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NEUTRALIZATION OF BACTERIOPHAGE ϕX174 BY RABBIT ANTIBODY

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NEUTRALIZATION OF BACTERIOPHAGE ϕX174 BY RABBIT ANTIBODY

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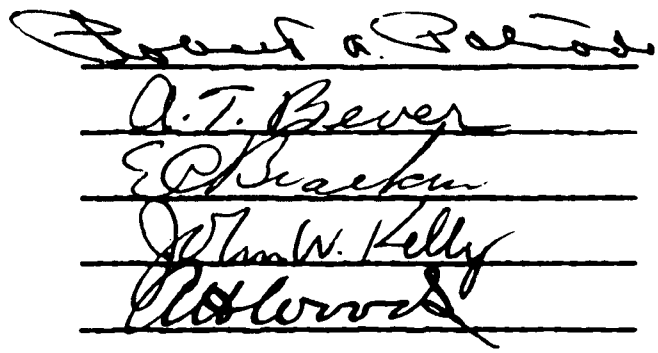

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CHAPTER I

INTRODUCTION AND HISTORY

Neutralization of Viruses by Antibody

The interaction between viruses and antibody is a kinetic process. The reaction is under the control of non-variable factors, such as the size, mass, and number of reactive sites of the two reactants, and variable factors, such as temperature, pH, and concentration, which affect the number of collisions between virus and antibody. Under fixed conditions, and with an excess of antibody, the number of collisions leading to neutralization is constant per unit time, thereby resulting in first-order kinetics of neutralization. Another aspect of the reaction of basic significance is that inactivation begins immediately after virus and antibody are mixed (Mandel, 1960). This absence of a lag phase can be interpreted as evidence that a single molecule of antibody is sufficient to cause neutralization of a virus particle.

There is no general agreement as to whether the virus-antibody reaction is reversible. The leading proponent of the concept of reversibility is probably Burnet (1955) who stated, "the virus antibody

reaction resulting in inactivation is irreversible in the case of the typical bacterial viruses and completely reversible in the case of the animal viruses." Later Burnet (1960) changed his opinion somewhat by stating, "the virus-antibody interaction resulting in inactivation was irreversible in the case of bacterial viruses and initially at least, wholly reversible for all the animal viruses we had tested." Earlier Burnet et al. (1937) had reported that 1:10 and 1:100 dilutions of a mixture of immune serum and human influenza virus produced the same number of "foci" on the chorio-allantoic membrane of the chick embryo as the original, undiluted mixture. They interpreted this finding as indicating dissociation of the virus and antibody. However Taylor (1941), working with influenza A virus, found that reactivation of neutral serum-virus mixtures was not complete and that approximately 90 per cent of the virus remained permanently neutralized. Tyrrell and Horsfall (1953), working with the WS strain of influenza A virus, obtained results supporting those of Taylor (1941). They tested the infectivity of serum-virus mixtures, after sufficient dilution, in the allantoic cavity of the chick embryo and detected reactivation of neutralized virus. However, only about ten per cent of the virus present in the original mixture became reactivated following dilution.

An example of reversible neutralization is found also in the tobacco mosaic virus (TMV). Rappaport (1959) found that TMV and specific rabbit antiserum combine in a manner characteristic of a reversible reaction. The results showed that the fraction of antibody remaining uncombined after prolonged re-equilibration increased approximately

threefold after the mixture was diluted 1:100. Rappaport (1961) subsequently observed that greater dissociation of the virus-antibody complex occurred at pH 2.0 than at pH 7.0.

Evidence favoring the concept that virus-antibody interaction is irreversible is just as convincing. The results of Dulbecco et al. (1956) showed no measurable dissociation of poliovirus-antibody complexes. Similarly, they observed no dissociation of Western equine encephalitis (WEE) virus from its complex with homologous antibody. In a critical analysis of the work of Dulbecco and his co-workers, Fazekas de St. Groth et al. (1958) and Fazekas de St. Groth and Reid (1958) concluded that, as a result of operational procedures employed by the former group, systematic errors were introduced that, if taken into account, would make the non-dissociation hypothesis untenable. Rubin and Franklin (1957) found that neutralized Newcastle disease virus (NDV) underwent between 0.1 and 1.0 per cent dissociation on dilution. Neutralization in this system therefore appears to be largely irreversible. After poliovirus and its specific antiserum react at pH 7.0 dilution and incubation of the virus-antiserum mixture results in only about 1.0 per cent dissociation (Mandel, 1959). In later studies with the same system, Mandel (1961) showed that the virus-antiserum complex could be made reversible by reducing the pH of the reaction mixture to between 2.5 and 2.0. That reactivation was the result of dissociation of the complex and not denaturation of the antibody was shown by the unimpaired capacity of the reactivated mixture to undergo reneutralization when the pH was adjusted to neutrality.

In a recent study with adenovirus type 5 and its specific antiserum Kjellén (1962) obtained results by two methods which indicated that the virus-antibody reaction was not reversible. The first was called the simultaneous inoculation method. By this technique antiserum and virus were inoculated simultaneously onto monolayers of MAS-cell cultures. The proportion of virions escaping into the cells was a function of (1) initial concentration of the virus; (2) the competition between combining sites of the virus particle for receptors of the cells and those of the antibodies; (3) the number of virions which never collided and reacted with antibody; and (4) the firmness of the union between virus and antibody in those which did collide and react. Kjellén found that monolayers treated with the virus-antiserum mixture for 15 to 240 minutes gave a constant number of plaques regardless of the incubation time. These results indicated that a completely stable, or irreversible, virus-antibody complex formed rapidly, since the number of cells infected in the test system did not increase. The second method measured the stability of the virus-antibody complexes that were formed after adding fractional parts of the total virus to fixed amounts of serum. This was a test of the Danysz effect. If the virus-antibody union were irreversible, the extent of inactivation of each increment of virus added would be dependent on the amount of free antibody remaining after the reaction of the prior virus with antibody. If the virus-antibody union were reversible, the extent of inactivation of each increment of virus added would be the same as when the virus was added in a single dose. To test this effect, Kjellén added different amounts of virus to a

constant amount of antiserum, incubated the mixture for various lengths of time, and measured the extent of neutralization. An additional amount of virus was then added to the mixtures and neutralization was again measured. Finally, virus was added a third time and neutralization was measured. The results supported the concept of a Danysz effect. There was no evidence for reversibility, even after incubation of the virus-antiserum mixtures for as long as 24 hours.

It is generally assumed that residual infectivity which follows virus-antibody interaction represents surviving virus particles with no, or very few, attached antibody molecules. The possible role of specific aggregates in the neutralization reaction and in infection has received little attention. In the foot-and-mouth disease virus-antibody system studied by Bradish et al. (1962), the fact that both residual infectivity and neutralizing activity existed simultaneously was interpreted in terms of distinct virus and antibody "regions" at the surface of the virus-antibody complexes. Neutralization was essentially irreversible. The authors envisioned that virus particles existing in virus-antibody complexes, the size of which varied with the serum/virus input ratio, were not inactivated but retained a potential infectivity. The results of these studies may help explain the discrepancies existing in the literature concerning the reversibility of virus-antiserum reactions.

The rate of neutralization of viruses is influenced by the temperature of incubation of the reaction mixture. In studies of poliovirus and its specific antiserum it has been found that the specific rate constant is changed by a factor of approximately 1.4 for each

10 C change of temperature (Mandel, 1960). Dulbecco et al. (1956) reported an energy of activation of approximately 6,000 calories $mole^{-1}$ for the neutralization of WEE by specific antiserum. This corresponds to a Q_{10} of approximately 1.4. From these two selected examples, it appears that neutralization of viruses represents a reaction in which the temperature dependence of the specific rate constant is a function of the diffusion rates of the reactants. The specific rate constant appears also to be dependent on the concentration of serum (Mandel, 1960).

Studies of influenza and NDV (Rubin and Franklin, 1957) viruses and their hosts cells have provided some insight into the mechanism of action of neutralizing antibody. Adsorption of the infective particle on the cellular surface occurs first. The entry of the infective virus into the host cell is, unlike that of bacterial viruses, a cellular function (Mandel, 1962). It has been shown to occur equally well with active or inactive virus, as long as the host cell is capable of pinocytosis or viropexis (Fazekas de St. Groth, 1948). The time required for passage of virus through the cell membrane is approximately one to two hours. Since the cell membrane must wet, flow around, and eventually engulf the virus particles the rate of engulfment is to some extent dependent on the charge of the two surfaces. In the earliest stages of viropexis, part of the virus is still exposed and hence accessible to antibody. Neuraminidase action at this stage can no longer prevent infection (Ackermann et al., 1955). The beginning of viral replication is marked by the disappearance of all viral properties recognized extracellularly. This is called the eclipse

phase. All components of the new generation of virus must be synthesized during the eclipse phase. Virus is formed in, or near, the cell membrane (Flewett, 1952) and released slowly as soon as it is formed (Cairns, 1952). The emerging virus particle must free itself from the cell surface via its neuraminidase before it can enter the surrounding medium or adsorb to another host cell (Cairns and Mason, 1953). The stages at which viral multiplication can be blocked by antibody have provided important information on the mechanism of neutralization. Antibody may bring about complete neutralization at one stage and yet give no apparent neutralization at another stage. Adsorption of the virus to the host cell can be prevented by pretreating the virus with antibody. Blocking of adsorption is favored either by pre-incubating the two components with antibody or by reducing the efficiency of the adsorptive surface. While the virus is on the surface of its host cell there is an early stage during which neuraminidase action is capable of liberating it (Fazekas de St. Groth, 1948). Stone and Ada (1952) showed that the virion probably moves about the surface of the cell in some form of two dimensional Brownian movement. It elutes from the cell when its position is shifted over an area which is free of neuraminic acid. During this stage the virion would be sensitive to neutralization by antiserum. During the second stage of viropexis the particle appears to be fixed on the cell surface. Complete removal of neuraminic acid by neuraminidase does not bring about elution of virus (Ackermann et al., 1955). However, its infectivity can be neutralized by antibody (Wagner, 1954). During viropexis, the virion would be sensitive to antibody only during the first stage

of its circumscription by the cellular membrane.

Once inside the cell, virus is beyond the reach of antibody. Rous and Jones (1916) demonstrated that an intracellular existence protects virus from antibody. In addition, antibody would be ineffective in systems where virus passes from cell to cell during cell division or through intercellular bridges.

The Phage-Antiphage Reaction

Since Bordet and Cuica (1921) first showed that bacteriophages were antigenic, the literature on the immunology of bacteriophages has grown enormously. Andrewes and Elford (1933a) showed that a 1:100 dilution of an antiserum against C36 phage neutralized 95 per cent of the homologous phage diluted 1:1,000,000 after four hours' incubation at 37 C. The same dilution of serum also neutralized 95 per cent of the undiluted phage under the same conditions. In further studies, Andrewes and Elford showed that the phenomenon held also for phages C16 and C13 and their respective antisera and they applied the term "percentage law" to the phenomenon. The percentage law states that in a neutralization experiment with a constant concentration of serum the fraction (P/P_0) of surviving phage, where P represents the actual number of surviving phages, is independent of the initial concentration of phage (P_0), over a wide range of the latter. Andrewes and Elford (1933b) concluded that unless there was a considerable excess of antibody in the fluid around each particle of phage, the phage could not receive a large enough coating of antibody for inactivation. They suggested that, if an excess of antibody was always necessary for in-

activation, the number of phage particles "must be few and far between compared with the available antibody." This interpretation is partially in error since later studies by Hershey (1941) and Mandell (1955) have shown that only one or two antibody molecules are necessary for inactivation of phage.

The percentage law can be expected to break down through at least two independent mechanisms (Burnet et al., 1937; Hershey et al., 1943). First, if the serum concentration/P_0 ratio is so low that the effective antibody concentration decreases during a fixed period of inactivation, one may expect P/P_0 to increase with P_0. Second, whenever P_0 is so high that the frequency of phage to phage collision allows significant aggregation, the surviving fraction, P/P_0, may be expected to decrease with increasing P_0.

The percentage law is applicable to the neutralization of animal viruses, as was shown by Mandel (1960) with poliovirus and by Bradish et al. (1962) with foot-and-mouth disease virus.

The classical studies of Burnet et al. (1937) on various phage-antiphage reactions showed clearly that the shapes of the phage survivor curves were influenced greatly by the concentration of serum used and that the differences in shapes of the curves were accentuated with progressive neutralization. When the experiments were performed with high concentrations of serum for one hour there was a precipitous fall in P/P_0, followed by a steady, slower rate of decline. At lower concentrations of serum there was an initial lag before an approximately steady rate of decline was reached. At high concentrations of serum there was a continuing action of the serum on the surface of the

plates. This effect increased with increasing serum concentration and accounted, in part, for the precipitous fall in P/P_0 at high serum concentration. To overcome this effect, the phage-antiphage reaction mixture was diluted in cold broth prior to plating. With this technique P/P_0 did not show the sudden, precipitous fall with a subsequent steady, slower rate of decline. The only deviation from an exponential decrease in P/P_0 was an initial lag period. The mathematical expressions for the neutralization reaction and the cause for the initial lag will be given below.

In general, over the usual experimental range, the percentage law may be used as a working rule which greatly simplifies the design of a neutralization experiment and the interpretation of its quantitative aspects. Because of the continuing action of serum on plates, it has become customary to work with dilute serum. The initial serum concentration/P_0 ratios must be such that, after incubation, a 1:50 to 1:100 dilution of the reaction mixture gives (1) essentially immediate cessation of the neutralization reaction, and (2) a residual P/P_0 fraction which may be plated directly (provided no more than a few thousand plaque-formers ml^{-1} are present) without risk of continuing action of the serum. This risk can be eliminated further by plating samples from the diluted reaction mixture by the soft-agar technique.

Since the phage-antiphage reaction is irreversible, or more strictly, does not reach equilibrium under practical conditions of study (Hershey, 1943), equilibrium constants have not been obtained for neutralization reactions. Therefore, essentially all phage neutralization experiments have been analyzed by the application of ki-

netic concepts. All kinetic approaches to the study of the neutralization reaction should take into account the percentage law. In the reaction

$$\text{Phage} + \text{Ab} \longrightarrow \text{Phage}\cdot\text{Ab}$$

Ab represents x molecules of antibody required to combine with, and to inactivate, a single phage particle. The rate of the reaction follows first-order kinetics down to one to ten per cent survivors. This means that the rate of neutralization is independent of the concentration of phage (percentage law). A plot of the logarithm of P/P_o against time yields a straight line, the slope of which is independent of P_o. Moreover, the slope is dependent on the concentration of antibody and the temperature of the reaction. The mathematical expressions for the reaction are

$$-dP/dt = Kt/D \text{ (differential form)} \qquad (1)$$

$$K = 2.3\ D/t \times \log P_o/P \text{ (integrated form)} \qquad (2)$$

$$P/P_o = e^{-Kt/D} \text{ (exponential form)} \qquad (3)$$

in which D represents the final dilution of serum in the phage-serum mixture, t is time, and K is the velocity constant (Adams, 1959). The K value is an extremely valuable characteristic of the serum.

