially valid provided that the serum used was so weak that the work was done in the region of excess antigen, but it seems more likely that the results were due to the unsatisfactory phage assay method used. It is suspected that other workers during this period who also obtained results supporting the hypothesis that phage and antiphage combined in definite proportions, did so because of characteristics inherent in their phage assay technique. Other investigators at that time who reported that inactivation proceeds in a logarithmic fashion based their conclusions on extremely flimsy kinetic evidence. In 1933 Andrewes and Elford (25) carried out a large series of inactivation measurements, varying phage input, serum concentration and time and using the plaque counting technique. The survival curves were steep at first down to about 10% survivors and then flattened out considerably. They found that in a given time for a given concentration of serum, the fraction of phage inactivated was independent of the concentration of input phage. This "percentage law" was found to hold with the highest dilution of serum producing measurable inactivation. It was concluded, therefore, that all the observed inactivation of phage took place in great antibody

excess. The fact that the rate of inactivation slowed down after 90% of the phage had been inactivated was not explained. More precise data on the time course of neutralization of phage were presented by Burnet (13), who showed that the course of inactivation may be logarithmic down to almost 0.1% survivors.

b. Flattening out of the curve at low survivors.

Andrewes and Elford (25) filtered out all phage from a phage serum mixture in which the rate of inactivation had dropped practically to zero and added a second input of phage. This second input was inactivated at the same rate as the first. From this fact and because of the percentage law, they concluded that the flattening out of the curve at low survivors was not due to depletion of antibody. Measurement of the rate of inactivation of the progeny of serum survivors showed that rate decrease was also not due to a genetically resistant fraction of the phage population. Suggestions have been made by several investigators that the falling off of the rate is due to a heterogeneity introduced into the phage population by the reaction with antibody. A satisfactory explanation of this phenomenon, however, has not yet been presented in the literature.

c. Initial lag of survival curves.

While studying the influence of serum concentration on the rate of phage inactivation, Burnet (13) observed that with very dilute serum there was a distinct lag before the rate

of reaction became logarithmic. A similar lag at higher concentrations of serum was not reported, but may easily have escaped detection with the greater rate of inactivation at the higher serum concentrations. Because the properties of serum survivors indicated the existence of partially inactivated phage, Burnet expressed the opinion that the observed lag reflected the requirement of more than one antibody molecule to inactivate a phage particle under these conditions. Kalmanson et al. (40) also observed an initial lag in the rate of inactivation of phage. They compared their experimental data with theoretical curves calculated on the basis that one, two, or three antibody molecules were required for inactivation and concluded that the experimental curves most closely resembled two or three target curves. Sagik (41) has reported the presence in T2 lysates of a fraction of phage particles which under the normal conditions of infection will not multiply. This fraction of phage particles appears to be inhibited by a component of the host bacterium. Treatment of a lysate containing such a fraction of inhibited phage particles, with dilute specific antiserum resulted first in a rapid rise in titer of about three fold followed by a decline. The titer declines at the same exponential rate as a lysate in which the phages are first activated to maximum titer by one of the other means available. Sagik concludes that the rise in titer is due to the removal of the inhibitor from phage particles by specific antibody without

inactivation. Non-inhibited phages are then inactivated by antibody in the usual manner. Cann and Clark (47) suggest that the initial lag in the serum inactivation curve of phages reported by other workers is due to the summation of activation as reported by Sagik (41) and the inactivation by antibody. For T2 this interpretation may be valid and since Kalmanson et al. (40) worked with T2, their results may reflect a similar situation. The existence in lysates of reactivable inhibited phages, however, cannot be demonstrated in the case of T4.

d. Rate constant of phage inactivation.

1) Introduction.

Practically all measurements of the rate of inactivation of phage have been done under conditions of antibody excess. Since under these conditions, most of the phage population is inactivated exponentially at a constant rate which is proportional to the serum concentration and independent of the phage concentration, the course of the reaction may be described by the following relation (34):

$$\frac{P}{P_o} = e^{-kDt}$$

P_o = concentration of phage at time 0.
P^o = concentration of phage at time t.
D = dilution of antiserum.
t = time in minutes.
k = rate constant of inactivation.

The titer of the serum is then conveniently expressed in terms of the single parameter k.

2) Influence of temperature.

The Q_{10} of the rate of inactivation of phage by antiserum in the range between 0° C and 37° C in a medium containing electrolytes has been reported to be about 2.0 by several investigators (34,38,40). In distilled water the Q_{10} was reported by Jerne and Skovsted (38) to be 1.4. This may mean that the phage particles and antibodies must have a greater kinetic energy in electrolyte containing medium than in distilled water in order for a collision to result in specific union and consequent inactivation of the phage particle.

3) Influence of pH.

Kalmanson et al. (40) report that in glycine-phosphate-acetate mixtures the rate of inactivation of phage is practically indifferent to changes of pH in the range of pH 5.0-10.0, but is markedly slower at pH 4.0. Jerne and Skovsted (38) find that at very low ionic strength the rate of inactivation is markedly decreased outside the range of pH 4.8-7.2.

4) Effect of ionic strength.

The rate of inactivation of T4r by specific serum is reported by Jerne and Skovsted (38) to be increased a thousand fold when the salt concentration of the medium is decreased from 1 N to 0.001 N. This remarkable effect is caused by the ionic strength of the medium rather than by any specific ions, since all anions and cations tested act in the same way.

This discovery has helped greatly to further our understanding of the phage antiphage reaction and has made available a valuable technique for kinetic studies. It will be discussed further in a later section.

2. Reaction rate constant from diffusion theory.

Hershey (32) calculated the expected rate of reaction between phage and antibody in antibody excess on the assumption that the rate of reaction is limited by diffusion. Unfortunately, the data then available for the size of phage were in error. Also, at that time it was not known that there exist two distinct antigens in phage, one on the tail and one on the head, and that the antigen capable of combining with neutralizing antibody is located exclusively on the tail. These calculations will, therefore, be corrected accordingly.

The rate of accumulation of antibody molecules on one phage particle according to Hershey (32) is as follows:

$$Q = 4\pi C_1(R_1D_2 + R_2D_1)$$

Q = number of antibody molecules adsorbed by one phage particle per second

C_1 = number of antibody molecules (per ml)

R_1 = equivalent radius of the antibody molecule (cm)

R_2 = equivalent radius of the phage particle (cm)

D_1 = diffusion coefficient of antibody (cm^2/sec)

D_2 = diffusion coefficient of phage (cm^2/sec)

The product R_1D_2 is negligible compared to R_2D_1 and may be omitted. The relation accordingly reduces to:

$$Q = 4\pi C_1 R_2 D_1$$

From electron micrographs of frozen-dried preparations, Williams (5) reports for T2, T4 and T6 a head shaped like an elongated hexagon of approximate dimensions 95 x 65 millimicrons and a tail 100 x 25 millimicrons. Since it has been shown that the antigens corresponding to neutralizing antibodies are present exclusively on the tail of the phage, only collisions with the tail will be considered. The radius of a sphere having the same surface area as the tail is 2.6×10^{-6} cm. The radius of a sphere having the same surface area as the tip of the tail assuming this tip to be a flat disc of 25 mμ diameter is 6.2×10^{-7} cm.

The diffusion coefficient of rabbit antibody of 3.8×10^{-7} cm^2/sec at 20° C used by Hershey is practically the same as that reported for the water soluble fraction of horse Diphtheria antitoxin of molecular weight 184,000 (42). This value is 3.9×10^{-7} cm^2/sec at 20° C which corresponds to about 6.9×10^{-7} cm^2/sec at 37° C.

From the values of the parameters given above:

$Q = 2.2 \times 10^{-11} C_1$ sec^{-1} at 37° C for the whole phage tail.

$Q = 5.4 \times 10^{-12} C_1$ sec^{-1} at 37° C for the tip of the phage tail.

IV. THEORY OF THE INACTIVATION OF PHAGE.

A. Previous theories.

In 1937, Burnet *et al.* (13) presented a quantitative description of phage neutralization. These authors assumed that both the phage and the antibody populations were homogeneous, that the reaction is irreversible and that antibody was present in great excess under their conditions of study. It was recognized that the reaction is complex and that many antibody molecules eventually combine with a phage particle during the course of the reaction. Also that more than one antibody molecule may be necessary to inactivate a phage. Nevertheless, as a first approximation it was assumed that a single antibody molecule is sufficient to inactivate a phage particle. It was then suggested that the course of the reaction could be described by the usual formula for a bimolecular reaction:

$$K = \frac{1}{t} \cdot \frac{1}{a-c} \ln \frac{(a-x)\,c}{(c-x)\,a}$$

a = initial concentration of phage.
x = phage inactivated in time t.
c = initial concentration of antibody.

When c is large compared to a or x then,

$$K = \frac{1}{t} \cdot \frac{1}{-c} \ln \frac{a-x}{a} \qquad \text{or}$$

$$Kc = \frac{1}{t} \cdot \ln \frac{1}{P}$$

where P is the fraction of surviving phage particles at time t. This equation is identical to that suggested by Delbrück (34) to describe the exponential form of the inactivation curve. (cf Section III, p 30 .)

No attempt has been reported in the literature to formulate a description of the inactivation of phage which is valid also when antibody is not in great excess. A first step in this direction was made by Hershey (32), who worked out a theory for the rate of deposition of antibody on the free surface of phage. He considered the reaction of phage (P) and antibody (A) to be a series of bimolecular reactions:

$$P + A \longrightarrow PA \qquad (a)$$

$$PA + A \longrightarrow PA_2 \ldots \qquad (b) \ldots$$

$$PA_{(n-1)} + A \longrightarrow PA_n \ldots \qquad (c) \ldots$$

$$PA_{(s-1)} + A \longrightarrow PA_s \ldots$$

where n is the number of antibodies molecules necessary to inactivate a phage particle and s the number required to saturate the surface. The reaction between phage and antibody was considered to be irreversible. In this scheme reaction (c) represents the inactivation of phage. Accordingly, the rate of attachment of antibody to a phage particle is

$$\frac{dx}{dt} = kC\,(1 - x/s)$$

k = rate of reaction at zero time and unit antibody concentration (molecules A per sec)
C = concentration of antibody
x = prevailing number of antibody molecules adsorbed
s = limiting value of x

Integrating for the conditions of antibody excess, i.e., C remaining constant, we obtain

$$1 - x/s = e^{-kCt/s} \quad \text{or} \quad k = -(s/Ct)[\ln(1-x/s)]$$

By experimentally determining three quantities, (1) the combined antibody, x, when the "average" phage is neutralized, (2) t_x, the time required to accomplish this degree of neutralization, and (3) s, the maximum number of antibodies capable of attaching to a phage particle, Hershey was able to evaluate k. Unfortunately, the estimates of these parameters were in error because of erroneous notions about the size of phage particles, because no method was yet available to avoid aggregation effects, and because the existence of non-neutralizing antihead antibody was still unknown. Aggregation effects tend to make the estimate of s too small, whereas the presence of non-neutralizing antibody tends to make the estimate of s too large.

B. Development of the present theory.

1. New aspect of the theory.

The theory to be proposed differs from existing theories in essentially one point. Two kinds of sites on a phage are postulated, viz., killing sites and non-killing sites both of which may combine with neutralizing antibody. Only when at least one of the killing sites is occupied is the phage inactivated. The number of killing sites per phage is intro-

duced as an unknown parameter to be determined from experiments.

The aim of the theory is to predict the time course of neutralization for any input ratio of phage and antibody.

2. Discussion of the assumptions.

Let it be emphasized that we shall consider only the potentially neutralizing antibodies and the corresponding antigens. Non-neutralizing antihead antibody and the corresponding antigenic sites play no part in the phage inactivation reaction.

a. The phage population is homogeneous.

The phage population is considered to be homogeneous in two respects. First, all the phage particles in the population are considered to be identical. Second, both killing sites and non-killing sites are considered to be identical in their ability to combine with antibody. The experimental justification for the first assumption has been brought out in the literature review.

b. The antibody population is homogeneous.

Though it is known that antibodies in a serum may vary considerably in their combining power and their specificity, the antibody population will be considered, as a first approximation, to be homogeneous with respect to combining power.

c. Phages possess killing sites and non-killing sites.

Phages will be considered to possess two kinds of sites

to which antibody can attach. The attachment of an antibody molecule to a killing site results in the inactivation of the phage particle. Attachment of an antibody molecule to a non-killing site does not result in the inactivation of the phage particle. Attachment to non-killing sites, however, results in depletion of antibody molecules, and becomes important when antibody molecules are not in great excess. The difference between killing and non-killing sites is thought to be due to their location on the phage particle.

Since phages are inactivated by antibody molecules, there can be no doubt of the existence of killing sites. However, the experimental evidence for the existence of sites to which potentially neutralizing antibodies may attach without inactivating a phage is still lacking. There is evidence that phages adsorbed to bacteria can still combine with neutralizing antibody. This suggests that neutralizing antibody molecules can attach to other parts of the tail of a phage particle than at the tip. Whether or not the attachment of antibody molecules to the sides of the tail will cause inactivation of unadsorbed phage is not known.

d. All sites on a phage particle react independently and with equal probability.

This assumption is made as a first approximation.

e. The attachment of antibody molecules to phage sites is irreversible.

It will be assumed that antibody molecules attach irre-

versibly to random sites on the phage particles. The experimental evidence is unambiguous that the combination with phage of antibody from a hyperimmunized animal is irreversible under ordinary conditions of study. The evidence for this irreversibility and for reversibility in the case of serum taken from the animal early in the course of immunization has been discussed in the literature review section.

f. Attachment of one antibody molecule to a killing site is sufficient to inactivate the phage particle.

Evidence for this assumption comes from the fact that the survivor curve usually does not show an initial lag. Suggestive evidence can also be derived from a comparison of the maximum inactivation rates measured in a medium of low ionic strength with the calculated collision frequency between phage and antibody. These rates turn out to be of the same order of magnitude, indicating that perhaps every collision between phage and neutralizing antibody may result in the inactivation of the phage.

The existence of incompletely neutralized phage would suggest that more than one antibody molecule may be required for inactivation. The evidence pointing to the existence of incompletely neutralized phage particles, however, may be interpreted in other ways. The altered properties of serum survivors may be due to aggregation effects and to temporary inactivation by reversible antibodies.

The initial lag of the inactivation curve found by Burnet (13) has been presented as evidence for the requirement of more than one antibody for the inactivation of phage. This observation may have had other causes. One possibility which has already been discussed is the activation of inhibited phages in lysates by a serum factor. A lag might also be due to complications arising from a serum-cofactor requirement as described by Jerne and Skovsted (38). Since the inactivation curve sometimes does and sometimes does not show an initial lag, it seems reasonable to assume that the lag is a secondary complication rather than an indication that more than one antibody molecule is required for inactivation.

g. Inactivation of phage does not affect the reactivity of the unoccupied sites.

It will be assumed that the reactivity of the unoccupied sites on a phage particle is independent of whether or not an antibody molecule has reacted with a killing site.

There is some experimental evidence that serum-inactivated phage is still able to combine with more molecules of neutralizing antibody. (J. D. Mandell, 1954, unpublished).

h. No aggregation of antibody or phage occurs.

These complicating effects can be largely avoided by the use of very low concentrations of phage and antibody.

3. Derivation of the theory

a. Restatement of the assumptions.

(We shall consider only the neutralizing antibodies and the corresponding antigens.)

1. The phage population is homogeneous.
2. The antibody population is homogeneous.
3. Phages possess killing sites and non-killing sites.
4. All sites on a phage particle react independently and with equal probability.
5. The attachment of antibody molecules to phage sites is irreversible.
6. Attachment of one antibody molecule to a killing site is sufficient to inactivate the phage particle.
7. Inactivation of phage does not affect the reactivity of the unoccupied sites.
8. No aggregation of antibody or phage occurs.

b. Definitions.

N = the total number of antigenic sites per phage.

n = the number of killing sites per phage, $(1 \leq n \leq N)$

P = the number of active phage at time $\underline{t}$ per unit volume.

P_o = the initial input of active phage per unit volume.

A = the number of free antibody molecules at time $\underline{t}$ per unit volume.

A_o = the initial input of free antibody molecules per unit volume.

A_u = the number of antibody molecules in undiluted serum per unit volume.

D = the dilution of antiserum.

K = the rate of attachment of antibody molecules to phage sites per minute.

$p = P/P_o$, the active fraction of phage.

$r = P_oN/A_o$, the input ratio of antigenic sites to antibody molecules.

$S = A_o - P_oN \equiv A_o(1-r)$, the antibody excess.

$A_o \equiv A_uD$

c. Attachment of antibody molecules.

1) The general case.

Since the rate K of attachment of antibody molecules to phage sites is proportional to the number of free sites and to the number of free antibody molecules, (cf. Hershey (32)) the disappearance of free antibody molecules may be expressed as follows:

$$\frac{dA}{dt} = -KA(P_oN - A_o + A) \qquad (1)$$

P_oN is the total number of sites in suspension.

$A_o - A$ is the number of antibody molecules fixed at time t.

$P_oN - A_o + A$ is the number of free sites at time t.

By integration of (1):

$$\frac{1}{A_o - P_oN} \ln \frac{A - A_o - P_oN}{A} + \ln \frac{A}{P_oN} = -Kt \qquad (2)$$

Solving (2) for A:

$$A = \frac{A_o(A_o - P_oN)}{A_o - P_oNe^{-Kt(A_o - P_oN)}} \qquad (3)$$

$$A \equiv \frac{s}{1 - re^{-Kst}} \qquad (3a)$$

2) The special case at equivalence.

For the special case at equivalence where $P_oN=A_o$, equation (1) becomes:

$$\frac{dA}{dt} = -KA^2 \qquad (4)$$

By integration of (4):

$$A = \frac{A_o}{A_oKt + 1} \qquad (5)$$

d. Surviving phage as a function of the attachment of antibodies.

1) The general case.

$$P/P_o = p = \left(1 - \frac{A_o-A}{P_oN}\right)^n \qquad (6)$$

A_o-A is the number of antibody molecules fixed at time t which is equivalent to the number of sites covered at time t.

$\frac{A_o-A}{P_oN}$ is the fraction of sites occupied at time t, or the probability that a site is covered at time t.

None of the n killing sites on a phage must be covered for phage to survive.

Substituting the value for A in (6) from (3) and simplifying:

$$p = \left(\frac{1 - r}{e^{KA_o(1-r)t} - r} \right)^n \qquad (7)$$

$$\equiv \left(\frac{1 - r}{e^{sKt} - r} \right)^n \qquad (8)$$

2) The special case at equivalence.

In the special case at equivalence where $P_oN=A_o$, equation (6) becomes:

$$p = \left(\frac{A}{A_o} \right)^n \qquad (9)$$

Substituting the value for A in (9) by (5):

$$p = \left(\frac{1}{1 + KA_ot} \right)^n \qquad (10)$$

3) The special case in antibody excess.

In strong antibody excess r is negligibly small compared to 1, and equation (7) reduces to the familiar form:

$$p = e^{-nKA_ot} \qquad (11)$$

4) The special case of antigen excess at infinite time.

In antigen excess, $\underline{r}$ is greater than 1. From equation (7):

$$\lim_{t \to \infty} p = \left(\frac{r - 1}{r}\right)^n \qquad (12)$$

$$p_\infty = 0 \text{ for } r \leq 1 \qquad (12a)$$

C. Experimental evaluation of parameters.

1. Introduction.

A few definitions that are implied in the foregoing development of the theory will be stated explicitly. The absolute rate constant for the attachment of antibody molecules to phage sites is represented by $\underline{K}$ and may be called the "attachment rate constant." It refers to unit concentration of antibody. The rate constant for the inactivation of phage in the presence of a unit concentration of antibody is represented by $\underline{nK}$ and may be called the "neutralization rate constant." The neutralization rate in serum of strength $\underline{A_u}$, diluted by the fraction $\underline{D}$, is $\underline{nKA_uD}$. The neutralization rate in undiluted serum, $\underline{nKA_u}$ is usually referred to as the "serum titer."

2. The serum titer.

The slope of the inactivation curve in serum excess determined experimentally gives a value for the product $\underline{nKA_o}$ or

$\underline{nKA_u}D$. Since D is known, we can calculate $\underline{nKA_u}$.