Recently Bradish *et al.* (1962) presented a formula for the percentage law which was generalized to include a term showing the dependence of the percentage law upon the initial concentration of antiserum:

$$(P_o/P)^{1/n} = K \times S_o/D \qquad (4)$$

Here K defines the units in which S_0 (original serum concentration) is expressed. The value of K was given as 1.0 since the concern for antibody concentration is only relative. The term 1/n defines the nature of the dependence of P_0/P upon the antibody concentration (S_0/D) and it was found by the expression:

$$n = \frac{\log P_1 - \log P_2}{\log D_1 - \log D_2} \qquad (5)$$

in which P_1 and P_2 represent survivors in neutralization mixtures where a fixed concentration of phage reacted with two dilutions, D_1 and D_2, of antiserum. When the logarithm of P_0/P was plotted against the logarithm of the dilution of antiserum, the slope of the resulting curve also gave the value for n. Bradish et al. (1962) called the constant n the "slope constant."

The expression:

$$\log S_0 = 1/n \times \log (P_0/P) + \log D \qquad (6)$$

was obtained by taking the logarithm of both sides of equation (4). This formula is important since the neutralizing activity of the undiluted antiserum may be obtained in terms which take the percentage law into account. Log S_0 was called the serum neutralization index (SNI). Equation (6) described adequately the neutralization of infectivity of foot-and-mouth disease virus by guinea pig or rabbit antiserum (Bradish et al., 1962) and, in general, confirmed the percentage law.

As mentioned above, early experiments by Burnet et al. (1937) on phage-antiphage systems gave neutralization curves with an initial

lag period. Careful studies by Kalmanson et al. (1942) on the T2 phage-antiphage reaction, using highly diluted antiserum, confirmed the initial lag noted by Burnet. The cause of the deviation from first-order kinetics was resolved by Cann and Clark (1954) who showed that activation of inhibited T2 phages by anti-T2 serum accounted for the initial lag. Sagik (1954) showed that freshly prepared T2 lysates contained inhibited phages which failed to bind to host cells. The failure was apparently due to the presence of a substance of bacterial origin which covered the attachment sites of the affected phages. Spontaneous activation of the inhibited phages occurred slowly. Rapid activation of the inhibited phages occurred when they were exposed to a medium of low salt concentration, to heat, or to low concentrations of antiphage serum. Cann and Clark (1954) demonstrated that in the absence of inhibited phages the phage neutralization reaction followed first-order kinetics down to five to ten per cent survivors.

In the neutralization of phage, a quantitative relationship exists between the specific rate constant, the energy of activation, and the absolute temperature (Hershey, 1941). This relationship is given by the Arrhenius equation

$$\ln K_2/K_1 = E/R(1/T_1 - 1/T_2) \qquad (7)$$

where K_1 and K_2 are the velocity constants for the neutralization reaction at absolute temperature T_1 and T_2, respectively. R is the gas constant and E (energy of activation) is a constant characteristic of the system. The temperature coefficient for the velocity constant, K, was determined for phage C16 by Burnet et al. (1937). The K values

at 0, 37, and 45 C were 45, 930, and 1350 minute^{-1}, respectively. The Q_{10} was thus about 2.1. Similar studies were made by Kalmanson et al. (1942) with the serologically related phage, T2. The K value was found to be 0.5 second^{-1} at 0 C and 0.7 second^{-1} at 37 C, which corresponded to a Q_{10} of about 2.0. In addition, Hershey (1941) reported that the rate of neutralization of T2 exhibits a strong temperature dependence, corresponding to a Q_{10} of approximately 2.0. The meaning of these values was placed in doubt by studies of Cann and Clark (1954). They found that the neutralization of activated T2 shows a Q_{10} of only about 1.4. It was suggested by Adams (1959) that the low Q_{10} value was due to the use of a concentration of sodium chloride lower than that (0.15 M) used by the earlier workers. However, Talmage and Cann (1961) showed that the experiments of Cann and Clark (1954) were carried out in a medium entirely consistent with those used by the earlier workers. In an extension of their original experiments, Cann and Clark (1956) found that the Q_{10} value was also 1.4 when the reaction was carried out in a medium containing only 3×10^{-3} M sodium chloride.

Assuming that the experiments of Cann and Clark (1954; 1956) gave results which led to the nearest correct estimate for the Q_{10} value of the T2 neutralization, it can be concluded that the neutralization of activated T2 shows only a small temperature dependence at 3×10^{-3} M or 0.15 M concentrations of sodium chloride.

A similar value ($Q_{10} = 1.4$) was found by Jerne and Skovsted (1953) for the neutralization of T4 in the absence of sodium chloride, but the value in 1.0 M sodium chloride was about 2.0. That the dependence

of Q_{10} upon the concentration of sodium chloride in this case was not due to activation of inhibited phages may be ruled out because during its purification the phage was held temporarily in a medium containing only 0.01 M sodium chloride.

Low Q_{10} values (about 1.4) are typical of short range forces such as coulomb and van der Waals forces or hydrogen bonds, all of which have small activation energies. A Q_{10} of 1.4 suggests a diffusion controlled reaction. On the other hand, high Q_{10} values (2.0 to 3.0) are characteristic of reactions which involve the making or breaking of covalent bonds (for example, —C—C- or -C—N—). Obviously the type of bonding mechanism involved in phage-antiphage reactions is not the same for every phage neutralization system. Wide differences between Q_{10} values suggest that the relationship between Arrhenius constants and the bonding mechanism involved in neutralization should be investigated.

Andrewes and Elford (1933a) observed that neutralization of phage proceeds at an exponential rate until 90 to 99 per cent of the phages are inactivated. The rate then decreases abruptly until no further change is seen. The terminal surviving phage appeared to be resistant to neutralization. Andrewes and Elford (1933b) and Kalmanson et al. (1942) demonstrated that the presence of such a large amount of terminally surviving phage could not be due to exhaustion of antibody. Previously, Andrewes and Elford (1933a) had suggested, but disproved that the resistance could be due to inhomogeneity of the phage population with respect to susceptibility to inactivation. Proof against the idea was obtained by showing that the resistance was a phenotypic

rather than a genotypic expression of the phages since progeny of the surviving phages proved to be normally susceptible to neutralization by antiserum.

Delbrück (1945) suggested that the resistance to neutralization in bacteriophage T1 preparations might be inherent in the phage population or that it might develop during the course of the reaction as a result of attachment of antibody at non-critical sites on the phage. Definitive studies to clarify these points have apparently not been done.

The possibility exists also that the terminally surviving phage could be accounted for on the basis of reversible attachment between phage and antibody. Jerne and Avegno (1956) reported that inactivation of bacteriophage T4 by primary-response serum was reversible on dilution, but that neutralization with secondary-response serum or hyperimmune serum was irreversible. Strong evidence against the idea of a reversible phage-antiphage reaction is found in the work of Hershey (1943) who, while studying lattice formation between phage and antibody, was unable to detect free antibody in supernatant fluids of specific precipitates. In addition, the infectivity of neutralized phage was not increased when the mixture of phage and antiserum was allowed to stand for six months at 4 C after being diluted 1,000 times beyond the concentration at which antibody had any measurable neutralizing effect. Similar results were obtained in diluent with or without salt and in a diluent containing a detergent. In addition, Hershey presented evidence that the infectivity of neutralized phage in specific precipitates did not increase following the addition of a large excess of

formalin-inactivated homologous phage. Thus the phage in the specific precipitates was irreversibly bound to antibody since it could not be replaced by the formalin-inactivated phage. The results of the above experiments support the idea of irreversibility of the phage-antiphage reaction. It thus appears that the high proportion of terminally surviving phage cannot be explained by reversibility of the neutralization reaction.

Anomalous neutralization behavior has been ascribed to effects of the properdin system and complement. Various viruses, for example influenza virus, NDV, herpes simplex, T2, and T7 have been shown to be sensitive to the properdin system (Ginsberg and Wedgwood, 1960). Van Vunakis et al. (1956) provided evidence that the neutralizing activity of fresh human serum for phage T2 may reflect properdin levels. They indicated that this activity was demonstrable only in the presence of all four components of complement, divalent cation, and properdin. Serum which contained no properdin failed to show phage neutralizing activity, whereas the addition of purified properdin to such serum restored T2-neutralizing activity. Ginsberg and Wedgwood (1960) state that the properdin system may act alone with some viruses or, with others, may act to potentiate the neutralization by antibody. Neutralization of some strains of dengue virus by specific antibody was shown to require the properdin system (Sabin, 1950). The extent of this requirement for the properdin system in all viral neutralization systems is not known. However, it is known that adeno-, Coxsackie, ECHO, and poliomyelitis viruses are resistant to the properdin system either alone or with antibody (Ginsberg and Wedgwood, 1960). In addition,

purified antibody from fractionated serum inactivates bacteriophage T2 in the absence of the properdin system (Cann and Clark, 1954). These facts tend to indicate that the requirement for properdin in viral neutralization systems is not a general one. According to Toussaint and Muschel (1962) the effect of complement in neutralization tests appears to be related to the nature of the antibody used. In their experiments on the neutralization of T2 by normal serum, inactivation depended on normal antibody and complement. In contrast, neutralization of phage by specific antiserum was independent of complement.

Relation Between Infectious Nucleic Acid (INA) and Neutralization

Due to recent findings concerning an unusual property of viruses (vide infra), a theory has been constructed which appears to offer a reasonable explanation for all or a part of the terminally surviving phage. Gierer and Schramm (1956) discovered that the ribonucleic acid (RNA) component of TMV was infectious, independent of its protein capsid. In subsequent years the same finding has been extended to Mengo encephalitis virus (Coulter et al., 1957), poliovirus (Alexander et al., 1958), foot-and-mouth disease virus (Brown et al., 1958), and influenza virus (Maassab, 1959). In addition, the work of Gierer and Schramm was confirmed by Fraenkel-Conrat (1956). Nucleic acid isolated chemically from bacteriophage ϕX174 was shown to be infectious for Escherichia coli protoplasts (Guthrie and Sinsheimer, 1960; Sekiguchi et al., 1960; Huppert et al., 1961). Since both types of nucleic

acids, RNA and DNA (deoxyribonucleic acid), are involved in the examples cited above, the occurrence of infectious nucleic acid (INA) is believed to be a general phenomenon (Herriott, 1961). However the efficiency of infection is usually lower with INA preparations than with intact viruses.

The possibility that both viral infectivity and neutralizing antibodies could co-exist in the same serum-virus mixtures was suggested many years ago. Todd (1928) found that a mixture of fowl plague virus and immune serum contained infective virus after an incubation period of four hours at 37 C. Andrewes (1928) found that vaccinia virus incubated with immune serum for $4\frac{1}{2}$ hours prior to testing was as infectious as vaccinia in freshly prepared serum-virus mixtures. Sabin (1935) added substantial support to this concept through his studies on several virus-serum systems. In one study he presented evidence suggesting that the reason for the varying infectivity of pseudorabies virus-serum mixtures given by different routes, or in different animals, was (1) not due to the fact that one route was more sensitive than another in detecting small amounts of non-inactivated virus and (2) not dependent on prior incubation in vitro. Sabin proposed that no inactivation could have occurred in vitro and that the character of the tissue used for the assay of the serum-treated virus appeared to affect the outcome of the tests. In another study with rabbits he found that more than 100 times the concentration of immune serum needed to neutralize 50 to 500 minimal infective doses of B virus inoculated by the intracutaneous route failed to protect against a single, minimal infective dose inoculated by the intracere-

bral route. In control tests, rabbits were found to be equally susceptible by both routes. Herriott (1961) explained Sabin's findings on the ground that immune serum neutralized whole virus but not INA. The fact that the mixtures were more infectious by the intracerebral than by the intracutaneous route was considered to be due to the greater nuclease activity of the skin.

Dulbecco et al. (1956) reported that excess antibody failed to neutralize completely the infectivity of either WEE virus or poliovirus. This observation suggested to Herriott (1961) that either the serum ribonuclease was ineffective or the viral nucleic acid was protected by some kind of envelope.

Studies on poliovirus (Alexander et al., 1958), influenza virus (Maassab, 1959), and tick-borne encephalitis virus (Sokol et al., 1959) showed that INA resists neutralization by antiserum which neutralizes the homologous whole virus. The fact that some sera from humans who are ill with viral infections will completely neutralize infectivity cannot be used as an argument against the existence of INA. The time used in preparing the sera and the increased levels of deoxyribonuclease (DNAase) in the serum could well militate against detecting INA in the blood (Herriott et al., 1961).

In work with poliovirus, the range of host cells sensitive to infection by INA was found to be greater than that for the whole virus (Holland et al., 1959). A similar situation has been found to prevail with INA of bacterial viruses. It has been shown, for example, that protoplasts derived from bacterial cells normally resistant to whole virions of ϕX174 are readily infected by INA isolated chemically from

ϕX174 (Sekiguchi et al., 1960). The INA from TMV (Gierer and Schramm, 1956) and ϕX174 (Guthrie and Sinsheimer, 1960) produce the usual type of infection, with the release of whole virions. The infectivity of DNA from T2 phage for protoplasts was destroyed by treating it with DNAase for 80 minutes (Fraser et al., 1957). The infectivity of INA from ϕX174 was destroyed by pancreatic DNAase but not by E. coli phosphodiesterase (Fiers and Sinsheimer, 1962).

The knowledge that antibodies to whole viruses are directed against the protein capsid and that INA would not be susceptible to antibodies directed against the whole, homologous phage suggests that terminal phage infectivity in neutralization reactions may arise through a mechanism involving INA. If it is assumed (1) that INA is exposed or liberated from the intact phage during its reaction with antibody and (2) that INA is infectious for a variable fraction of the host bacteria, the terminal phase of a neutralization reaction could represent the simultaneous existence of antibody and INA.

Bacteriophage ϕX174

An early report on bacteriophage ϕX174 by Sertic and Boulgakov (1935) gave its plaque size, thermo-resistance, and its antigenic relationship to other typhi phages. The first studies on its size utilized ultrafiltration techniques (Sertic and Boulgakov, 1935), ultracentrifugation techniques (Lépine et al., 1944), and the technique of irradiation with alpha rays (Bonét-Maury and Bulgakov, 1944; Frilley et al., 1944). More recently, electron microscopy has been applied to studies of ϕX174 in an attempt to elucidate more precisely

its size and structure (Sinsheimer, 1959a; Hall et al., 1959; Tromans and Horne, 1961; MacLean and Hall, 1962; Daems et al., 1962). In shadowed electron micrographs the phage appears to consist of twelve capsomeres, about 70 Å in diameter, arranged as at the vertices of an icosahedron. The external shape of the virus appears to be in the form of a polygon with a diameter of about 250 Å. No tail structure has been seen.

Sinsheimer (1957) presented a preliminary report on the physical and chemical properties of ϕX174. Further studies (Sinsheimer, 1959a) of highly purified virus revealed that ϕX174 had a sedimentation constant of 114 S, a particle weight of 6.2×10^6, and a diameter of about 250 Å. No RNA was present, but DNA and protein accounted for approximately 25 per cent and 75 per cent of the particle weight, respectively.