3. Depletion experiments.

From antibody depletion experiments, estimates of $\underline{n}$ and of the ratio $\underline{A_u}/\underline{N}$ may be determined as will be shown below. The evaluation of $\underline{N}$ and $\underline{A_u}$ separately would require an independent measure of one of these quantities. It cannot be derived from kinetic data on inactivation.

It is feasible to estimate the largest input of phage which is completely inactivated in infinite time by a fixed dilution of antiserum. This measurement, however, is subject to error for two reasons. It is difficult to extrapolate to infinite time because aggregation might produce artifacts. Also, it is difficult to determine complete inactivation of input phage, because a small proportion of phage particles which are temporarily inactivated by reversibly attaching antibody molecules later appear as survivors and mask the actual survival level. The reversible antibody effect, however, can be largely eliminated by the use of the strong serum plating technique (cf. p. 53). The maximal phage input which is completely inactivated should represent the input phage at r = 1, (see equation 12a), from which $\underline{A_u}/\underline{N}$ could be calculated because when r = 1, $P_oN = A_o = A_uD$.

A better estimate of $\underline{A_u}/\underline{N}$ is obtained by determining the phage inputs causing depletion of about 20-80% of the neutralizing antibody of a given antiserum and estimating by extrapolation the amount of phage necessary for 100% deple-

tion. With this method the reaction goes to completion in a relatively short time because serum is present in sufficient excess, and also, complications due to reversible antibody molecules can be disregarded, since it is not necessary to measure survivors down to extremely low levels.

Once a value for A_u/N is known, then also r is known for any given mixture of this antiserum and phage, and n may be estimated by measuring the survivors at infinite time in conditions of phage excess, (r greater than 1) according to equation (12). The measurement of p_∞ will be most accurate in the range of about 1-50% survivors because at these higher survival levels the value of r is larger, the reaction goes to completion more quickly, and reversible antibody complications are avoided.

Values of n and r may also be estimated simultaneously from equation (12) by measuring two different survival levels at infinite time corresponding to two different phage inputs.

V. EXPERIMENTAL SECTION.

A. Introduction.

Investigation of the phage-antiphage reaction under conditions of antibody depletion and phage excess presents some difficulties. In order to avoid complications of aggregation of phage by antibody, it is necessary to work at low concentrations of phage. However, low phage concentrations can only deplete correspondingly low concentrations of antiserum, and at low antiserum concentration the inactivation rate is so slow that instability of phage and antiserum due to non-specific causes becomes important, and experimental variability is increased. A promising way to attack the problem became available when Jerne and Skovsted (38) discovered that the rate of inactivation was a thousand times faster in distilled water than at the usual ionic strengths. In addition, other aspects of this phenomenon seemed interesting. For these reasons, we undertook to study the phage-antiphage system in solutions of low ionic strength. Very soon many technical problems appeared and had to be eliminated to obtain reproducible results. Many experiments were done before the few important variables were discovered and could be controlled. It is felt that the solution of these technical problems is as important as the results to be reported here from the application of the technique.

B. Materials and methods.

1. Phage and bacteria.

Strain B of Escherichia coli was used as the host bacterium for plating the phage and for growing phage stocks.

An r (rapid lysis) mutant of phage T4 was the phage used for most of the work. The r mutant is preferred to the wild type because it produces plaques that are clearer, better defined, and easier to count. This T4r stock did not require tryptophane for adsorption as evidenced by its ability to form the same number of plaques on synthetic media without tryptophane, as on the complete T medium. The tryptophane independent strain was chosen rather than a requiring strain in order to avoid possible complications from this source.

2. Media

a. Bacterial growth media.

T medium: Difco Bacto - tryptone 10 g, NaCl 5g per liter. The solid medium contained 10g per liter Difco Bacto-agar for bottom layer and 7g per liter for the top layer.

M9: Na_2HPO_4 7g, KH_2PO_4 3g, NH_4Cl 1g per liter. Before use 50 ml autoclaved 10% glucose solution and 1 ml autoclaved 1 M $MgSO_4$ were added per liter.

b. Buffers.

Phosphate: Na_2HPO_4 7.1g, KH_2PO_4 27.2g per 500 ml solution to make a stock solution at 0.5 M, pH 5.86. This buffer changes to pH 6.50 when diluted beyond 0.0025 M. The pH does not change with further dilution until the buffer capacity is negligible in comparison to other substances in distilled water. This limiting capacity is approx. 0.00005 M.

Citrate: Citric acid, 0.1 M and sodium citrate, 0.1 M were mixed to give buffers of different pH's from 4.2-6.4.

Pyridine-acetate: Pyridine was added to 0.1 M acetic acid to pH 5.70 to make 1 liter of buffer. Further dilution of this buffer caused no change in pH greater than 0.05 unit.

Anthranilate: Anthranilic acid, 1.37g plus 1 N NaOH to bring the pH to 5.5, plus water to make 200 ml. This buffer is 0.05 M as anthranilate.

H buffer: Na_2HPO_4 3.0g, KH_2PO_4 1.5g, NaCl 4g, K_2SO_4 5g per liter. Before use autoclaved $MgSO_4$ was added to 0.001 M, $CaCl_2$ to 0.0001 M and gelatin to 10 micrograms per ml. ("H" stands for Hershey who proposed this buffer, (46).)

3. Antiserum.

The antiserum was obtained from Dr. N. K. Jerne at the State Serum Institut, Copenhagen. It had been prepared by injecting a horse with a total of about 10^{15} T4r particles The injections had been given every two days for three periods of three weeks, allowing two weeks between injection periods. The dose injected was increased gradually to about 5×10^{13} particles toward the end of the immunization period, and the animal had been bled about 10 days after the last injection. The serum had then been lyophilized in 3 ml aliquots and sent from Copenhagen in this form. The dry material (267 mg per ampoule) was rehydrated to the original volume by the addition of 2.8 ml of sterile distilled water and was then frozen in 0.1 ml quantities for storage. For daily experiments the serum was kept as a 1:100 dilution in H buf-

fer. It is stable in this dilution for at least a month in the refrigerator.

4. Glassware.

To avoid undesired effects of small amounts of contaminating materials in a distilled water medium, tubes, flasks and pipettes were cleaned by a rigorous procedure. The glassware was treated with concentrated sulfuric acid plus sodium nitrate in a boiling water bath for at least one hour, rinsed seven times in tap distilled water, drained, rinsed once with boiling distilled water, drained again, and dry sterilized. The pipettes and tubes were cleaned, rinsed, sterilized and stored in glass containers throughout.

When the experimental work was first begun, it was not known how rigorous a cleaning procedure would be necessary. To be on the safe side the procedure just described had been adopted. During about the first year of experimental work, the pipettes were sterilized in copper cannisters. When it was suspected that erratic instability of the phage in distilled water was caused by copper salts contaminating the pipettes from the cannisters, large glass tubes were used instead. The acid cleaning procedure may not be necessary if the use of copper cannisters is avoided.

5. Distilled water.

Glass distilled water was purchased from the Arrowhead and Puritas Waters, Inc. in 5 gallon bottles. It was found to be of uniform quality with a pH of 6.4, and was used

routinely for rinsing and for preparing buffers and synthetic media. When distilled water was tried as the reaction medium, erratic results were obtained with different samples from different sources. In an effort to avoid this source of variation, the use of commercial distilled water was adopted with the hope that it would be more uniform. Inactivation experiments in one liter volumes require fairly large quantities of distilled water. Later, dilute buffer was substituted for distilled water as the reaction medium. With the use of dilute buffer, the uniformity of the distilled water may not be important.

6. Preparation of phage stocks.

Lysates were made by inoculating young 30 ml aerated cultures of B in M9 with T4r obtained by stabbing single plaques. Five of these lysates which contained about 7×10^{10} plaque forming particles per ml and which appeared to be homogeneous with respect to plaque type were pooled. The pooled lysates were purified by filtration through a Berkefeld candle followed by two cycles of high and low speed centrifugation in the cold. The purified phage was suspended in H buffer at a titer of 4×10^{11} per ml and stored in the refrigerator. The overall recovery from the crude lysate was 60%. Most of the loss could be accounted for in the supernatants from the high speed centrifugations, indicating that little phage, if any, was killed by the purification procedure. The stock was stable during storage in the refrigera-

tor. No drop in titer was detected over a period of 10 months.

7. Methods of plating phage.

a. Direct plating.

For routine assay of phage stocks or serum survivors, the agar layer method (43) was used. In the case of serum survivors, direct plating measures the number of phages which have escaped the attachment of antibody molecules to killing sites, plus the number of phages which have been reactivated by detachment of reversible antibody during the incubation of the plate.

b. Strong serum plating technique.

The fraction of antibody molecules in strong immune serum which will form a reversible union with a phage particle is very small. However, at low survival levels this becomes important, and an assay method is needed that will not score as survivors, phages that were reactivated after inactivation by reversible antibody. For this purpose Jerne (1954, personal communication) developed the "strong serum" plating technique. It consists of adding a 0.1 ml sample of the reaction mixture containing serum survivors to 0.2 ml of a suspension of young B cells, allowing 10 minutes for adsorption of the phage, adding 0.1 ml of 1:100 "strong" serum to kill within five minutes any phage particles still susceptible to serum and, finally, mixing the 0.4 ml with 2.5 ml of melted soft agar and pouring over the solid bottom layer of agar.

Soon after adsorption to a bacterium, a phage can no longer be inactivated by antiserum. Reversibly inactivated phage particles will have only a small chance to become reactivated during the ten minutes allowed, and are then irreversibly inactivated by the strong serum. The strong serum will not inactivate the survivors which adsorbed to the bacteria during the ten minutes, and will not prevent formation of plaques on the plate.

After pouring, all plates were incubated at 37° C for 16-18 hours and then counted.

C. Experimental results.

1. Choice of the reaction medium.

In an attempt to find a medium in which the rate of inactivation is rapid and both the antiserum and phage are sufficiently stable, the serum titer was measured in different concentrations of buffers over a range of pH's. Jerne and Skovsted (38) showed that the rate of inactivation is maximal in distilled water and is progressively reduced in solutions of higher ionic strength. Consequently, the use of any buffer would decrease the rate of inactivation. It turned out, however, that 0.0005 M buffer is sufficient to maintain a constant pH with the concentration of phage and antiserum required in experiments. Also, both the antiserum activity and the phage are fairly stable in this medium, and the rate of inactivation is not greatly reduced.

a. Experimental procedure.

Serum titers under different conditions of pH and ionic strength were measured by the same procedure. From a stored 1/100 dilution of antiserum, 0.05 ml was further diluted into 1000 ml at 37° C to make a final dilution of $1:2 \times 10^6$. At zero time, 0.5 ml of T4r diluted to contain 2×10^7 phage particles/ml was added to the liter of diluted antiserum to give an initial phage input of 10,000/ml. Every minute (during the first five minutes) 0.05 ml - 1.0 ml samples of the mixture were plated in duplicate by rapid mixing with 2.5 ml of melted soft T agar plus bacteria and pouring over a sublayer of solid T agar. The serum action is immediately arrested by the salt concentration in T agar, making it unnecessary to dilute the mixture before plating. The serum titer was then calculated from the slope of the line obtained by plotting log survivors against time.

b. Ionic strength and pH.

Fig. 1 shows rates of inactivation at varying ionic strength and constant pH; Fig. 2, at varying pH and constant ionic strength. Phosphate, citrate, anthranilate, and pyridine acetate buffers were used to maintain stable pH over the range of 4.9 to 7.6 and ionic strength from 0.015 to 0.00018. The rate reaches a maximum around pH 5.7. The height of this maximum depends on the ionic strength. The rate of inactivation is difficult to measure below pH 5.0 because the phage is not stable. Above an ionic strength

of 0.001, the rate of inactivation rapidly decreases. The maximum serum titer of approximately 2.5 x 10^6/min measured in these experiments agrees well with the maximum titers measured in distilled water reported by Jerne and Skovsted (38). It turned out not to be practical to work at pH 5.7, however, because the phage was not sufficiently stable. In 0.0005 M phosphate buffer at pH 6.5, the phage was reasonably stable--80% surviving after three hours at 37° C. This medium was adopted for most of the subsequent experiments.

c. Stability of antibody activity.

1) Dilution of the antiserum.

In addition to the salt effect, Jerne and Skovsted (38) described a cofactor effect on the rate of inactivation of T4r by serum diluted in distilled water. When antiserum against T4r was diluted in a small volume of distilled water (about 10 ml), the inactivation was found to be very rapid in a dilution of 1:50,000, but in serum diluted 1:100,000 or more, no inactivation was observed. This sudden inhibition of inactivation was considered to be due to the adsorption onto the glass surface of the vessel of a cofactor present in antiserum which is necessary for inactivation of the phage in distilled water. This experiment was repeated, and results similar to those reported by Jerne and Skovsted (38) were obtained. The maximal dilution which was still active without the addition of extra cofactor was found to be variable from day to day. Sometimes a dilution of

1:40,000 was inactive, and occasionally a dilution of 1:2,000,000 was active. This variability may be due to variations in the cofactor-adsorbing properties of the glass surface and in the length of time the diluted antiserum was allowed to remain in contact with it. These complications could be avoided by making a large dilution step into a large volume. When 0.05 ml of antiserum diluted 1:100 in H buffer is delivered directly into one liter of swirling 0.0005 M phosphate buffer and rapidly mixed by pouring back and forth into another flask, the antiserum activity is stable without the addition of cofactor, and the rate of inactivation is reproducible from day to day. Antiserum diluted in low ionic strength medium must not be allowed to stay in contact with a large glass surface or activity will be lost. For example, when 0.05 ml of antiserum 1:100 remains for twenty minutes in the bottom of a flask wet with dilute buffer before diluting, about half the serum activity is lost.

2) Effect of volume on the stability of antibody.

The stability of the phage neutralizing activity of antiserum highly diluted in low ionic strength medium depends on the volume. Antiserum was diluted quickly in a large volume as described above. After dilution it was allowed to stand for increasing times at 37° C in different volumes. The inactivation rate was then measured as described above, with the concentration of input phage adjusted to make a final con-

centration of 10,000/ml in all cases.

It was found that there was no measurable loss of serum activity after three hours at 37° C in 1000 ml volume. In 100 ml volume the serum activity gradually decreased to about 70% of the initial activity in three hours. In attempting to measure the stability of serum diluted 1:2 x 10^6 in 10 ml volume, irreproducibility was again encountered. The serum activity was erratic from day to day. Sometimes the activity was stable for an hour, while other times in the control 10 ml sample even at zero time it was less than 10% of that expected. As mentioned above, it is suspected that this phenomenon is due to unpredictable variations in the surface properties of the test tubes and pipettes. As long as large volumes are used, however, the difficulty is avoided and the serum titer is constant and reproducible.

2. Survival curves.

a. Dependence on phage input.

The survival with time of T4r was followed in one liter mixtures of antiserum diluted 1:10^6 in 0.0005 M phosphate buffer pH 6.5 at 37° C. The survivors were assayed by direct plating. The resulting survival curves are represented in Figs. 3,4,5,6 and 7. Initial phage inputs of 10^4, 10^5 and 10^6/ml corresponding to $\underline{r}$ values of 0.00125, 0.0125 and 0.125, (large antibody excess) are represented in Fig. 3. The slopes of these three curves are the same. The value of $\underline{nkA}_u$ calculated from these slopes is 1.6 x 10^6/min. Initial

phage inputs of 8×10^6, 1×10^7, 2×10^7 and 4.5×10^7, corresponding to $\underline{r}$ values of 0.5, 0.625 (small antibody excess) 1.25 and 2.8 (phage excess) are represented in Figs. 4, 5, 6 and 7 respectively. Theoretical curves are plotted together with the experimental curves. The basis of the estimates of $\underline{r}$ and the construction of the theoretical curves will be discussed later. Within the limits of the assay technique, the initial slope of the survival curves remains the same. At progressively higher phage inputs the curves flatten out at progressively higher survivor fractions, indicating an exhaustion of antibody. This interpretation is confirmed by other experiments. The curves do not extrapolate to the level of the input phage at zero time, but show a delay of about 30 sec. An explanation for this may be found in the experimental technique. Since it took 20-30 seconds to thoroughly mix 0.5 ml of phage with 1000 ml of diluted antiserum, it is difficult to actually pinpoint zero time. In these experiments the stopwatch was started when the phage began to flow from the pipette. Other experiments done in smaller volume show shorter lags and in several cases no lag at all. The curves are interpreted, therefore, as one-target curves. The bend of the curves at low survivor levels is complicated by the presence of the small fraction of antibodies in the antiserum which inactivate the phage reversibly. These antibodies detach during the incubation of the plate, allowing phage particles to form plaques which should not be

scored as survivors.

b. Dependence on serum concentration in serum excess.

The rate constant of inactivation was measured by the same procedure described in section C,1 above, except that the concentration of antiserum was varied. Table 1 shows that the serum titer is independent of the serum dilution at which the test is carried out. This confirms that the neutralization rate constant is directly proportional to the first power of the antiserum concentration.

3. Antibody depletion.

a. Partial depletion of antiserum.

A 1: 100 dilution of antiserum was further diluted to 1: 10^6 by mixing 0.1 ml with a liter of 0.0005 M phosphate buffer, pH 6.5. The buffer contained a 1: 10^5 dilution of normal rabbit serum to insure against cofactor depletion. One hundred ml aliquots were measured into 5 small flasks and placed in a 37° C waterbath. At zero time, inputs of 0.5, 1.0, 2.0 and 3.0 x 10^7 T4r/ml were added to four flasks and the fifth was kept as a control. After three hours, 0.05 ml from each flask was plated by the "strong" serum plating technique. A second input of phage of 10^4/ml was then added. The rate of inactivation of this second phage input was measured by plating 0.05 ml aliquots by the "strong" serum plating method in duplicate every minute during the first five minutes of the reaction. The results of this experiment are

presented in Table 2. Judging from the remaining serum activity in flasks 2 and 3 three hours after this first input of phage, all antibody should have been depleted in flasks 4 and 5. Evidently, the reaction in flasks 4 and 5 had not gone to completion. (This point will be discussed later.) From the values obtained from flasks 2 and 3, it can be calculated that it requires approximately 1.6×10^7 phages to just deplete the neutralizing antibody in antiserum diluted 1: 10^6. Therefore, r = 1 for $D = 10^{-6}$ and $P_o = 1.6 \times 10^7$/ml. For the undiluted serum we thus obtain $A_u = 1.6 \times 10^{13}$ N antibody molecules/ml.

b. Survivors at infinite time.

Phage inputs from 1×10^7/ml to 1.6×10^8/ml were mixed with antiserum diluted 1: 10^6 in one liter volumes at 37° C and assayed two and three hours later by direct plating and by strong serum plating. The results are tabulated in Table 3. Since the survival level did not decrease from two to three hours, the reaction probably had gone to completion. For phage inputs greater than 2.0×10^7, there is no difference between the assay of survivors by direct plating and by strong serum plating. This suggests that the contribution to the survivors by phage particles temporarily inactivated by reversibly attaching antibody molecules and subsequently reactivated, is negligible at survival levels of 3% or greater. At lower survival levels, the difference in survivors as measured by the two plating methods is very

great. Since the strong serum plating technique is not completely efficient in eliminating a last remnant of spurious survivors, it cannot be decided with certainty whether the survival levels obtained by the strong serum plating technique for phage inputs of 1.0 and 2.0 x 10^7/ml are real or apparent. It is suspected, from the small and variable size of the plaques, however, that the values are spurious and that the survival should be considered to be zero.

The data in Table 3 indicate that it requires between 2 and 4 x 10^7 T4r/ml to deplete antiserum diluted 1:10^6. Considering the element of uncertainty in interpreting the measurement of extremely low survival levels, this estimate is in reasonably good agreement with the estimate of 1.6 x 10^7 for the same quantity in section a above.

Using the value of 1.6 x 10^7 from section a to calculate r for the three highest phage inputs of Table 3, we obtain r values of approximately 10, 5, and 2.5 respectively. From these values of r and from the average survivors at infinite time given in Table 3, n can be calculated by equation 12. The n values thus found are 16.2, 10.6 and 11.5 respectively. Considering the greater experimental variation of the data for the survivors from the highest phage input, and the greater likelihood that aggregation may have contributed to the survival level measured at this phage concentration, the value 16.2 is likely to be a poorer estimate of n than the other two.

The data, therefore, indicate that n (the number of killing sites on a phage particle) is larger than 10, and is certainly not 1 or 2.