Recent studies have shown that the probability of inactivation of ϕX174 by decay of incorporated P^{32} is 1.0 (Tessman et al., 1957; Kozinski, 1961). Disintegrations of P^{32} in ϕX174 have ten times the efficacy of that observed with other bacterial viruses (Hershey et al., 1951; Stent and Fuerst, 1955). Sinsheimer (1959a) suggested that this very high efficiency of inactivation was due to an unusual structure in the DNA molecule. In subsequent work Sinsheimer (1959b) was able to show from chemical and physical studies on the DNA extracted from ϕX174 that each virion contains one molecule of single-stranded DNA. The fact that the DNA molecule is single stranded accounts for the high efficiency of inactivation of ϕX174 virions by decay of incorporated P^{32}, since each disintegration would result in scission of

the DNA strand.

The kinetics of neutralization of purified ϕX174 by antibody has apparently not been studied. Zahler (1958) described the neutralization of ϕX174 by antiserum against a related phage, S13. Several reports have appeared recently on the antigenicity of ϕX174 in guinea pigs (Uhr et al., 1962a), newborn premature infants (Uhr et al., 1962b), and non-mammalian vertebrates (Uhr et al., 1962c). Although these last three publications contain highly significant data concerning the production of antibody molecules, they are not concerned specifically with the kinetics of neutralization.

The objectives of the present research were (1) to study in detail the kinetics of neutralization of purified ϕX174 bacteriophage by specific antibody, (2) to determine whether neutralization obeys the percentage law, and (3) if so, to characterize the phage which escapes neutralization.

CHAPTER II

MATERIALS AND METHODS

Media

The following media were used:

(1) Broth.

Tryptone[1]	10.0 gm
Potassium chloride	5.0 gm
Magnesium sulfate (1 M)	1.0 ml
Calcium chloride (1 M)	0.5 ml
Distilled water	1,000.0 ml

For diluent purposes, normal rabbit serum[1] (NRS) was added to the broth to a final concentration of 1:100.

(2) 3XD medium (Fraser and Jerrel, 1953).

Dihydrogen potassium phosphate	4.5 gm
Disodium phosphate	10.5 gm
Ammonium chloride	1.0 gm
Magnesium sulfate	0.3 gm
Casamino acids[1]	15.0 gm
Glycerol	30.0 gm

[1]Obtained from Difco Laboratories, Detroit, Michigan.

Calcium chloride . . . . . 0.3 ml

Gelatin (1.0 per cent solution). . . 1.0 ml

Distilled water. . . . . 1,000.0 ml

(3) Modified 3XD medium (Guthrie and Sinsheimer, 1960). This was the same as 3XD medium except that 0.9 gm dihydrogen potassium phosphate and 2.1 gm disodium phosphate were used.

(4) Protoplast nutrient broth (PNB).

Casamino acids. . . . . 10.0 gm

Nutrient broth[1] . . . . . 10.0 gm

Glucose . . . . . 1.0 gm

Sucrose . . . . . 100.0 gm

Distilled water . . . . . 1,000.0 ml

After this solution was autoclaved at 110 C for 15 minutes, 10 ml of a sterile, aqueous 10 per cent solution of magnesium sulfate were added.

(5) Bottom agar.

Tryptone . . . . . 10.0 gm

Sodium chloride . . . . . 2.5 gm

Potassium chloride . . . . . 2.5 gm

Agar[1]. . . . . 11.0 gm

Sodium hydroxide (1 N) . . . . . 6.0 ml

Distilled water . . . . . 1,000.0 ml

After the solution was autoclaved at 110 C for 15 minutes, 1.0 ml of sterile, aqueous 1 M calcium chloride was added.

(6) Top agar.

Tryptone	10.0 gm
Sodium chloride	5.0 gm
Agar	8.0 gm
Distilled water	1,000.0 ml

(7) Protoplast top agar (Guthrie and Sinsheimer, 1960). This medium consisted of PNB with the addition of 8.0 gm of agar per liter and, at the time of use, the addition of 30 per cent bovine serum albumin (BSA)[2] to give a final concentration of 2.0 per cent.

(8) Hard agar. This medium, consisting of broth with 2.0 per cent agar, was used exclusively to prepare slants.

Host Organisms

Bacteria. Escherichia coli, strains C[3], H[3], and K12S[4] were used. On receipt, each strain was streaked on bottom agar and a single colony was picked and restreaked on agar. This procedure was repeated twice and a single colony was then picked and transferred to a hard agar slant. All experiments were carried out on bacteria derived from these single-colony isolations. Transfers of stock cultures were made once weekly on hard agar slants. The slants were then incubated at 37 C overnight and stored at 4 C. The plating bacteria, E. coli C, were grown in broth overnight at 37 C, with aeration.

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[2]Obtained from Armour Pharmaceutical Company, Kankakee, Illinois.

[3]Generously provided by Dr. Irwin Tessman, Department of Biological Sciences, Purdue University, Lafayette, Indiana.

[4]Obtained from Dr. John Drake, Department of Microbiology, University of Illinois, Urbana, Illinois.

The culture was then diluted 1:5 in broth and 0.3 ml (1.2×10^8 colony-formers) of the diluted bacterial suspension was used per plate.

Protoplasts. 0.5 ml of an overnight broth culture of E. coli K12S was inoculated into 19.5 ml of modified 3XD medium and the culture was incubated at 37 C, with aeration, until the cell count reached 5×10^8 cells ml^{-1} as determined with a Petroff-Hauser bacteria counter. The cells were then subcultured by inoculating one ml into 24 ml of pre-warmed 3XD medium. This culture was then incubated in the usual fashion until the cell count reached 5×10^8 ml^{-1}. Twenty ml of the culture were then centrifuged at 1,000 g for ten minutes at room temperature. The supernatant fluid was discarded and the pellet was taken up in 0.35 ml of 0.5 M sucrose. 0.2 ml of 0.25 M Tris (hydroxymethylaminomethane) buffer, pH 8.1, and 0.1 ml (0.2 mg) lysozyme[5] in distilled water were then added, followed by 0.02 ml of 4.0 per cent EDTA (disodium ethylenediamine tetraacetate). The mixture was incubated for 15 minutes at 37 C and diluted fivefold in PNB containing two per cent BSA. After five minutes, a further fourfold dilution was made in PNB without BSA. This material served as the stock suspension of protoplasts.

Lysates. Mass cultures of E. coli C to be infected with phage were prepared in 0.5 to 15 liters of the 3XD medium. An overnight culture of the organism grown in 3XD medium was diluted 1:33 in the same medium at 37 C. The resulting culture was incubated, with

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[5]Obtained from Sigma Chemical Company, 3500 DeKalbe, St. Louis, Missouri.

aeration, at 37 C and when the concentration of bacteria reached 10^9 cells ml^{-1}, according to a count made with a Petroff-Hauser bacteria counter, the culture was infected with ϕX174 to a final concentration of 10^{10} plaque-formers ml^{-1}. At the moment of infection, one gm of magnesium sulfate was added aseptically per liter of culture. Foaming, which occurred about 45 minutes after inoculation, was controlled by the addition of antifoam[6]. Lysis was usually assisted by the addition of one ml of chloroform per 25 ml of lysate. Incubation was continued for 30 minutes, with aeration.

Preparation of Bacteriophage ϕX174

The bacteriophage ϕX174[3] used throughout these studies was derived from a single plaque on E. coli C. Three different lots of phage were prepared. In each case, lysates as described above were used.

Purification and concentration of lot I phage (ϕX174d). A pool of several lysates, totaling 3,900 ml, and containing approximately 1×10^{10} plaque-formers ml^{-1}, was filtered through an F, then through a UF fritted glass filter. The filtrate was concentrated approximately ten times by dialyzing it against carbowax 4,000[7] at 4 C (Soller, 1961). The concentrated phage was then refiltered through UF

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[6]Dow-Corning Antifoam A Spray, Dow Corning Corporation, Midland, Michigan.

[7]Obtained from Union Carbide Chemical Company, Division of Union Carbide Corporation, New York, New York.

glass and subjected to five alternate cycles of high-speed (100,000 g for three hours) and low-speed (1,000 to 10,000 g for 15 minutes) centrifugations. All high-speed centrifugations were done in a Spinco Preparatory Ultracentrifuge, Model L[8]. After the first high-speed centrifugation, the 3XD medium was decanted and replaced by 1/10 its volume of sterile, 0.9 per cent sodium chloride containing 10^{-3} M magnesium sulfate (Mg-saline). Mg-saline was then used throughout the remainder of the concentration procedure. The pellet obtained after the final high-speed centrifugation cycle was taken up in 22 ml of the Mg-saline and stored at 4 C.

Purification and concentration of lot II phage (ϕX174a). Eight liters of lysate were prepared in 3XD medium as described above and the phage was purified and concentrated according to the method of Tromans and Horne (1961). For this purpose bacterial debris was first removed from the lysate by centrifuging it at 2,000 g for 30 minutes. The pellet was then discarded and the supernatant fluid, while being maintained at 4 C, was brought slowly to 40 per cent saturation with ammonium sulfate. The solution was allowed to stand at 4 C overnight and the precipitate which formed was removed by centrifugation at 10,000 g for 30 minutes and was resuspended in 100 ml Tris-EDTA buffer (0.1 M Tris plus 0.01 M EDTA) at pH 8.0. The suspension was then centrifuged at 1,000 g for 15 minutes to remove any large, non-viral material. Approximately 11 ml of the supernatant fluid was placed in each of nine lusteroid tubes and centrifuged at 100,000 g for 90

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[8]With the assistance of Mr. Carl Haygood, Veterans Administration Hospital, Oklahoma City, Oklahoma.

minutes. A four-inch needle and syringe were used to remove the supernatant fluids from each pellet. The phage pellets were covered immediately with 1.0 ml of fresh Tris-EDTA buffer and placed at 4 C overnight for elution of the phage into the buffer. The supernatant fluids containing the phage were pooled and recentrifuged at 1,000 g for ten minutes to remove debris. The resultant phage suspension was stored at 4 C.

Purification and concentration of lot III phage (ϕX174b). A lysate of ϕX174 was produced in 15 liters of the 3XD medium. The purification and concentration procedures used were the same as those described above for the lot II phage (ϕX174a), except that each of the pellets recovered after a second centrifugation at 100,000 g were resuspended in 1.0 ml of 0.074 M sodium tetraborate, pH 9.1 (Sinsheimer, 1959a). 5.4 gm of rubidium chloride were then added slowly to ten gm of the virus suspension (Sinsheimer, 1959a) and the material was centrifuged to equilibrium (20 hours at 4 C) at 90,000 g with an SW 30 rotor in a Spinco Preparative Ultracentrifuge[8]. The centrifuge was allowed to come to rest without use of the electronic brakes. The centrifuge tube containing the phage was then carefully removed from the rotor. The grey colored material floating on the surface of the fluid was removed with a small wooden spatula and discarded. A needle and syringe were used to remove about three ml of the fluid covering the virus band. A second needle and syringe were used to remove approximately one ml of fluid containing the virus band. The remainder of the fluid in the tube was decanted into a sterile test tube. The virus content of the three fractions was assayed on E. coli C by the

method described below. Each fraction was then stored at 4 C. In addition, a sample of the fraction containing the ϕX174b virions was diluted 1:100 in broth containing 1:100 NRS and stored at 4 C.

Determination of sedimentation coefficients of ϕX174a and ϕX174b. The sedimentation coefficient is defined as the velocity of the sedimenting particles per unit field. The value is found by the following equation:

$$s = 1/w^2 \cdot dx/dt \qquad (8)$$

where x is the distance of the boundary in centimeters from the axis of rotation, t is the time in seconds, and w is the angular velocity in radians per second. The dimension of sedimentation coefficients is in seconds and the unit 1×10^{-13} second has been termed 1 S, where S stands for the Svedberg unit.

Sedimentation coefficients of ϕX174a and ϕX174b were determined in the Spinco Ultracentrifuge, Model E^8. The AN-A rotor, equipped with a 3.0 mm thick, 4.0^o, single-sector cell, was used. The cell was loaded with the phage preparation and positioned in the rotor. The rotor was then hung on the drive shaft and the chamber was closed. When the vacuum in the chamber reached 5×10^{-4} cm Hg the rotor was accelerated to a velocity producing 38,900 g's. During acceleration the sedimentation pattern was examined through the viewing screen to determine if any material present in the phage suspension sedimented across the cell before the rotor attained the desired operating speed. The temperature of the chamber was 21 C ± 3.0 C during the determination. Photographs of the moving boundary were taken every four min-

utes with the automatic photographic device. Values for x were then determined, permitting the calculation of the sedimentation coefficients for the various peaks. Sedimentation coefficients are generally reported as $S_{20,w}$, the value the material would have in a solvent with the density and viscosity of water at 20 C. Corrections of the observed sedimentation coefficient, S_{obs}, to this standard state can be performed. In the present study, however, all of the data necessary for these conversions were not available and the results are therefore reported as observed sedimentation coefficients.

Plaque counts. The soft agar overlay technique was used for all plaque counts (Adams, 1959). With this procedure, 0.3 ml of plating bacteria (E. coli C), prepared as described above, and 0.05 to 0.1 ml of a suitable dilution of ϕX174 were added to three ml of melted top agar held at 44 C. The mixture was poured immediately into a plate containing approximately 30 ml of solidified bottom agar. The plates were allowed to remain on a level surface until the top agar solidified. This usually required about 15 minutes. They were then incubated four to six hours at 37 C and plaques were counted with the aid of a Quebec Colony Counter.

Duplicate platings from the same diluted sample of phage generally agreed to within ten per cent when the average plaque count was about 250. Duplicate assays from separate dilutions of the same phage stock generally agreed to within about ten per cent when each assay was based on two or four plates.

Stability of ϕX174d at 37 C and at 4 C. Since the stock ϕX174

was stored at 4 C but was used in neutralization experiments at a 10^3 or 10^4 times smaller concentration at 37 C, it was important to compare the stability of the diluted phage at 4 C and 37 C. Accordingly, ϕX174d was diluted 1.20×10^3 times in broth containing 1:100 NRS, placed at 4 C, and assayed for phage immediately, after 30 minutes, and after 60 minutes. The phage was also diluted 1.20×10^3 times in the following media:

(1) Broth with 5×10^{-2} M magnesium sulfate.

(2) Same as (1), but with 1:100 NRS.

(3) 0.15 M sodium chloride with 10^{-3} M magnesium sulfate.

(4) Same as (3), but with 1:100 NRS.

(5) 0.15 M sodium chloride with 5×10^{-2} M magnesium sulfate.

(6) Same as (5), but with 1:100 NRS.

The tubes were incubated at 37 C and after 30 and 60 minutes a sample was taken from each tube and assayed for its content of phage.

Stability of various concentrations of ϕX174b at 37 C and 44 C. Since, in the plating procedure, ϕX174 was held temporarily at 44 C, it seemed desirable to measure its stability at this temperature. Serial dilutions of ϕX174b were prepared in broth containing 1:100 NRS, at 37 C, to contain 6.26×10^6, 7.43×10^5, 7.08×10^4, and 9.11×10^3 plaque-formers ml^{-1}. Each concentration of phage was contained in a volume of five ml. The tubes were incubated further at 37 C and samples were removed after one, two, four, eight, and 48 hours and assayed for phage. An identical set of phage dilutions was incubated at 44 C and samples were removed for assay at the same time intervals.