D. Discussion.

1. Comparison of theoretical and experimental curves.

In Figs. 4, 5, 6, and 7 are plotted the theoretical curves of the time course of phage inactivation together with the corresponding experimental curves. The theoretical curves were calculated from equation 7 by using the experimentally determined values, $\underline{nKA}_u = 1.6 \times 10^6$, $A_u/N = 1.6 \times 10^{13}$ and $n = 11$. The experimental curves are all found to deviate from the calculated curves in essentially the same way; they flatten out sooner and faster. The deviation is greater with greater depletion of antiserum. One might expect this sort of deviation if not all of the antibody molecules reacted at the same rate, but some reacted more slowly.

The assumption in the development of the theory that the antibody population is homogeneous is probably not a very good approximation of the actual situation. It is well known from many antigen-antibody systems that an antibody population is heterogeneous with respect to the combining power and specificity of the antibody molecules. Let us analyze, therefore, how a heterogeneity in rate of reaction among the antibody population would affect the theory.

Instead of a homogeneous antibody population let us consider a population made up of fractions differing in the rate constant of inactivation. The experiments in large serum excess measure, then, a value of $\underline{nKA}_u$ which represents the sum of the contributions of all the fractions, the relative contribution of each fraction remaining constant throughout the experiment, and for each fraction its contribution being in proportion to its size and reactivity. The value of $\underline{nKA}_u$ under these circumstances is not a valid estimate of the concentration of antibody in a serum. A large remaining fraction of slower reacting antibody, for example, may be estimated by this criterion as being equal to a much lower remaining concentration of faster reacting antibody. One might explain the results obtained in the partial depletion experiments on this basis. Consider the case, for instance, where after incubating a large input of phage with a dilution of antiserum, it is found that the $\underline{nKA}_u$ has been reduced, because the rate at which a second small input of phage is inactivated is only 30% of the control. It does not necessarily follow that 70% of the antibody has been removed. It may mean that the fraction of more rapidly reacting antibody has preferentially been removed, leaving behind a slower reacting population. The data, in fact, appear to support this interpretation. Incubation with almost three times the amount of phage necessary to remove 70% of a $1{:}10^6$ dilution of antiserum leaves about 10% of the antibody activity after the

reaction should have gone to completion. Therefore, heterogeneity of antibody will cause an underestimation of $\underline{A}_u/\underline{N}$ by the partial depletion experiments and consequently an underestimation of $\underline{r}$. In the calculation based on the experiment determining survivors at infinite time, an underestimate of $\underline{r}$ will result in too high an estimate of $\underline{n}$ from equation 12. The interpretation of the experimental determination of the survivors at infinite time with an excess of phage, however, is not affected by heterogeneity in the antibody population. By using two values of finite survivors for two corresponding phage inputs, it is possible from equation 12 to evaluate both $\underline{n}$ and $\underline{r}$ simultaneously. The three values available from Table 3 were plotted and compared with Fig. 8. This comparison yielded values for $\underline{n}$ between 10 and 30, and values of $\underline{A}_u/\underline{N}$ between 1.5 and 3.5×10^{13}.

Since these values are in good agreement with those obtained from the partial depletion experiments assuming uniform antibody, the fraction of slower reacting antibody cannot be very large. Judging from the activity left in the partial depletion experiment when all the activity should have been taken out, a slower reacting fraction of the antibody population of about 10-30% may be enough to account for the anomalous residual activity.

2. Summary of the literature review of the phage-antiphage reaction.

Phage is antigenic. Inactivated phage may retain its

antigenicity. Phage antigens are different from bacterial antigens and are easily separable from them. The phage antigens are specific. This specificity is not changed by mutations affecting other characters, and mutations affecting a serological character are not known. Serological classification is of value in establishing mutual relationships between viruses. Some phages show serological cross reactions. Cross absorption of antisera by serologically related phages removes all neutralizing activity for the related phage and leaves almost full activity for the homologous phage.

Phage preparations can be obtained which are remarkably uniform in their neutralizability by antiserum. Phage lysates contain material which resembles separated heads and tails. Purified preparations of the head precursors, called doughnuts, have complement fixing activity, but no blocking power for the neutralizing activity in the serum. Phage has been demonstrated to have two antigens, one present on the head and one on the tail. Tail antigen is not located exclusively on the tip of the tail. Phage particles adsorbed to dead bacteria are antigenic and are still able to combine with neutralizing antibody.

The reactions between head and tail antigen and their corresponding antibodies may both be accompanied by complement fixation and precipitation. Neutralization is caused exclusively by the reaction between tail antigen and antibody (except for aggregation effects). Complement seems not

to be needed for the neutralization of phage by antibody.

Serum survivors may in some instances have altered properties. Serum inactivated phage may adsorb without infecting its host or may be prevented from adsorbing altogether. Adsorbed phage is completely unaffected by neutralizing antiserum.

Strong immune serum reacts irreversibly with phage. Early serum contains a large fraction of reversible antibody molecules. Late serum contains a smaller fraction of such reversible antibody. Phage inactivated by serum may be completely reactivated by digestion of the antibody with papain. Reactivability by papain decreases to zero as more and more antibody is allowed to attach to the phage.

In low ionic strength medium the rate of neutralization of phage is 1000 times faster than in broth or buffer of ionic strength around 0.1. The neutralization reaction of phage and antiphage highly diluted in low ionic strength medium requires a cofactor present in normal serum. The natural cofactors in serum and other substances that show cofactor activity have a high isoelectric point.

The time course of the serum neutralization of phage is exponential. The exponential rate of inactivation may or may not be preceded by a short time lag. This lag may be due to secondary complications and does not necessarily imply that combination with more than one antibody molecule is necessary for inactivation. The inactivation curves usually

flatten out when most of the phages have been inactivated. The inactivation rate in electrolyte containing medium has a Q_{10} of 2.0. In distilled water it has a Q_{10} of 1.4. In buffered solutions the inactivation rate is indifferent to pH between 5.0 - 10.0. In very low ionic strength, the rate is decreased outside the range of pH 4.8 - 7.2.

The absolute rate of attachment of antibody molecules to a site on a phage has been recalculated from kinetic theory according to a relation worked out by Hershey (32). This value at 37° C is 2.2×10^{-11} C_1 per sec calculated for the whole phage tail or 5.4×10^{-12} C_1 per sec calculated for the tip of the phage tail (C_1 is the number of antibody molecules per ml).

3. Estimates of parameters.

a. The number of antibody molecules that can fit on the tail of a phage particle.

We begin with a few purely geometrical considerations. The dimensions of a water soluble horse Diphtheria antitoxin molecule are 286 x 39 Å calculated as anhydrous prolate ellipsoid of revolution (42). Assuming that the antibody molecules attach to a phage tail by their ends and that they occupy a circular area corresponding to their largest cross section, viz., 39 Å, each molecule occupies an area of about 1.2×10^{-13} cm^2. Assuming the phage tail to be a cylinder 25 millimicrons in diameter and 100 millimicrons in height, the area of the exposed surface is 8.3×10^{-11} cm^2, and the

area of the tip of the tail is 4.9×10^{-12} cm^2. From the ratio of the areas, the maximum number of antibody molecules able to fit on the entire phage tail is about 700, and on the tip of the tail is about 40. Other estimates of the number of molecules fitting on the tip of the tail were made with the aid of a physical model. The antibody molecules were approximated as cylinders and the tip of the tail as a circle. With closest packing, the maximum number fitting on the tip of the tail is 30. From the arrangement giving the smallest number that can exclude others from finding a sufficient area, the minimum number is 13.

b. The number of antibody molecules in antiserum and the collision frequency between phage and antibody.

The experimental estimate of $\underline{A}_u/\underline{N}$, from depletion data, is 1.6×10^{13}. By estimating $\underline{N}$, a value for $\underline{A}_u$ may be calculated. The upper limit of $\underline{N}$ is equal to the number of antibody molecules fitting on the tail of the phage, or about 700. Thus, the number of inactivating antibody molecules in undiluted antiserum must not be greater than 1.1×10^{16}/ml. The horse serum obtained from Dr. Jerne contains 89 mg total solids per ml. If all the solid material represented antibody molecules of molecular weight of 180,000, it would be equivalent to about 3×10^{17} molecules/ml. The actual number of antibody molecules must obviously be much less than this. The estimate of 10^{16} molecules/ml derived above as an upper

limit, is compatible with it.

Next, let us consider the implications of the upper limit estimate of $A_u = 10^{16}$ molecules/ml for the neutralization rate. We assume that neutralization results from successful collision between antibody and the tip of the phage tail.

The calculated number of collisions with the tip of the phage tail (end of section III) is 5.4×10^{-12} C_1 per sec. Substituting 10^{16} molecules/ml for C_1 gives a value of about 5×10^4 collisions per second with the tip of the phage tail. The value estimated for the rate of inactivation by undiluted horse serum under optimum conditions is about 2.5×10^6/min or about 4×10^4/sec. With this estimate of the number of antibody molecules, the killing efficiency per collision with the tip of the tail is about 0.8. This is a remarkably high efficiency. It agrees with the fact that the rate of inactivation in distilled water is temperature independent. Moreover, it shows that our upper limit estimate of A_u is also a lower limit estimate. A smaller A_u would lead to the absurd conclusion of a collision efficiency greater than unity. Thus, our estimate of A_u, constituting both an upper and a lower limit, must be approximately correct. Therefore, also the number of antigenic sites on the phage tail must be close to the geometrical maximum, N = 700.

4. Speculations on the phage-antiphage reaction in low ionic strength medium.

a. The mechanism of phage inactivation.

The number of killing sites on a phage estimated experimentally turns out to be roughly equal to the calculated number of antibody molecules capable of fitting on the tip of the phage tail. Since it is known that phages adsorb to their host by the tip of the tail and that this adsorption is specific, one may imagine that the attachment of an antibody molecule anywhere on the tip of the tail prevents phage adsorption to the host bacterium, or, when adsorption is still possible, prevents injection of the DNA. When more antibody molecules have attached to the tail even adsorption of the phage is prohibited.

b. The increase in rate of inactivation in low ionic strength medium.