Production of ϕX174 Antibodies in Rabbits

Five albino rabbits, weighing approximately two kg, were used for the production of antibodies. Three of the rabbits were inoculated intravenously in a marginal ear vein with approximately 1 x 10^{12} plaque-formers of ϕX174d contained in one ml of the Mg-saline diluent. Twelve days later, each of the three rabbits was re-injected intravenously with the same dosage of phage. Blood samples were taken from each rabbit by cardiac puncture on the fifth, seventh, and ninth days after the first injection and on the fourth, sixth, and eighth days after the second injection of phage. The blood was collected in sterile tubes and allowed to clot at room temperature. The clots were then ringed and the tubes were stored overnight at 4 C. The following day the tubes were centrifuged at 1,000 g for 15 minutes and the sera were collected, treated with merthiolate (1:10,000), and stored at 4 C in rubber-stoppered test tubes.

The other two rabbits received culture filtrate material from E. coli C prepared as follows: 20 ml of broth were inoculated with cells from a hard agar slant and incubated at 37 C overnight, with aeration. The culture was then centrifuged at 1,000 g for 15 minutes and the supernatant fluid was passed through a UF sintered glass filter. Each of the two rabbits was injected intravenously in the marginal ear vein with one ml of the filtrate. Each rabbit received a second injection of one ml of the filtrate 12 days later. Serum samples were collected at the same time intervals, and by the same

technique, as described above.

Method of Measuring Neutralization of ϕX174 by Rabbit Antibody

The following method was used in the routine neutralization experiments. The stock phage was diluted in broth containing 1:100 NRS to a concentration ten times greater than that to be used in the ϕX174-anti-ϕX174 reaction mixture. The antiserum was diluted in broth to the concentration to be used in the neutralization reaction mixture and pre-incubated at 37 C. 0.1 ml of the diluted phage preparation was then added to 0.9 ml of the pre-warmed antibody solution and incubation was continued at 37 C. At the moment of mixing (time zero) a stop watch was started to time the experiment. At various intervals, the concentration of surviving phage in the reaction mixture was determined by removing a sample, diluting it 1:50 or 1:100 to stop neutralization, diluting further if necessary, and plating a sample from the last dilution of the mixture in duplicate or quadruplicate, as described above. Since it was technically impossible to obtain a sample of the input phage from the neutralization reaction mixture at zero time, a phage control was prepared for each experiment. 0.1 ml of the diluted phage described above was mixed with 0.9 ml of pre-warmed broth containing 1:100 NRS and incubated at 37 C. Two samples were removed immediately. Each sample was diluted separately and plated in quadruplicate. This process was repeated at the end of each experiment and the mean plaque counts from all 16 plates was used to calculate P_o. When K values were to be determined, formula

(2) given in Introduction and History was used. Slight modifications of the above procedures were required frequently. These changes will be described with the appropriate experiments.

Method of Infecting Protoplasts

ϕX174 was diluted 1:200 in an aqueous solution of 0.1 M potassium chloride and 10^{-3} M Tris, pH 8.1 (KCl-Tris buffer). Rabbit antiserum, which had been previously diluted 1:50 in broth, was diluted 1:900 in the KCl-Tris buffer. The final dilution of serum was thus $1:4.5 \times 10^{4}$. 0.1 ml of the diluted phage was added to 9.9 ml of the diluted, pre-warmed antibody solution and the mixture was incubated at 37 C for five hours. The phage control mixture consisted of 0.1 ml of the diluted phage plus 9.9 ml of the KCl-Tris buffer. The control mixture was also incubated at 37 C for five hours. The P_o and the terminal phage concentration in the phage-antiphage reaction mixture were determined in the usual manner. At the end of the five hour incubation period, 0.5 ml of 0.1 M Tris was added to 4.5 ml of the phage-antiphage reaction mixture in order to increase the Tris concentration to 0.01 M. To reduce the phage concentration in the phage control suspension to a level approximating that in the phage-antiphage mixture, it was diluted 1:70 in 0.01 M Tris. Two ml of the adjusted (0.01 M Tris) phage-antiphage mixture were then added to two ml of the stock protoplasts suspension. This material, designated Adsorption Mixture A, was incubated at 37 C. Adsorption Mixture B consisted of two ml of the diluted phage control suspension plus two ml of the stock protoplast suspension. It also was incubated at 37 C. After

ten minutes a sample taken from Adsorption Mixture A was diluted 1:5 in pre-warmed PNB (Growth Mixture A). At the same time another sample was diluted 1:10 in PNB with two per cent BSA, and a third sample was diluted 1:10 in chloroform-water[9]. To determine the total number of infectious centers (free phage plus infected protoplasts) in Adsorption Mixture A, 0.1 ml of the PNB diluted mixture was plated in 2.5 ml of the protoplast top agar. 0.1 ml of the chloroform-water diluted mixture was plated as usual to determine the number of free phage in Adsorption Mixture A. The contents of Adsorption Mixture B were treated exactly as described for Adsorption Mixture A. Eighty minutes after Growth Mixtures A and B were prepared they were assayed for their content of phage.

Lupus Erythematosus (LE) Sera in Neutralization Studies

Fifteen sera[10] obtained from patients with lupus erythematosus (LE) were used in a modification of the neutralization experiments described above. The source and description of the sera are presented in Table 1. Suitable dilutions of S24, an anti-ϕX174 rabbit serum which will be described later, were mixed with ϕX174 at a P_o of 3.28×10^7 plaque-formers ml^{-1} and the mixture was incubated at 37 C. At the end of varying periods of incubation, 0.1 ml volumes of the various LE sera were mixed with 0.9 ml volumes of the ϕX174b-S24 mixture and in-

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[9] 500 ml of distilled water was mixed with 20 ml chloroform. Before use the mixture was stirred on a magnetic stirrer to suspend the chloroform in small droplets.

[10] Obtained from Dr. George J. Friou, Departments of Medicine and Microbiology, University of Oklahoma Medical Center, Oklahoma City, Oklahoma.

TABLE 1

LUPUS ERYTHEMATOSUS SERA USED
IN NEUTRALIZATION EXPERIMENTS

Serum number	Patient	Date patient was bled	Results of tests for antinuclear antibodies	
			Spot[a]	Complement fixation[b]
LE 1	S. J.	2-24-62	-	-
LE 2	N. P. K.	-	-	N
LE 3	L. B.	3-12-62	N	N
LE 4	N. P. K.	1-10-60	-	±
LE 5	B. M.	1-10-61	1:8	N
LE 6	J. K.	10-31-62	1:32	N
LE 7	H. H.	9-14-62	1:25	AC
LE 8	N. P. K.	11-7-62	-	N
LE 9	C. F.	12-12-62	1:256	AC
LE 10	J. K.	12-12-62	1:16	+
LE 11	C. A.	11-10-62	1:4	+
LE 12	J. K.	1-10-62	-	N
LE 13	K. P.	11-12-62	1:16	N
LE 14	R. G. V.	12-1-62	1:64	+
LE 15	J. K.	11-21-62	1:256	+

[a]Performed by Dr. George J. Friou. Results given as highest dilution of serum giving a positive test. N is negative test.

[b]Performed by Dr. George J. Friou, by method of Robbins et al. (1957). N is negative test; ± is doubtful test; + is positive test; AC is anticomplementary serum.

-, not recorded.

cubation was continued. Sera from ten normal persons and hospitalized individuals who did not have LE were also tested. Control reaction mixtures were prepared by mixing 0.9 ml volumes of the ϕX174b-S24 mixture with either 0.1 ml of broth containing 1:100 NRS or 0.1 ml of a suitable dilution of S24. The concentration of S24 used was designed to yield, on dilution of 1:10, the same concentration present at the beginning of the neutralization period. At 30, 60, 90, and 120 minute intervals samples were removed, diluted if necessary, and assayed for surviving phage.

DNAase in Neutralization Studies

DNAase was tested for its effect on phage which survived neutralization. In a typical experiment, P_o was 6.48×10^7 plaque-formers ml^{-1} and S24, diluted 6×10^4 times, was used. After a three hour incubation period at 37 C, 2.5, 25, and 250 micrograms of pancreatic DNAase[11], contained in 0.1 ml distilled water, were added to 0.9 ml portions of the phage antiphage mixture and to 0.9 ml portions of a control suspension containing no S24. Incubation was continued at 37 C and samples were removed at 30, 60, and 120 minute intervals and assayed for their content of phage.

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[11]Obtained from California Corporation for Biochemical Research, Los Angeles, California.

CHAPTER III

RESULTS

Purification and Concentration of Bacteriophage ϕX174

Lot I phage (ϕX174d). The pooled lysates (3,900 ml), comprising lot I phage, contained a total of 3.9×10^{13} plaque-formers. After purification and concentration by the methods described, a total yield of 2.2×10^{12} plaque-formers in 22 ml of Mg-saline was obtained. A 180-fold concentration of the phage was thus effected and the recovery represented 56 per cent of the phage contained in the original lysate.

Lot II phage (ϕX174a). The concentration of ϕX174a virions at various stages of purification, and the volumes in which they were contained, are given in Table 2. An 85-fold concentration of the phage was obtained, with a recovery of 55.4 per cent of the phage present in the original lysate. The purified phage was contained in 9.0 ml Tris-EDTA and the suspension had a titer of 3.63×10^{13} plaque-formers ml^{-1} or a total of 3.27×10^{14} plaque-formers.

Lot III phage (ϕX174b). The concentrations of ϕX174b virions and the volumes in which they were contained at various stages of concentration and purification are given in Table 3. It can be seen that the volume of the original lysate was reduced nearly 11,000-fold dur-

TABLE 2

PURIFICATION AND CONCENTRATION OF ϕX174a

Virus preparation	Volume (ml)	Plaque count (per ml)	Total Plaque-formers
Mass lysate, bacteria free	8,000	8.20×10^{10}	6.56×10^{14}
Ammonium sulfate pellet resuspended in Tris-EDTA [a]	100	2.88×10^{12}	2.88×10^{14}
100,000 g pellet resuspended in Tris-EDTA	9	3.63×10^{13}	3.27×10^{14}

[a] 0.1 M Tris + 0.01 M EDTA, pH 8.0

TABLE 3

PURIFICATION AND CONCENTRATION OF ϕX174b

Virus Preparation	Volume (ml)	Plaque count (per ml)	Total Plaque-formers
(A) Mass lysate	16,400[a]	1.20×10^{11}	1.97×10^{15}
(B) Mass lysate after removal of bacteria and debris	16,000	2.10×10^{11}	3.36×10^{15}
(C) 40% ammonium sulfate fractionation of (B)	18,900	1.60×10^{11}	3.02×10^{15}
(D) 10,000 g supernatant of (C)	18,500	4.10×10^{9}	7.50×10^{13}
(E) 10,000 g pellet of (C) resuspended in Tris-EDTA[b]	123	7.23×10^{11}	9.00×10^{13}
(F) 100,000 g pellet of (E) resuspended in Tris-EDTA	15	3.86×10^{12}	5.80×10^{13}
(G) 1,000 g supernatant of (F)	15	3.10×10^{12}	4.65×10^{13}
(H) Density gradient centrifugation of (G), virus band	1.5	1.22×10^{13}	1.83×10^{13}

[a]This figure represents 15 liters of original lysate to which magnesium sulfate and chloroform were added.

[b]0.1 M Tris + 0.01 M EDTA, pH 8.0.

ing the preparation of ϕX174b. Concomitantly, the phage titer increased approximately 100-fold. However, only about one per cent of the phage contained in the original lysate was recovered.

Analytical ultracentrifugation sedimentation characteristics of ϕX174a and ϕX174b. An ultracentrifuge sedimentation pattern of ϕX174a virions in Tris-EDTA, pH 8.0, is shown in Figure 1. Two peaks were obtained. The sedimentation constant, S_{obs}, of the fast moving component was found to be 114.7 and that of the slower moving component, 76. Although the two peaks were short, each appeared to be homogeneous.

The ultracentrifuge sedimentation pattern of ϕX174b virions in borate buffer, pH 9.0, is shown in Figure 2. One peak was obtained with this phage preparation. The S_{obs} was found to be 113.

Stability of ϕX174d and ϕX174b

Stability of ϕX174d at 4 C and 37 C. The results of studies in which broth-diluted ϕX174d virions were incubated in various media for 30 and 60 minutes at 37 C are presented in Table 4. It can be seen that the phage was stable at 4 C and, in general, it appeared to be somewhat more stable in media supplemented with 1:100 NRS. For this reason, broth containing 1:100 NRS was used in all subsequent studies.

Stability of various concentrations of ϕX174b at 37 and 44 C. The results of incubating various concentrations of ϕX174b at 37 C for various lengths of time in broth with 1:100 NRS are given in Table 5. It can be seen that at all concentration levels the phage was stable for eight hours. However, after 48 hours at 37 C the titer

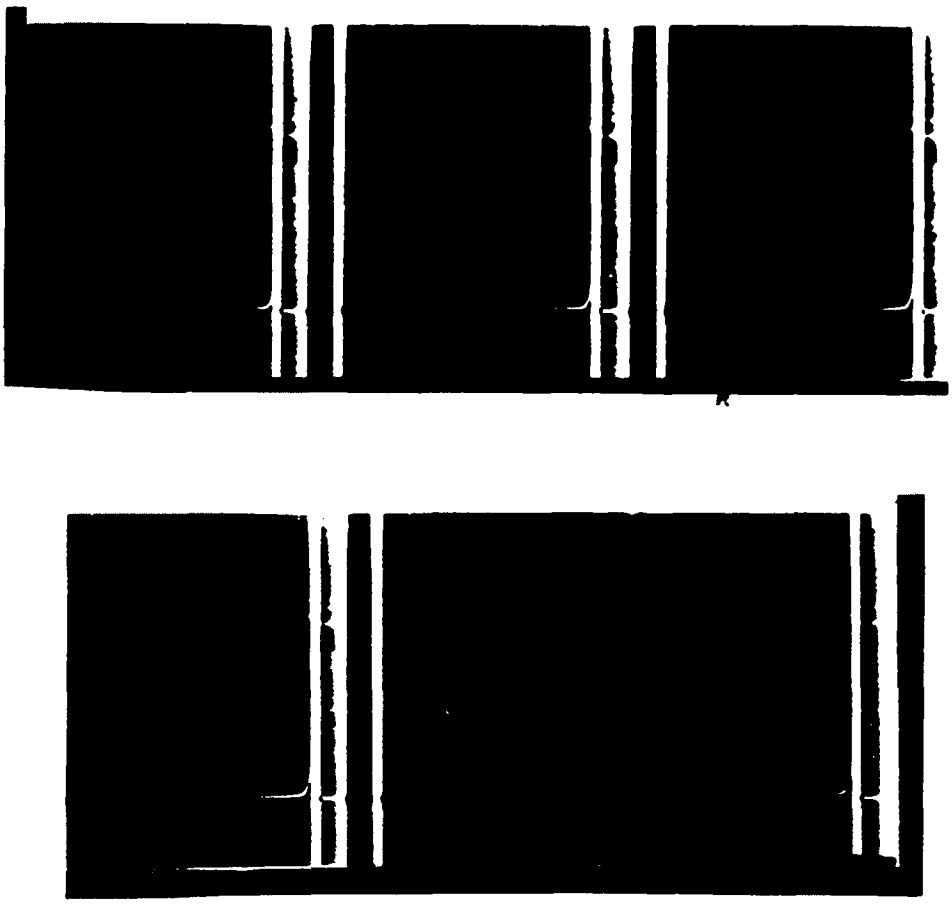

Figure 1. Ultracentrifuge sedimentation pattern for ϕX174a virions in Tris-EDTA buffer, pH 8.0. The sedimentation constant for the faster moving component is 114.7 S and for the slower moving component is 76 S. Pictures were taken every four minutes. 38,900 x g.

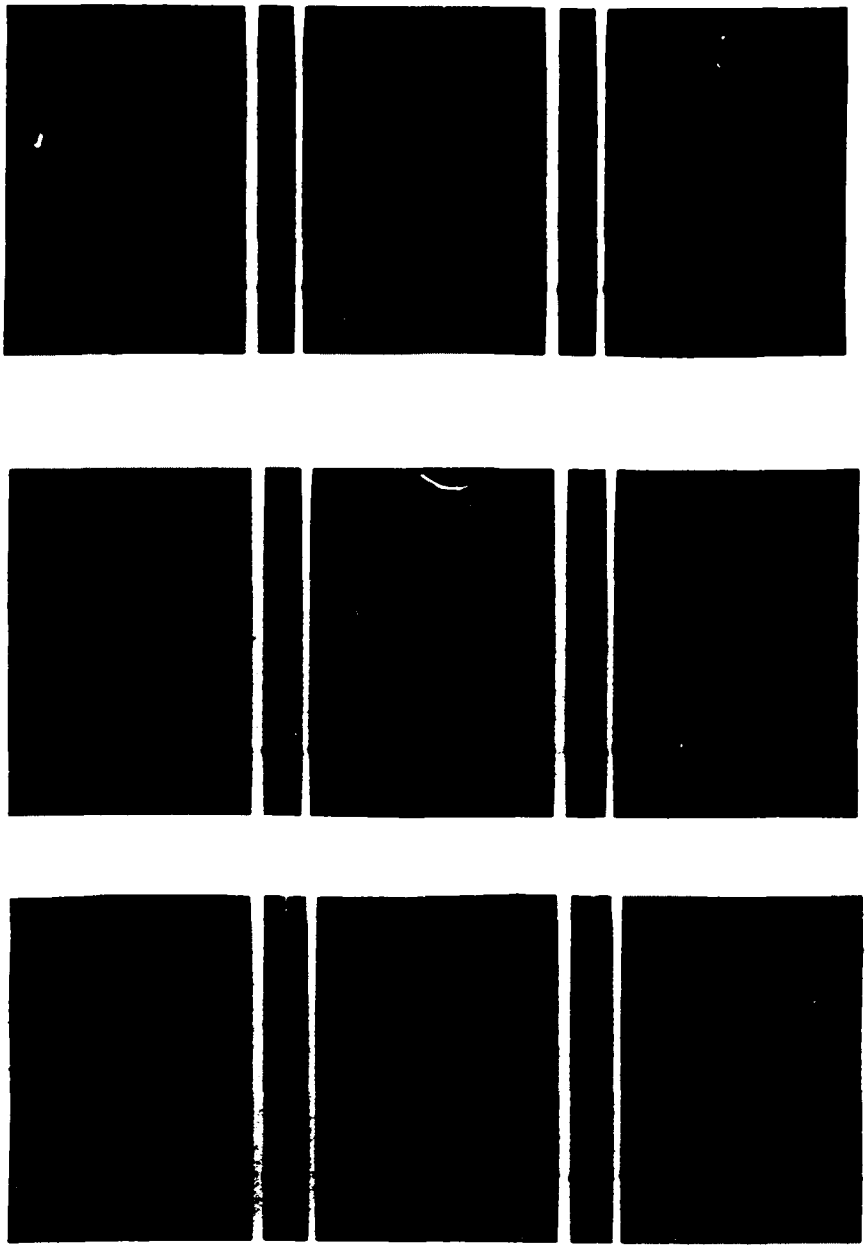

Figure 2. Ultracentrifuge sedimentation pattern for ϕX174b virions in borate buffer, pH 9.0. The sedimentation constant for this component is 113 S. Pictures were taken every four minutes. 38,900 x g.

TABLE 4

STABILITY OF ϕX174d IN VARIOUS MEDIA AT 37 C

Medium	30 Minutes		60 minutes	
	P [c]	P/P_o [d]	P	P/P_o
Control[a], broth with 1:100 NRS[b] at 4 C	1.38		1.20	
(1) Broth with 5×10^{-2} M magnesium sulfate	1.20	0.82	1.04	0.68
(2) Same as (1), but with 1:100 NRS	1.53	1.00	1.29	0.85
(3) 0.15 M sodium chloride with 10^{-3} M magnesium sulfate	1.41	0.93	1.25	0.82
(4) Same as (3), but with 1:100 NRS	1.22	0.80	1.09	0.72
(5) 0.15 M sodium chloride with 5×10^{-2} magnesium sulfate	1.41	0.93	1.25	0.82
(6) Same as (5), but with 1:100 NRS	1.26	0.83	1.39	0.92

[a] Plaque-formers at zero time = 1.52×10^7, ml^{-1}.

[b] Normal rabbit serum.

[c] P = plaque-formers $\times 10^7$, ml^{-1}.

[d] P_o = mean value of plaque-formers ml^{-1} for control at zero, 30, and 60 minutes.

TABLE 5

STABILITY OF ϕX174b IN BROTH WITH 1:100 NRS[a] AT 37 C

Time (Hours)	Plaque-formers ml^{-1}			
	$\times 10^6$	$\times 10^5$	$\times 10^4$	$\times 10^3$
Zero	6.26	7.43	7.08	9.11
1	6.38	7.06	7.05	8.50
2	8.85	8.80	9.50	6.69
4	7.50	8.50	9.20	9.60
8	9.30	6.92	8.40	8.60
48	2.49	2.95	3.80	3.00

[a]Normal rabbit serum.

of phage decreased in all four mixtures.

The results of incubating various concentrations of ϕX174b at 44 C for various lengths of time in broth with 1:100 NRS are given in Table 6. It may be seen that the phage was extremely unstable at 44 C. Inactivation of the phage was noted as early as the second hour. After 48 hours of incubation, no active phage was found in the tubes which originally contained the two lowest concentrations of phage. Only about three per cent of the phage survivied at the two higher levels studied.

Neutralization Studies

Production of anti-ϕX174 antibodies. Sera obtained from all of the rabbits prior to their inoculation with ϕX174d or with the host material preparation showed no neutralizing activity for ϕX174. In addition, the sera from the rabbits which received the host material showed no neutralizing activity for ϕX174. One of the rabbits which received bacteriophage ϕX174d died during the immunization procedure. The neutralizing activity for ϕX174 of the sera from the two surviving rabbits showed characteristic primary and secondary antibody responses. The neutralizing activity of the sera was measured and their K values were determined. The results are shown in Table 7. It can be seen that the K values of sera from both rabbits increased after the first injection of phage and that the values increased more rapidly and to a greater extent following the second injection of phage. The serum obtained from rabbit #2 on the sixth day after the second injection of phage was labelled S24.

TABLE 6

STABILITY OF ϕX174b IN BROTH WITH 1:100 NRS[a] at 44 C

Time (Hours)	Plaque-formers ml^{-1}			
	$\times 10^6$	$\times 10^5$	$\times 10^4$	$\times 10^3$
Zero	6.00	6.47	7.31	7.73
1	5.28	6.33	9.78	-
2	4.58	6.57	5.52	4.40
4	4.93	5.40	7.90	7.50
8	2.20	3.00	2.60	3.00
48	0.08	0.10	0	0

[a]Normal rabbit serum.

-, not done.

TABLE 7

ANTI-ϕX174 ANTIBODY PRODUCTION IN RABBITS

	Day on which sera were collected after:					
	First Injection			Second Injection		
	5	7	9	4	6	8
Rabbit 1	31[a]	52	41	125	3110	2367
Rabbit 2	126	374	243	8	2990	3262

[a] K value ($minute^{-1}$).

Neutralization of ϕX174d by S24. In preliminary experiments the K value for S24 was determined to be 2990 minute^{-1}. By using formula (2) it can be estimated that after one hour's incubation of a mixture of ϕX174d and diluted (2.8 x 10^4 times) S24 there should be approximately 10^{-3} surviving phage:

$$K = 2.3\ D/t \times \log_{10} P_o/P$$

$$2990 = 2.3\ (2.8 \times 10^4)/60 \times \log_{10} P_o/P$$

$$\log_{10} P_o/P = 2990/1080 = 2.77$$

$$P_o/P = 598$$

$$P/P_o = 1.65 \times 10^{-3}$$

An experiment was therefore performed to determine how closely an experimentally determined value would correspond with the theoretical. The neutralization experiment was performed with four phage-antiphage reaction mixtures in order to investigate the effect of initial phage concentration, P_o, on the survivor fraction. The P_o ranged from 1.22 x 10^8 to 1.22 x 10^5 plaque-formers ml^{-1}. The P_o for the control reaction mixture without antiserum was 1.22 x 10^8 plaque-formers ml^{-1}. The results of the experiment are shown in Figure 3. It can be seen that the fraction of phage surviving neutralization was about the same with each P_o for the first 20 minutes of incubation. After this time, the reaction mixture which initially contained 1.22 x 10^8 plaque-formers ml^{-1} (curve A) showed a decline in its rate of neutralization. The two reaction mixtures (curves B and C) containing the intermediate P_o's showed greater rates of neutralization than was shown with reaction mixture A and the extent of neutralization was greater by a fac-

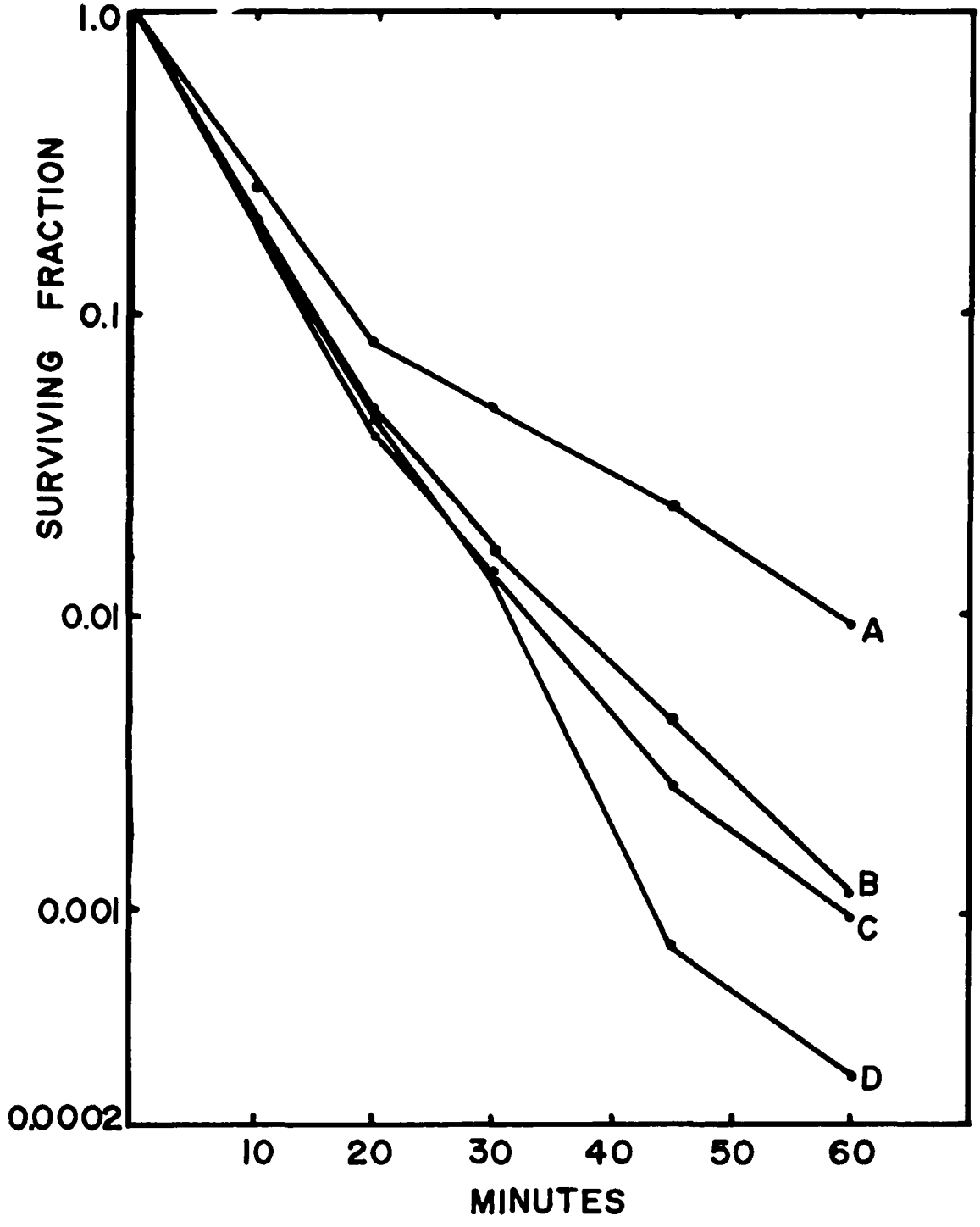

Figure 3. Neutralization of ϕX174d by S24 diluted 2.8×10^{4} times. Incubated at 37 C in broth with 1:100 NRS. P_o for reaction mixtures (plaque-formers ml^{-1}):

A = 1.22×10^{8} C = 1.22×10^{6}
B = 1.22×10^{7} D = 1.22×10^{5}

tor of approximately 10. Curves B and C were not exponential. The rate of neutralization occurring in the reaction mixture (curve D) which initially contained 1.22×10^5 plaque-formers ml^{-1} was exponential only during the first 45 minutes of the experiment. This experiment also showed that, with P_o in a range from 1.22×10^7 to 1.22×10^6 plaque-formers ml^{-1}, the fraction (approximately 10^{-3}) of P_o surviving neutralization after 60 minutes was the same as that calculated on a theoretical basis.