One might expect a rate of reaction to increase with reduction of ionic strength if the reaction took place at a pH between the isoelectric point of the two reactants. The absence of additional charges would allow the charges of the reactants to display themselves, increasing their effective attractive force. The isoelectric point of normal horse gamma globulin (and presumably also of immune globulin) is 5.7 (44) and that of phage is estimated to be around 4.3 (45). Fig. 2 shows that the rate of inactivation reaches a maximum around pH 5.7, a little higher than in the range

between pH 4.3-5.7. However, the isoelectric point of the antibody-cofactor complex, which according to Jerne and Skovsted (38) is the actual inactivating agent, would be expected to have a higher isoelectric point than globulin alone because of the high isoelectric point of cofactor. Jerne and Skovsted (38) gave evidence that salt acts antagonistically with cofactor and have suggested that increased salt concentration increases the dissociation of antibody and cofactor. Evidence from the Q_{10} of the reaction in high and low salt medium suggests that in high salt concentration the neutralization of phage requires an activation energy of the particles involved, but not in low salt concentration. The increase in the reaction rate in low ionic strength medium may be considered, then, as an increase in the probability that a collision between antibody and phage will result in specific union.

5. A model of the phage-antiphage reaction.

In conclusion, the picture of the phage-antiphage system may be described as follows. A phage particle consists of a head and a tail. The antigenic attachment sites for antibody are different on the head and on the tail. The head antigens and their corresponding antibodies do not take part in the phage inactivation reaction. All antitail molecules, however, are capable of inactivating the phages. On the tail of the phage particle there is room for not more than 700 antibody molecules. On the tip of the tail there is room

for about 30 antibody molecules. A phage is inactivated when at least one antibody molecule attaches to a site on the tip of the tail. These sites on the tip of the tail are the "killing sites" of the theory. Attachment of antibody molecules to the sites along the sides of the tail does not interfere with the infectivity of the phage particle, but depletes neutralizing antibody from the medium. In the undiluted hyperimmune antiserum used in the experiments, there are about 10^{16} specific antitail antibody molecules per ml. The killing efficiency of the collisions between phage particles and antitail antibody in distilled water is very high, almost every collision resulting in the inactivation of a phage particle.

Table 1

The Serum Titer in Antibody Excess as a Function of Antiserum Concentration

Dilution of antiserum	Serum titer (nKA_u)
$1: 1 \times 10^6$	$.99 \times 10^6$
$1: 2 \times 10^6$	1.1×10^6
$1: 4 \times 10^6$	1.1×10^6
$1: 8 \times 10^6$	$.95 \times 10^6$

Table 2

Partial Depletion of Antiserum Diluted 1: 1×10^6 and Determination of Residual Serum Titer

Flask No.	First phage input/ml	Second phage input after 3 hrs at 37° C	Serum titer for second input	Fraction of serum titer depleted	Calc P_o for 100% depletion
1	none	1×10^4	7.6×10^5	1.00	--
2	0.5×10^7	1×10^4	5.4×10^5	0.30	1.7×10^7
3	1.0×10^7	1×10^4	2.8×10^5	0.64	1.6×10^7
4	2.0×10^7	1×10^4	1.2×10^5	0.84	2.4×10^7
5	3.0×10^7	1×10^4	7.7×10^4	0.89	3.4×10^7

Table 3

Survivors at Infinite Time with Antiserum Diluted 1: 1×10^6

Input phage	Survivors					
	After 2 hours		After 3 hours			
	Direct plating	Strong serum plating	Direct plating	Strong serum plating	Average survivors	p
1.6×10^8	2.7×10^7	1.6×10^7	3.5×10^7	3.7×10^7	2.9×10^7	0.18
8.0×10^7	7.0×10^6	7.1×10^6	7.4×10^6	8.2×10^6	7.5×10^6	0.09
4.0×10^7	1.1×10^5	9.8×10^4	1.1×10^5	1.2×10^5	1.1×10^5	0.0027
2.0×10^7	6.8×10^3	$1 \times 10^{2*}$	6.2×10^3	< 10	0 (?)	--
1.0×10^7	7.0×10^3	$8 \times 10^{1*}$	6.6×10^3	$3 \times 10^{1*}$	0 (?)	--
control without antiserum 4.0×10^7	3.8×10^7	3.8×10^7	2.4×10^7	3.0×10^7	--	--

* Inaccurate, based on very few plaques.

FIGURES

1. Rate of neutralization of phage T4r in antiserum diluted 1: 2×10^6, varying ionic strength (μ) and constant pH.
2. Rate of neutralization of phage T4r in antiserum diluted 1: 2×10^6, varying pH and constant ionic strength (μ).
3. Survival curves of phage T4r in serum excess. Antiserum diluted 1: 2×10^6 in 0.0005 M phosphate buffer, pH 6.50, 1000 ml volume, 37° C
4,5. Survival curves of phage T4r in serum excess with serum depletion. Solid curve is theoretical curve fitted to the experimental. Antiserum diluted 1: 2×10^6 in 0.0005 M phosphate buffer, pH 6.50, 1000 ml volume, 37° C.
6,7. Survival curves of phage T4r in phage excess. Solid curve is theoretical curve fitted to the experimental. Antiserum diluted 1: 2×10^6 in 0.0005 M phosphate buffer, pH 6.50, 1000 ml volume, 37° C.
8. Theoretical curves of p_∞ versus $\underline{r}$ for different values of $\underline{n}$ according to equation 12, Section IV.
9. An illustration of size and form relations between a phage particle and antibody molecules.

FIGURE 1

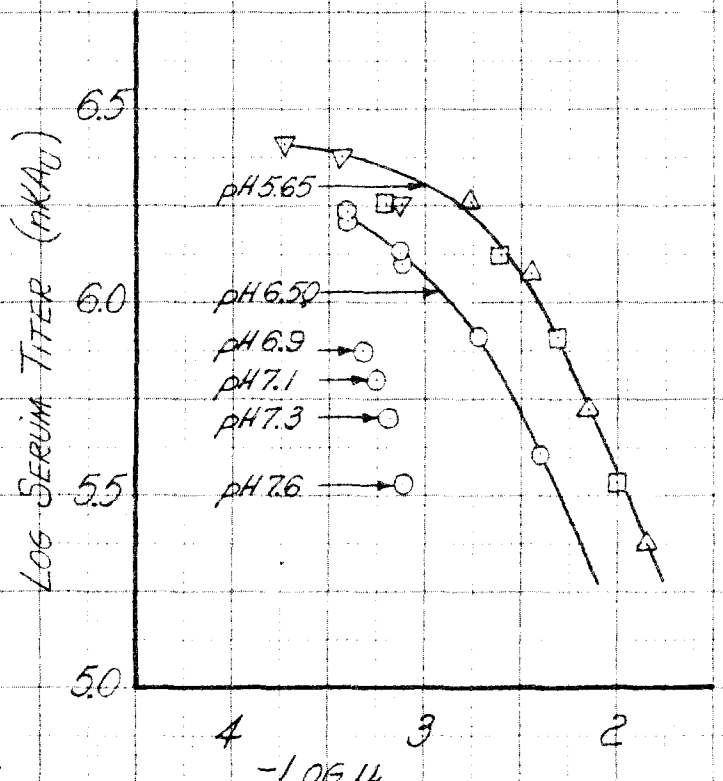
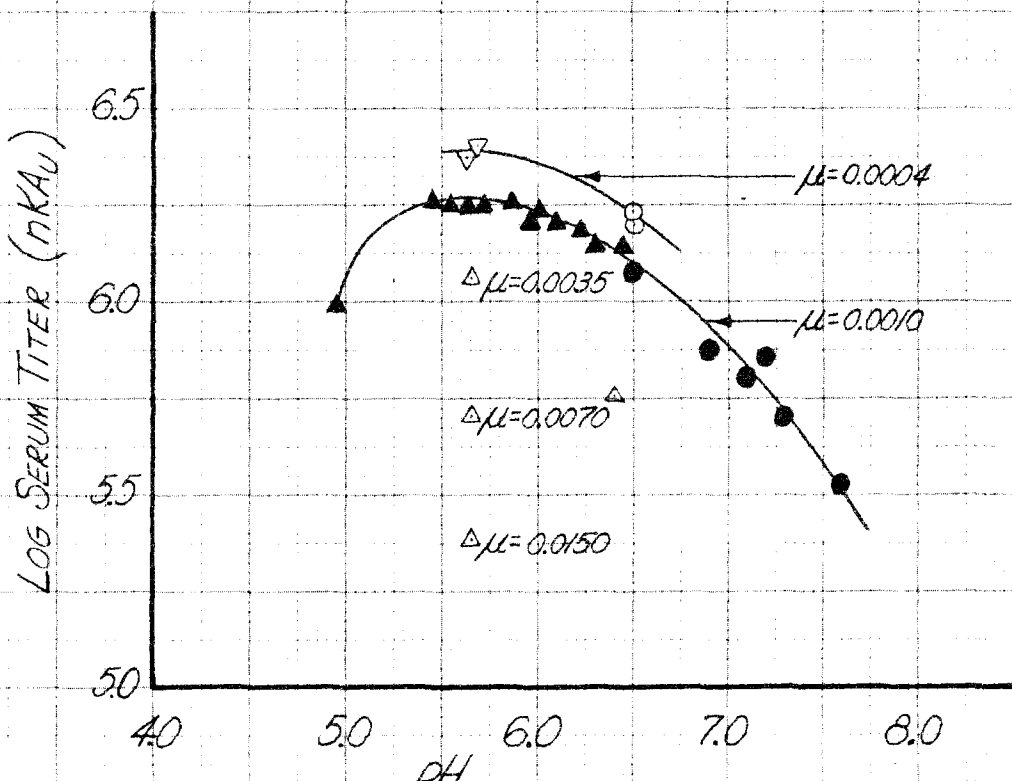