The data presented in Figure 3 were derived from samples taken at 10, 20, 30, 45, and 60 minute intervals. Since it seemed possible that more definitive information might be gained by taking samples more frequently during the neutralization period, an experiment was performed in which samples were taken every 2.5 minutes over a period of one hour. In this experiment, P_o ranged between 1.03×10^8 and 7.54×10^4 plaque-formers ml^{-1}. S24 was used at a dilution of 2.8×10^4. The results are shown in Figure 4. Curve A represents a reaction mixture with a P_o of 1.03×10^8 plaque-formers ml^{-1}. The rate of neutralization in this system was not exponential and the fraction of P_o surviving after 60 minutes of incubation at 37 C was 0.1. Curve B, representing a P_o of 9.70×10^6 plaque-formers ml^{-1} appeared to be logarithmic for about the first ten minutes after which time the rate of neutralization slowly decreased. Curve C represents a reaction mixture with a P_o of 9.51×10^5 plaque-formers ml^{-1}. At this serum concentration/P_o ratio the system appeared to have exponential neutralization characteristics for about the first 30 minutes of neutralization, followed by a slower rate of neutral-

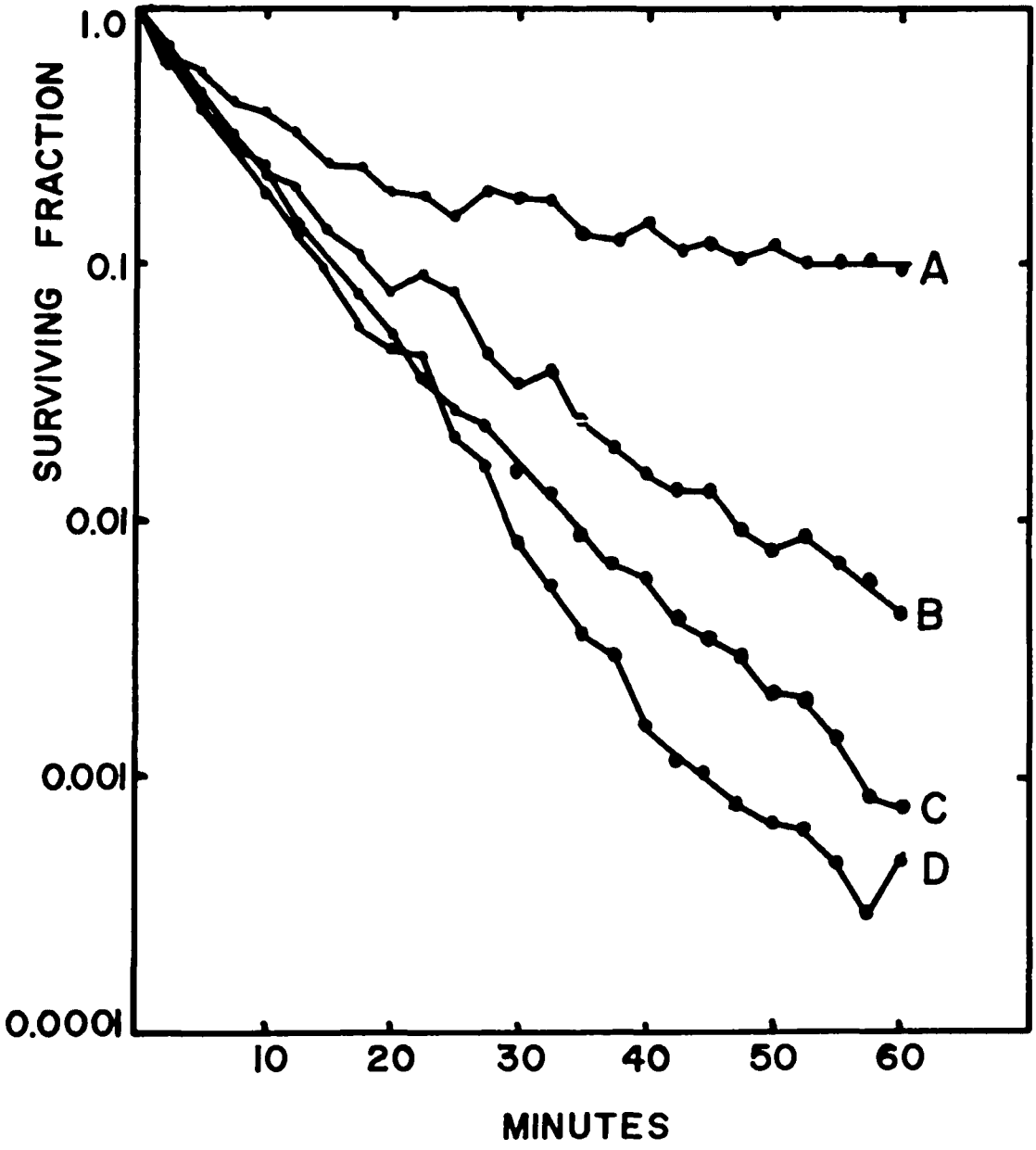

Figure 4. Neutralization of ϕX174d by S24 diluted 2.8×10^4 times. Incubated at 37 C in broth with 1:100 NRS. P_o for reaction mixtures (plaque-formers ml^{-1}):

A = 1.03×10^8
B = 9.70×10^6
C = 9.51×10^5
D = 7.54×10^4

ization. Curve D, which represents a reaction mixture containing 7.54×10^4 plaque-formers ml^{-1} initially, appeared to be exponential for the entire 60 minutes. It can also be seen that, with P_o in a range of 9.70×10^6 to 7.54×10^4 plaque-formers ml^{-1}, the fraction of P_o surviving neutralization after 60 minutes was approximately 10^{-3}.

In further study of the neutralization of ϕX174d by S24 diluted 2.8×10^4 times, the kinetics of neutralization was determined by doing eight, rather than four replicate platings on each sample taken from the reaction mixtures. In this experiment, the samples were taken at ten minute intervals during a one hour incubation period. The results are shown in Figure 5 where it will be seen that P_o ranged from 1.23×10^5 (curve D) to 7.80×10^6 plaque-formers ml^{-1} (curve A). All of the neutralization curves were exponential for about the first 15 to 20 minutes of incubation. The subsequent rate of neutralization appeared to be slightly less in all of the mixtures, except in the mixture represented by curve D. Curve D appeared to be exponential throughout the entire incubation period. After 60 minutes of incubation, the fraction of P_o surviving neutralization was approximately 4×10^{-3} and 10^{-4} in the reaction mixtures represented by curve A and curve D, respectively. For reaction mixtures represented by curves B and C, the fraction of P_o surviving was approximately 10^{-3}.

To this point the studies on the neutralization of ϕX174d by S24 had depended entirely on a one hour incubation period. Attention was therefore focused next on the fraction of phage surviving neutral-

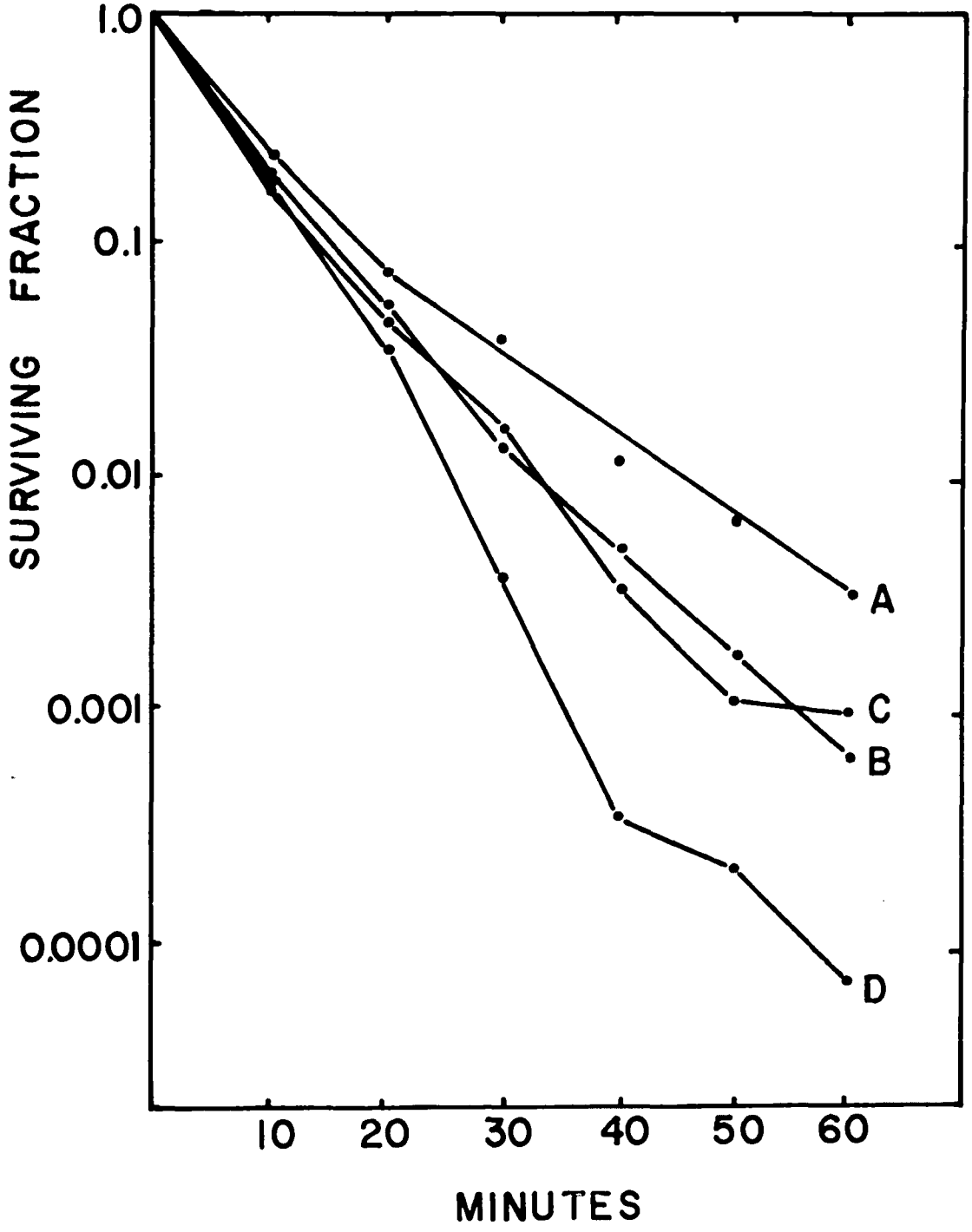

Figure 5. Neutralization of ϕX174d by S24 diluted 2.8×10^4 times. Incubated at 37 C in broth with 1:100 NRS. P_o for reaction mixtures (plaque-formers ml^{-1}):

A = 7.80×10^6 C = 8.80×10^5
B = 9.66×10^5 D = 1.23×10^5

ization after 6 to 12 hours of incubation. It seemed possible that with prolonged incubation the extent of neutralization might be found to be independent of P_o. For this purpose S24, diluted 2.8×10^4 times, was incubated with ϕX174d with P_o ranging between 6.20×10^5 and 6.20×10^8 plaque-formers ml^{-1} Samples were removed from the reaction mixture after one, two, four, six, nine, and 12 hours of incubation and assayed for surviving phage. The results of this experiment, shown in Figure 6, indicate that the differences observed previously in the fraction of P_o surviving neutralization in various reaction mixtures were magnified remarkably by increasing the incubation period. After 12 hours of incubation a 10,000-fold difference in the fraction of P_o surviving neutralization was observed between curve B and curve C. An insignificant difference was observed between curves C and D. About a tenfold difference was observed between curves A and B. The fraction of P_o surviving neutralization in the reactions represented by curves C and D was approximately 3×10^{-6}; for curve B, 6×10^{-2}; and for curve A, 0.8. The general shapes of curves C and D were essentially the same throughout the course of the experiment. The shapes of curves A and B were different with respect to each other and with respect to curves C and D.

It seemed of interest next to determine whether a tenfold increase in serum concentration would affect the kinetics of neutralization. S24 diluted 2.8×10^3 times was incubated with ϕX174d at P_o concentrations ranging between 2.80×10^8 and 2.80×10^5 plaque-formers ml^{-1}. Samples were removed from the reaction mixtures at one, two, four, six, and nine hour intervals and assayed for surviving

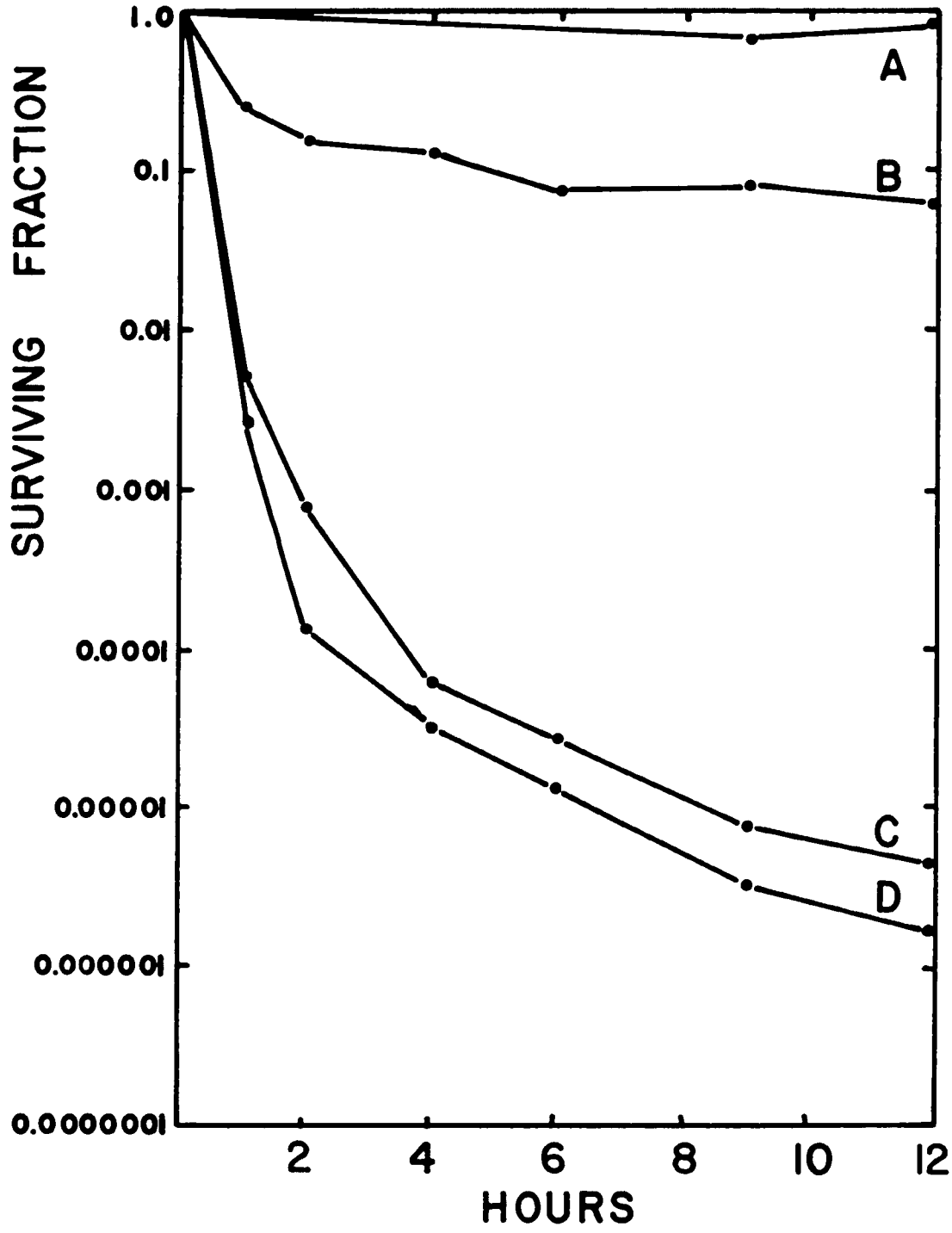

Figure 6. Neutralization of ϕX174d by S24 diluted 2.8×10^4 times. Incubated at 37 C in broth with 1:100 NRS. P_o for reaction mixtures (plaque-formers ml^{-1}):

A = 6.20×10^8 C = 6.20×10^6
B = 6.20×10^7 D = 6.20×10^5

phage. The results presented in Figure 7 reveal that the fraction of phage surviving neutralization was approximately the same (10^{-6}) when P_o was 2.80×10^7, 2.80×10^6, or 2.80×10^5 plaque-formers ml^{-1}. In marked contrast, when P_o was 2.80×10^8 plaque-formers ml^{-1} the surviving fraction was 6×10^{-2} after nine hours of incubation.

Another experiment was performed in which the concentration of S24 was ten times less than the usual concentration. S24 diluted 2.8×10^5 times was incubated for six hours with ϕX174d at concentrations of P_o ranging between 4.94×10^8 and 4.94×10^5 plaque-formers ml^{-1}. Samples were removed from the reaction mixtures at one, two, three, four, and six hour intervals and assayed for surviving phage. It can be seen from the results presented in Figure 8 that when P_o was 4.94×10^5 plaque-formers ml^{-1} the surviving fraction was 0.1. With reaction mixtures having P_o of 4.94×10^7 and 4.94×10^8 plaque-formers ml^{-1} no appreciable neutralization occurred during the six hour incubation period. When P_o was 4.94×10^6 plaque-formers ml^{-1} a very slow rate of neutralization occurred and the fraction of P_o surviving neutralization after six hours of incubation was approximately 0.55.

Examination of the results of neutralization studies illustrated in Figures 6, 7, and 8 reveals one characteristic which appears to be fairly uniform throughout. In those reaction mixtures in which significant neutralization occurred the initial rate of neutralization was fairly constant, and rather marked, down to 10^{-5} to 10^{-6} surviving phage. Later the neutralization rate decreased greatly and eventually approached zero. It seemed possible that reversible neutralization might account for this decreasing rate of neutralization. The

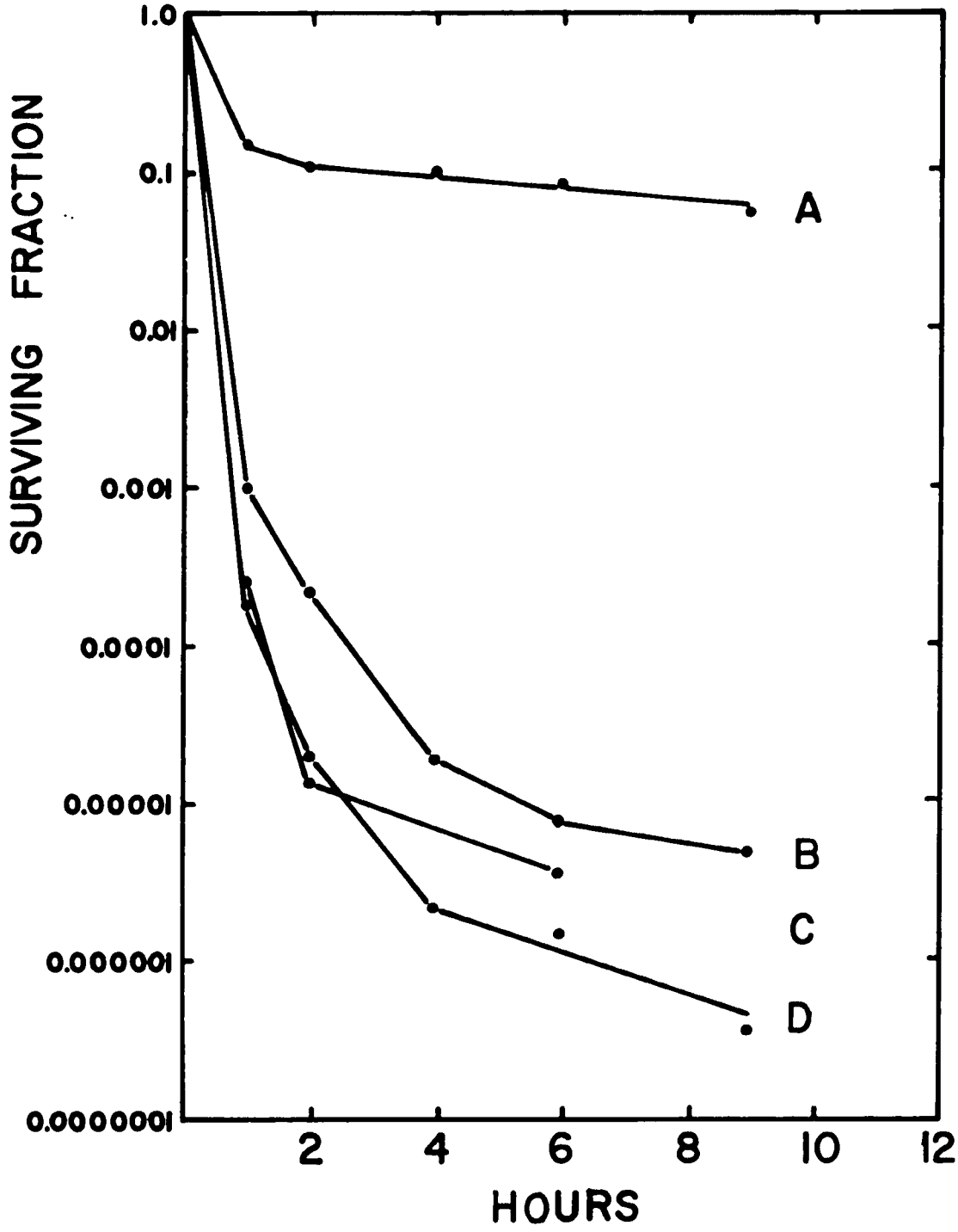

Figure 7. Neutralization of ϕX174d by S24 diluted 2.8 x 10^3 times. Incubated at 37 C in broth with 1:100 NRS. P_o for reaction mixtures (plaque-formers ml^{-1}):

A = 2.80 x 10^8 C = 2.80 x 10^6
B = 2.80 x 10^7 D = 2.80 x 10^5

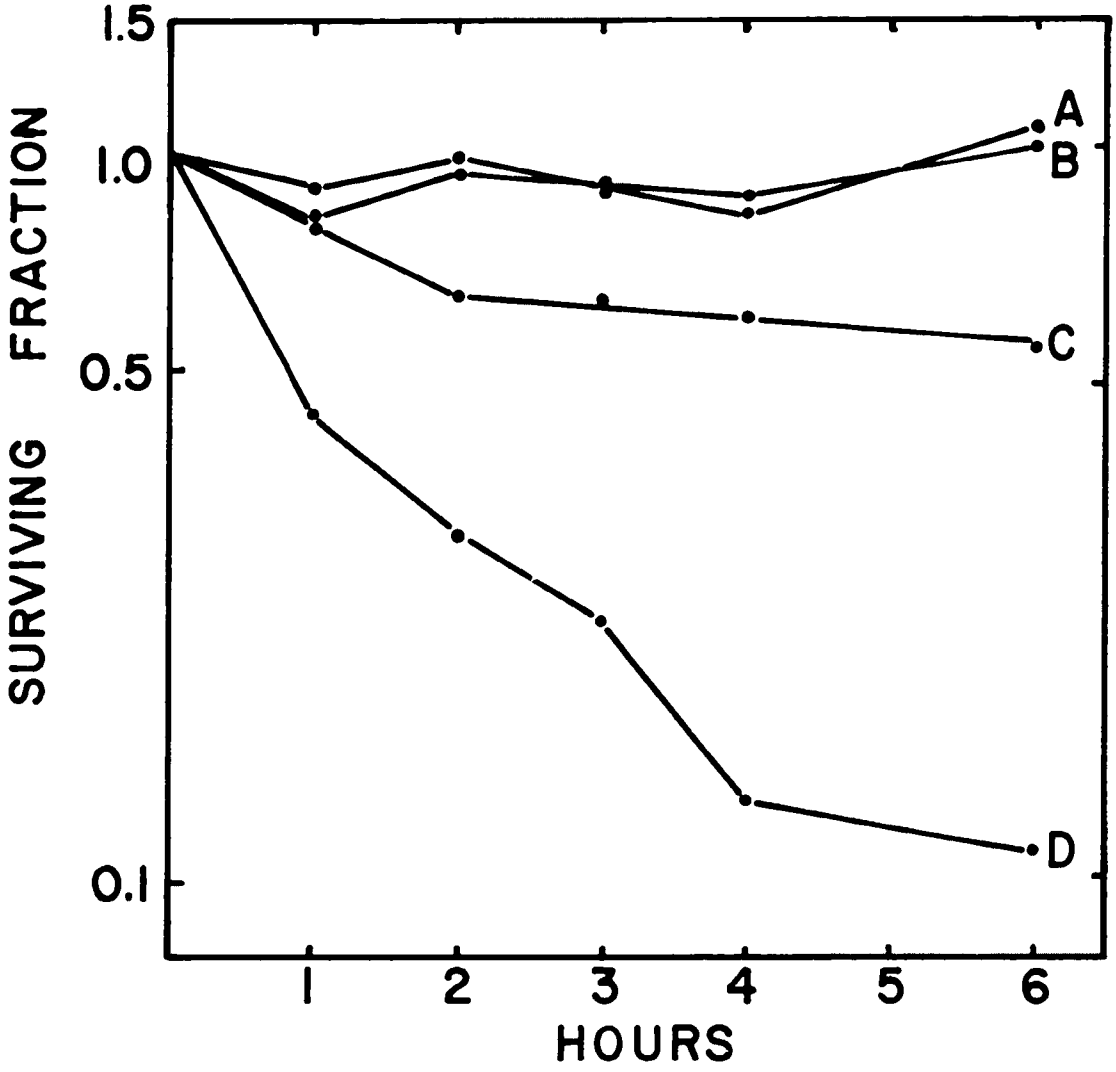

Figure 8. Neutralization of ϕX174d by S24 diluted 2.8 x 10^5 times. Incubated at 37 C in broth with 1:100 NRS. P_o for reaction mixtures (plaque-formers ml^{-1}):

A = 4.94 x 10^8 C = 4.94 x 10^6
B = 4.94 x 10^7 D = 4.94 x 10^5

phenomenon appeared to be most apparent when the P_o was approximately 10^8 plaque-formers ml^{-1}. To explore this phenomenon further, S24 was diluted 2.8×10^4 times and mixed with ϕX174d such that P_o was 1.04×10^8 plaque-formers ml^{-1}. Two identical mixtures were prepared. One of the mixtures, serving as the control, was incubated at 37 C continously for 60 minutes. The other mixture was incubated for ten minutes, diluted 1:50 in pre-warmed broth containing 1:100 NRS to stop neutralization, and incubated for an additional 60 minutes. At various intervals samples were removed from the two reaction mixtures, diluted, and assayed for surviving phage. The results, as given in Figure 9, showed that the neutralization reaction mixture serving as the control (solid curve) gave a non-exponential survivor curve. After ten minutes of incubation, the fraction of P_o surviving neutralization was 0.31 in the control reaction mixture and 0.32 in the reaction mixture that was diluted 1:50. On continued incubation the latter reaction mixture (broken curve) showed neither an increase nor a decrease in the fraction of P_o surviving neutralization. Apparently no reversible neutralization occurred in the partially neutralized, diluted phage-antiphage mixture.

It also seemed possible that the strain of host bacterium used for assay of surviving phage might account for the irregular neutralization curves seen above. To test this possibility samples taken from a phage-serum mixture were assayed for surviving phage on the usual host strain (E. coli C) and on strain H. In this experiment, S24 was used at a dilution of 2.8×10^4. P_o was 8.93×10^6 plaque-formers ml^{-1} when assayed on E. coli C, but when measured on E. coli

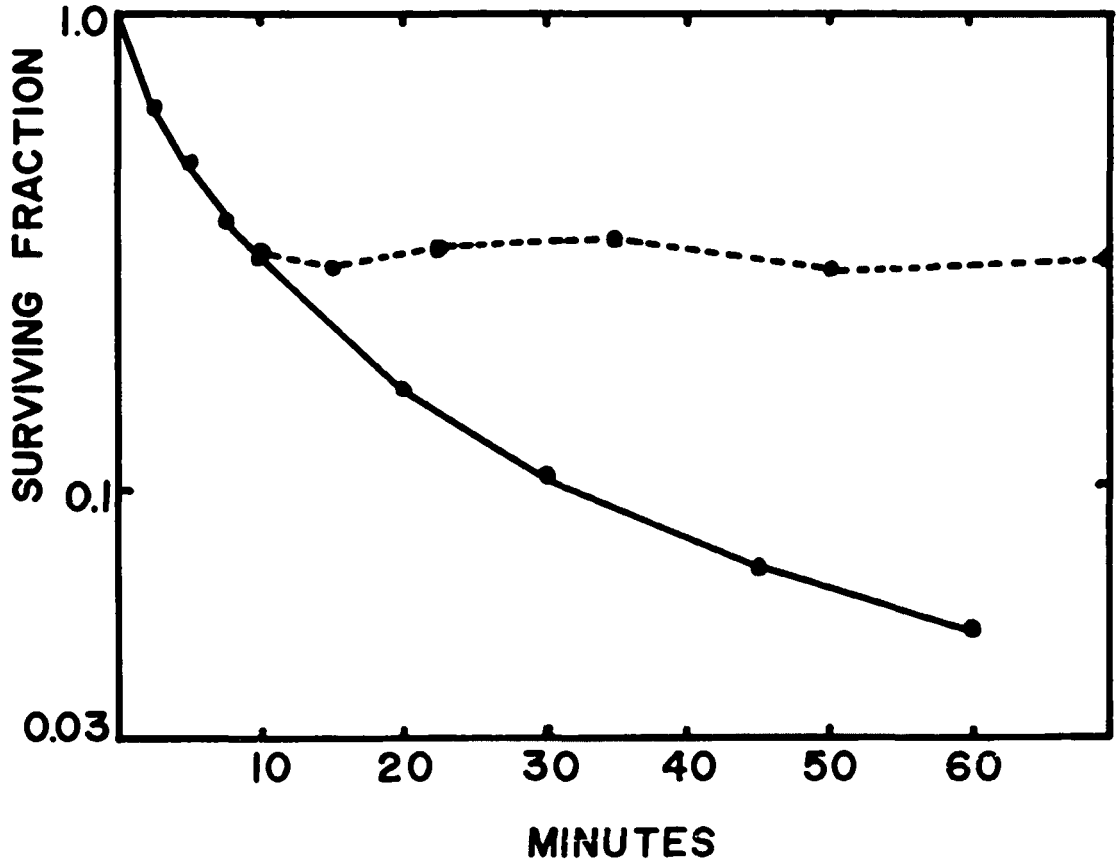

Figure 9. Failure of dilution to reverse the ϕX174d-S24 neutralization. Two identical reaction mixtures, containing S24 diluted 2.8 x 10^4 times and 1.04 x 10^8 plaque-formers ml^{-1} of ϕX174d, were incubated at 37 C in broth with 1:100 NRS. After ten minutes of incubation, one mixture was diluted 1:50 in broth and incubation was continued (broken curve). The other reaction mixture was untreated (solid curve).