FIGURE 2



●,○ PHOSPHATE
▲,△ CITRATE
▽ ANTHRANILATE
□ PYRIDINE ACETATE

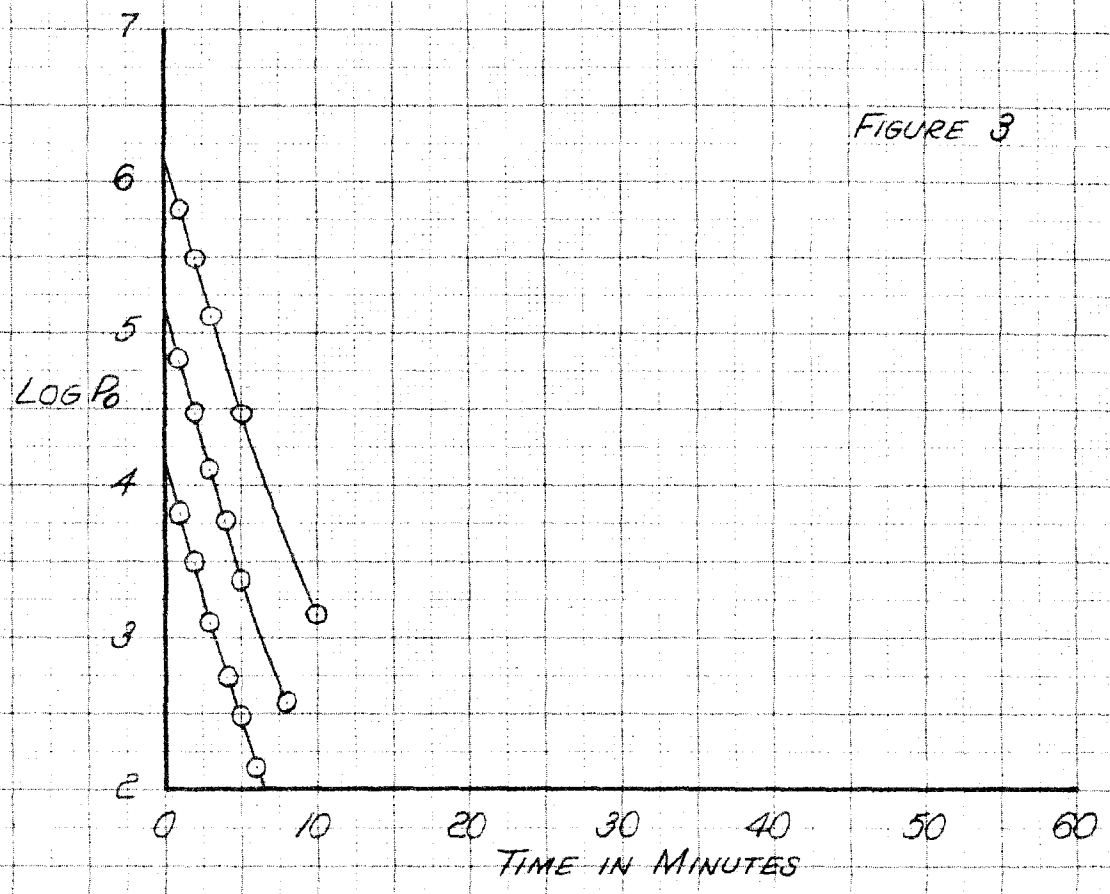


FIGURE 3

FIGURE 5

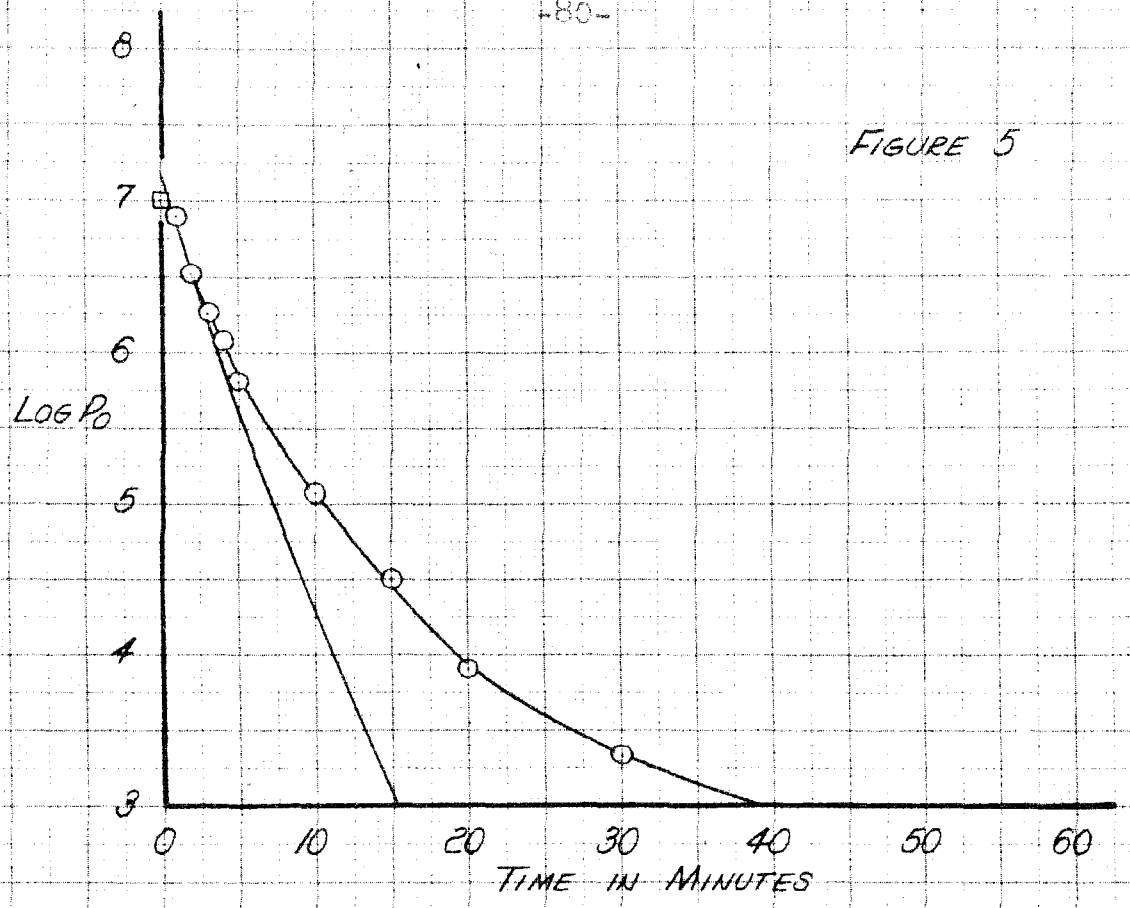
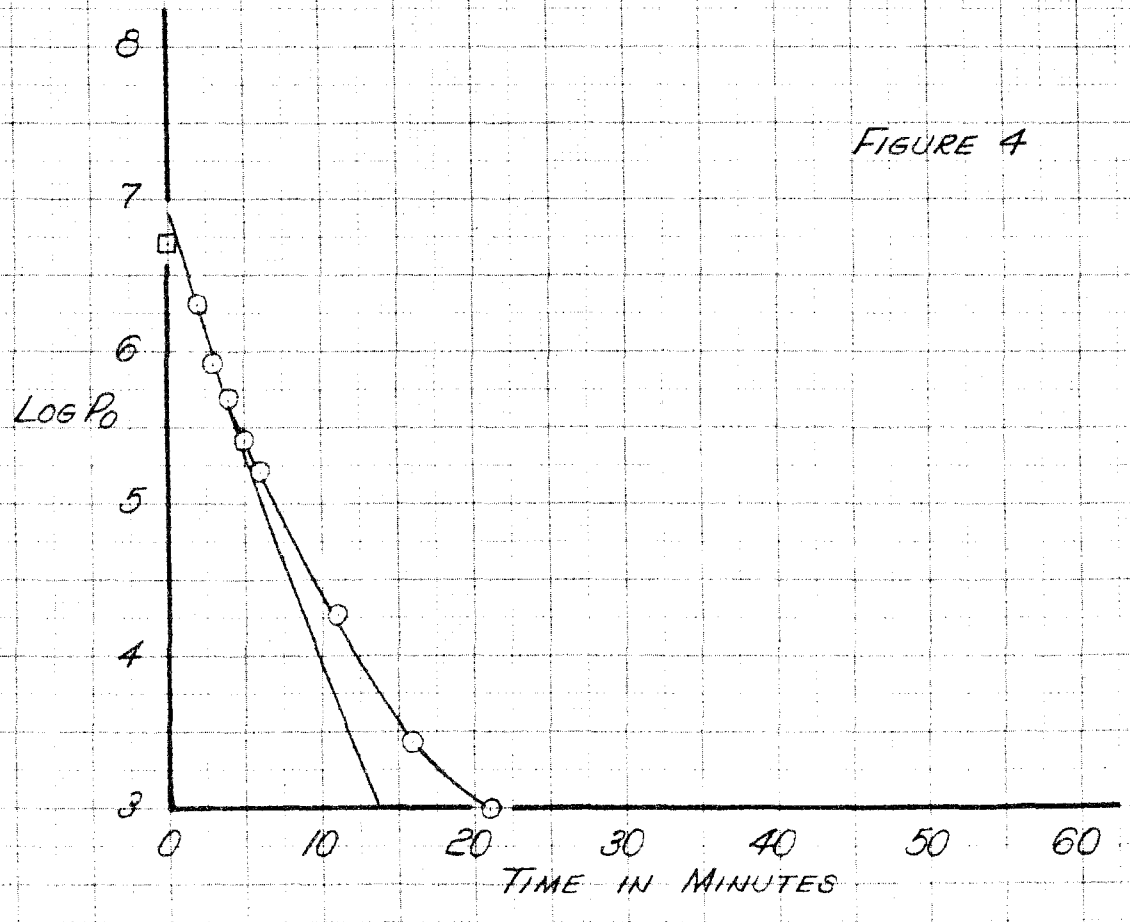


FIGURE 4



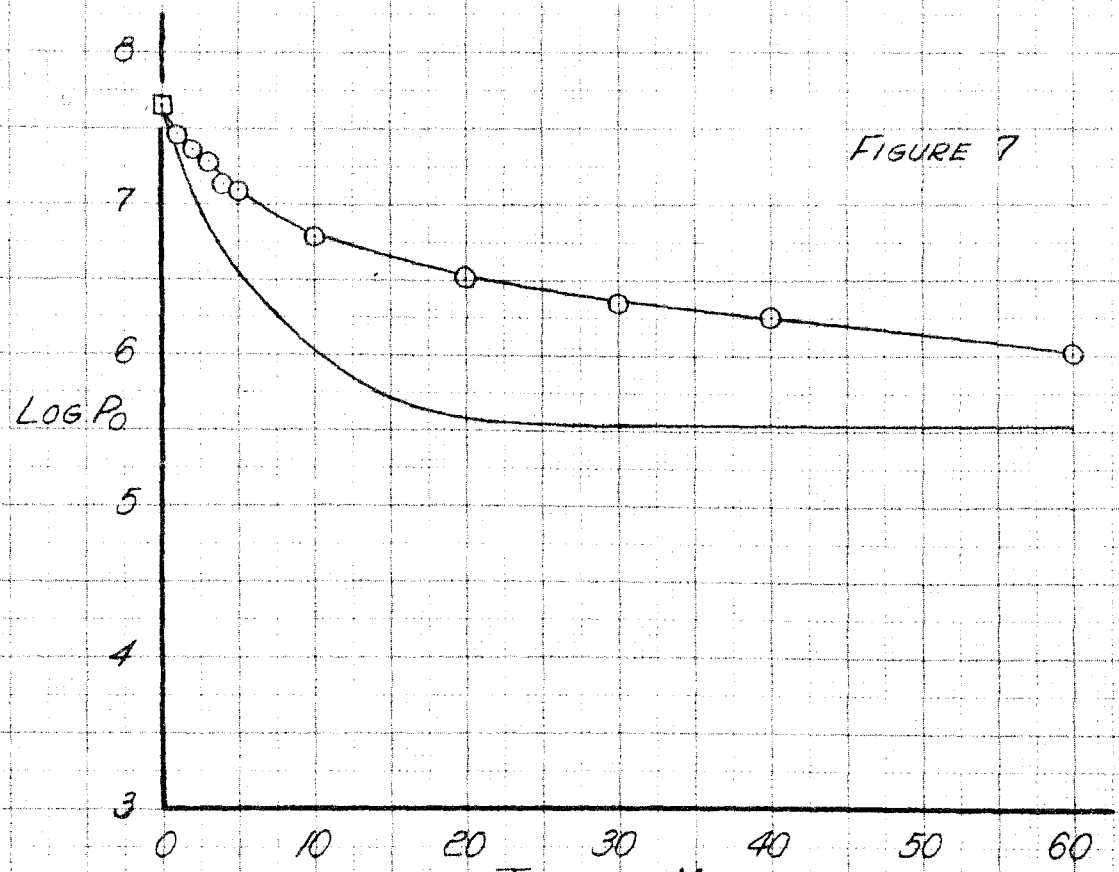


FIGURE 7

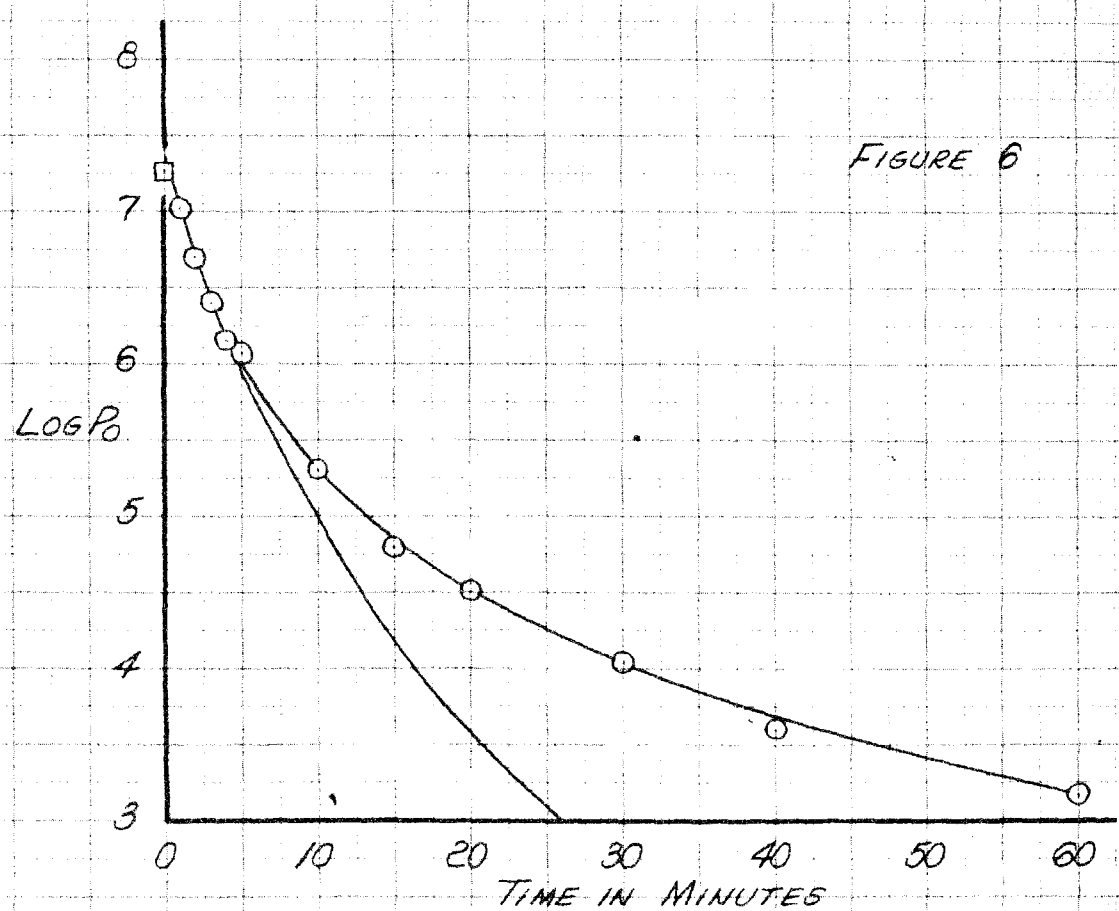
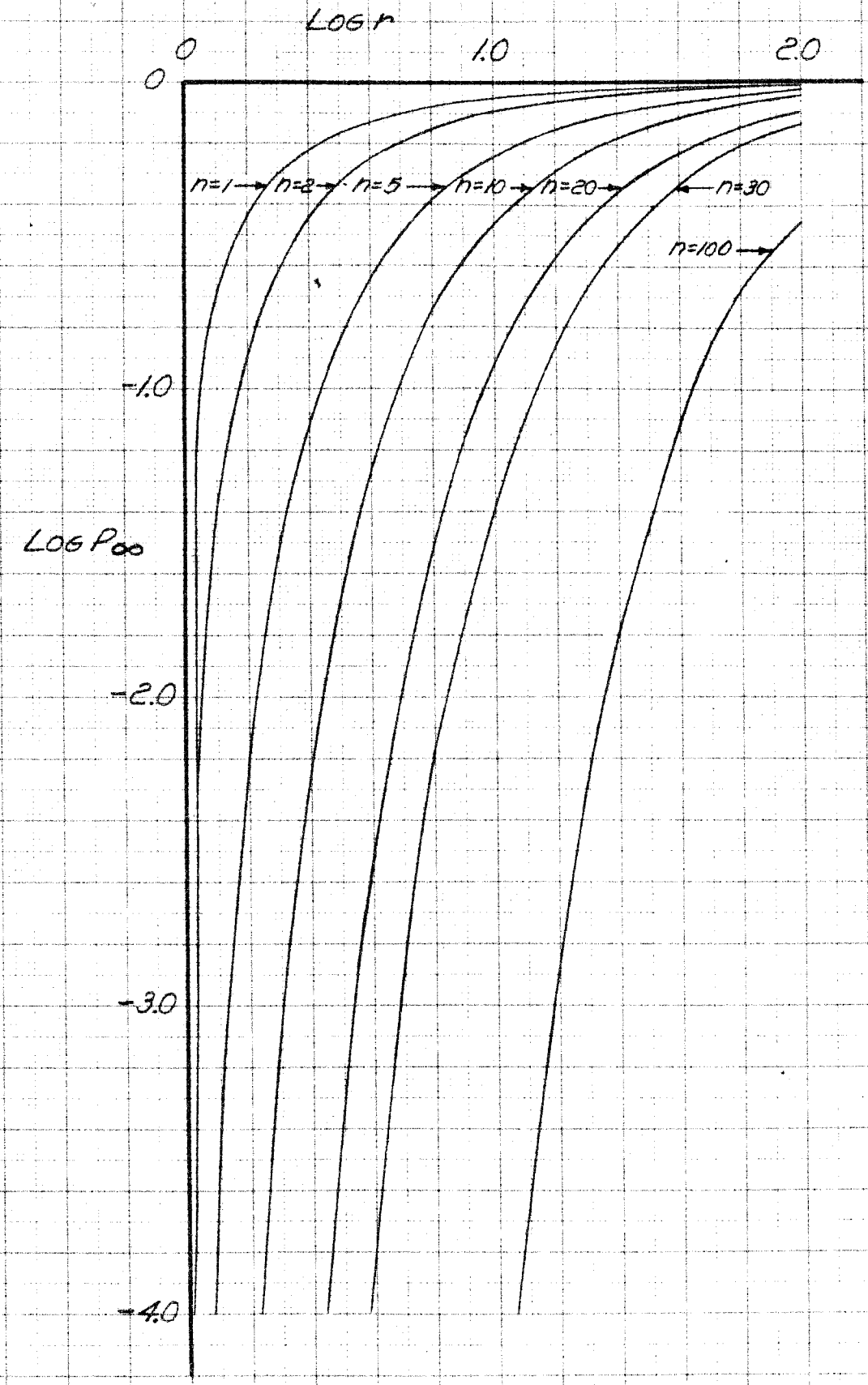


FIGURE 6

FIGURE 8



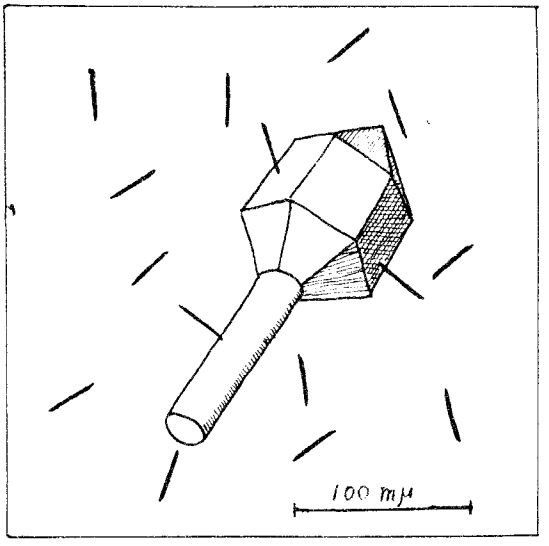


FIGURE 9

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