H the P_o was 5.60×10^6 plaque-formers ml^{-1}. Thus the efficiency of plating (e.o.p.) of ϕX174 on E. coli H was 0.63. A single phage-antiphage reaction mixture was prepared and incubated at 37 C for 60 minutes. At ten minute intervals samples were removed, diluted, and assayed on the two host strains. The results are shown in Figure 10. It can be seen that the host strain had no effect on the rate or extent of neutralization of ϕX174d. The fraction of P_o surviving neutralization was about 2.70×10^{-3} on both strains after 60 minutes of incubation at 37 C.

Most of the preceding experiments had been so designed that the fraction of P_o surviving neutralization after 60 minutes of incubation was between 10^{-2} and 10^{-3}. In the next experiment an attempt was made to increase the fraction of P_o surviving neutralization up to a range of from 10^{-1} to 10^{-2} by reducing the concentration of S24. S24 was diluted 6×10^4 times and the P_o ranged between 6.14×10^8 and 6.14×10^5 plaque-formers ml^{-1}. From the results presented in Figure 11 it will be seen that each of the reaction mixtures gave about the same rate and extent of neutralization. However, each of the four curves was curvilinear, suggesting that the rate of neutralization for each of the four reaction mixtures decreased with time. After 60 minutes of incubation the fraction of P_o surviving neutralization was approximately 2×10^{-2}.

Demonstration of the percentage law. In this experiment the phage used was that designated lot III, ϕX174b. S24 was diluted 6×10^4 times and the P_o ranged between 4.08×10^8 and 4.08×10^5 plaque-formers ml^{-1}. The results presented in Figure 12 show that all of the

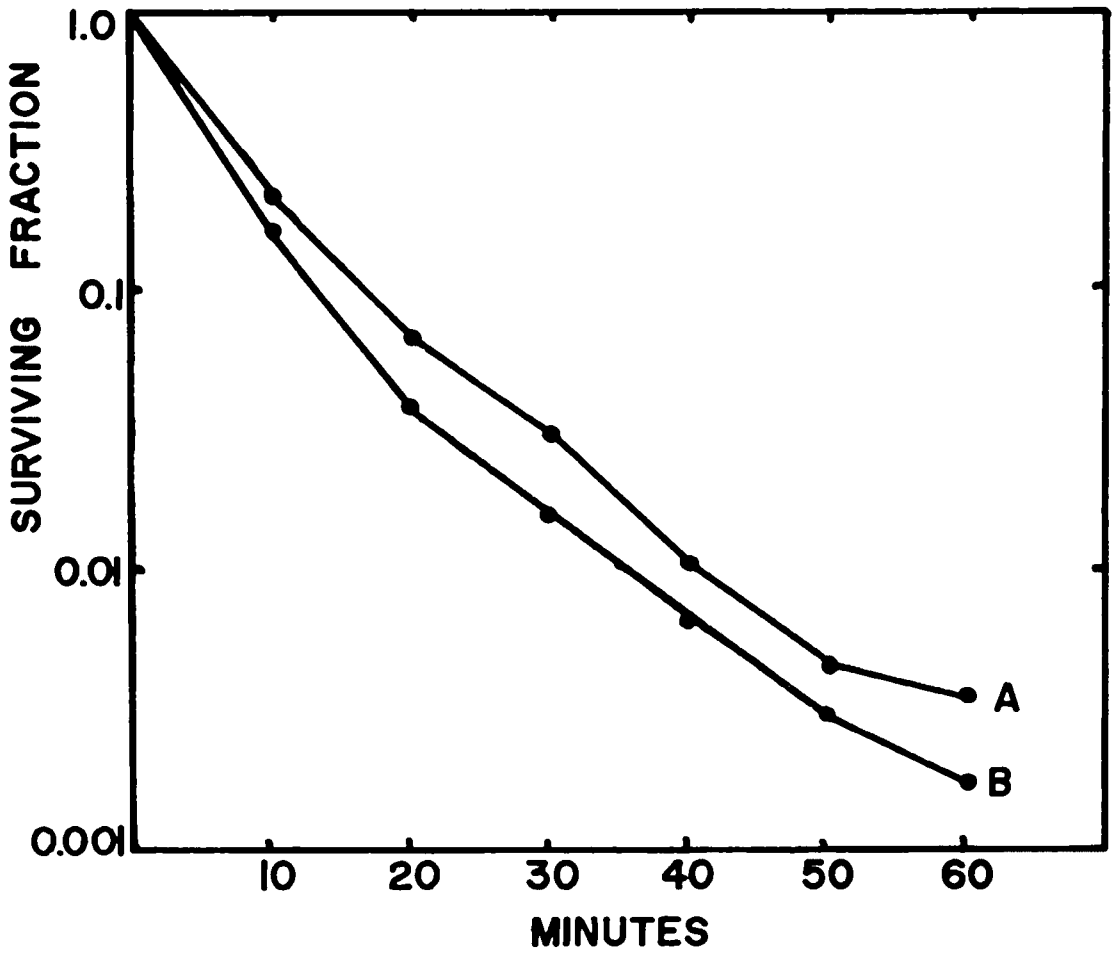

Figure 10. Neutralization of ϕX174d by S24 diluted 2.8×10^4 times. Incubated at 37 C in broth with 1:100 NRS. P_o was 8.93×10^6 plaque-formers ml^{-1}. Samples taken from the reaction mixture were plated on E. coli C (curve A) and on E. coli H (curve B).

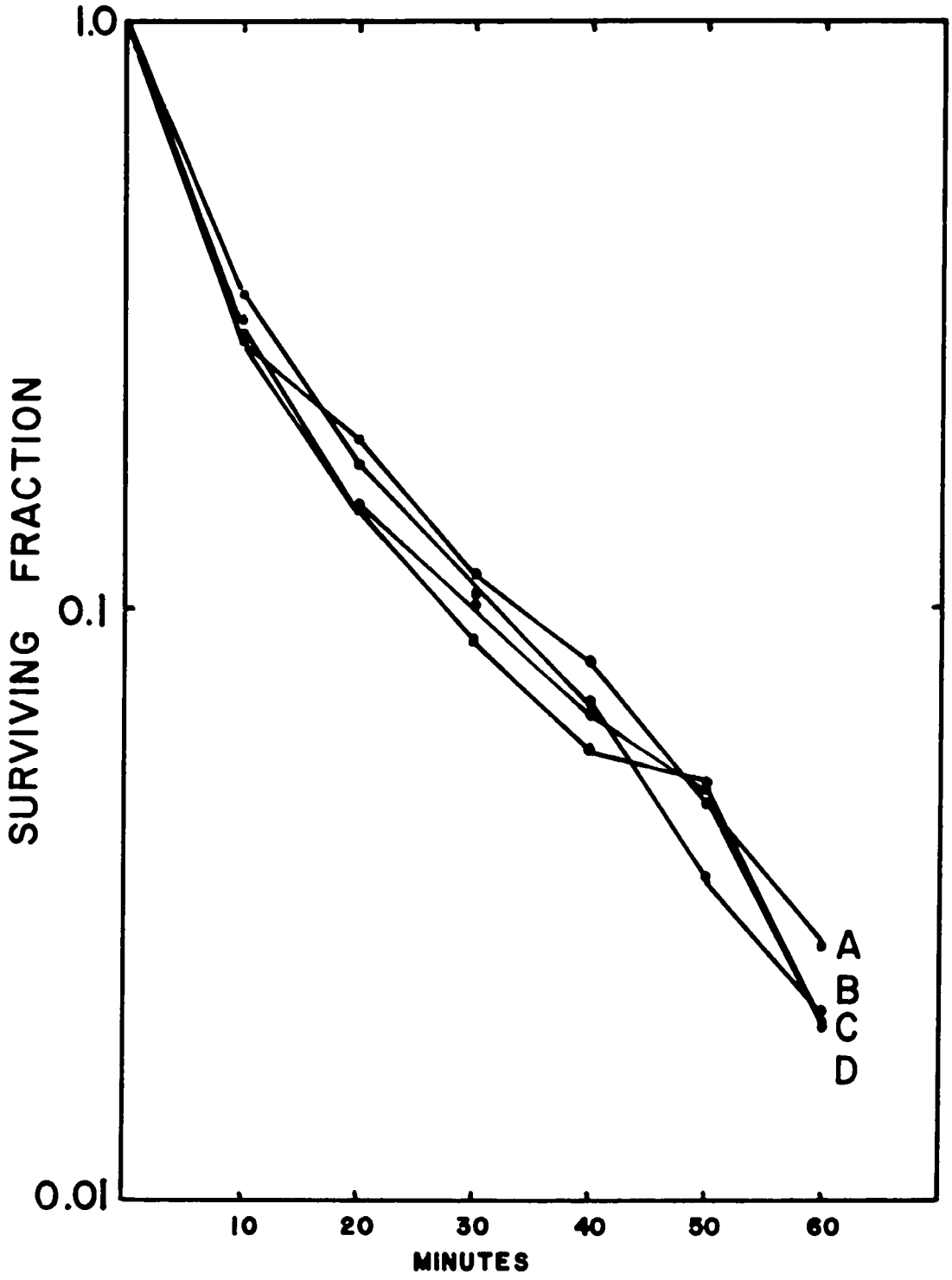

Figure 11. Neutralization of ϕX174d by S24 diluted 6 x 10^4 times. Incubated at 37 C in broth with 1:100 NRS. P_o for reaction mixtures (plaque-formers ml^{-1}):

A = 6.14 x 10^8 C = 6.14 x 10^6
B = 6.14 x 10^7 D = 6.14 x 10^5

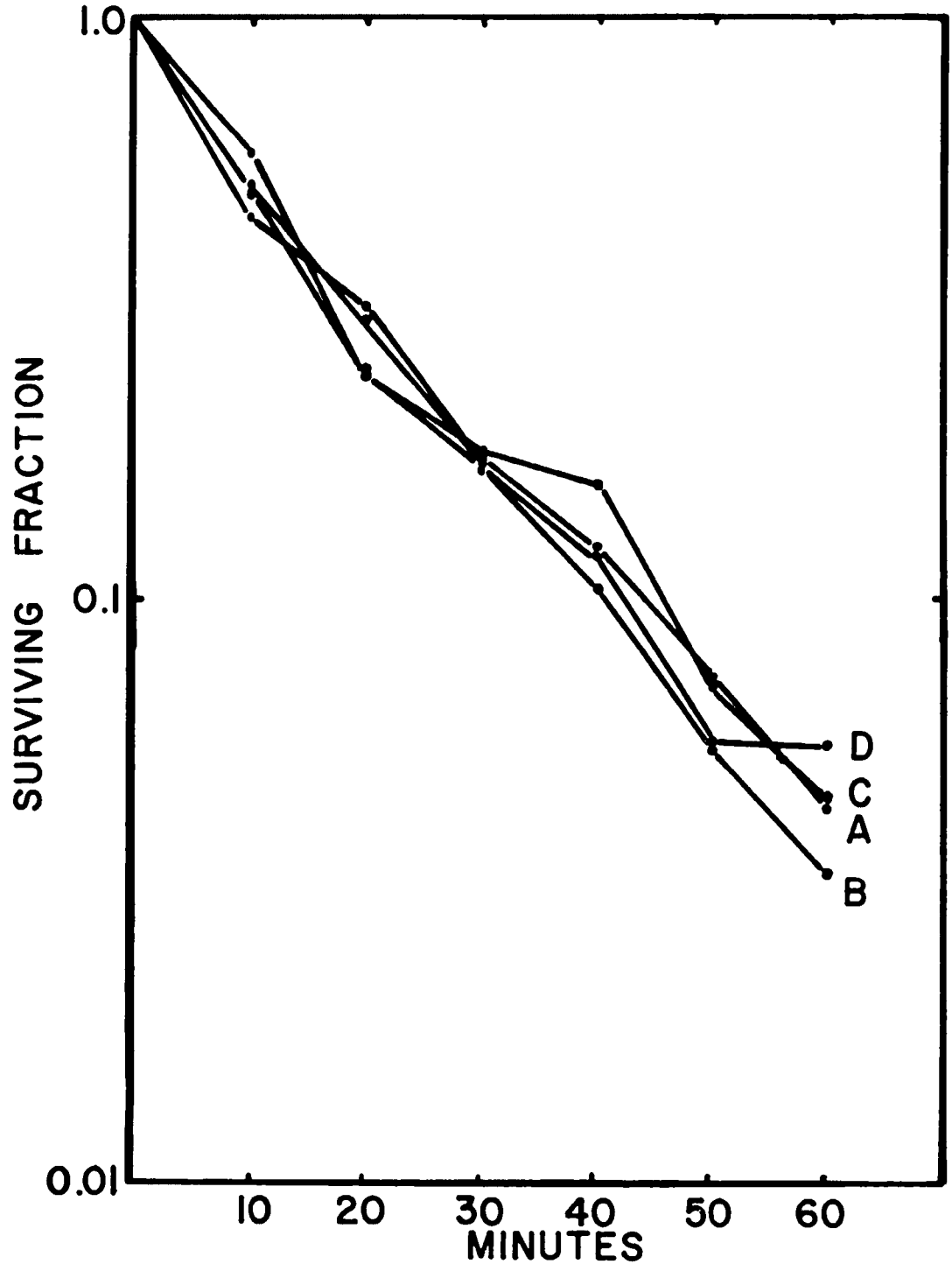

Figure 12. Neutralization of ϕX174b by S24 diluted 6×10^4 times. Incubated at 37 C in broth with 1:100 NRS. P_o for reaction mixtures (plaque-formers ml^{-1}):

A = 4.08×10^8 C = 4.08×10^6
B = 4.08×10^7 D = 4.08×10^5

neutralization reactions exhibited first-order kinetics over the 60 minute incubation period and the fraction of P_o surviving neutralization was approximately 4×10^{-2}. In addition, the fraction of P_o surviving neutralization at any particular time was approximately the same in each of the reaction mixtures. The results of this experiment demonstrated that the ϕX174-anti-ϕX174 system obeys the percentage law over a 1,000-fold concentration range of P_o, with the highest P_o being 4.08×10^8 plaque-formers ml^{-1}. In the next experiment, the kinetics of neutralization of ϕX174b was studied with a single P_o which had been shown in the previous experiment to be covered by the percentage law. In addition, ten replicate platings were made from each sample taken from the reaction mixture. S24 was diluted 6×10^4 times and the P_o was 3.93×10^6 plaque-formers ml^{-1}. The results are given in Figure 13. The neutralization curve showed first-order kinetics, with only slight deviations, during the course of one hours' incubation. The results of this and the previous experiment show clearly that the use of ϕX174b in neutralization reactions obliterated the curvilinear characteristic seen with ϕX174d.

Dependence of the rate of neutralization on the serum concentration. The rate of inactivation of phage by antiserum is directly proportional to the concentration of antiserum used (Andrewes and Elford, 1933a). It seemed of interest to determine whether the ϕX174-anti-ϕX174 system possessed this characteristic. To test this possibility S24 was diluted 6×10^5, 3×10^5, 1×10^5, and 6×10^4 times. To each dilution of serum was added ϕX174b at a P_o of 5.36×10^6 plaque-formers ml^{-1}. At ten minute intervals after preparing the re-

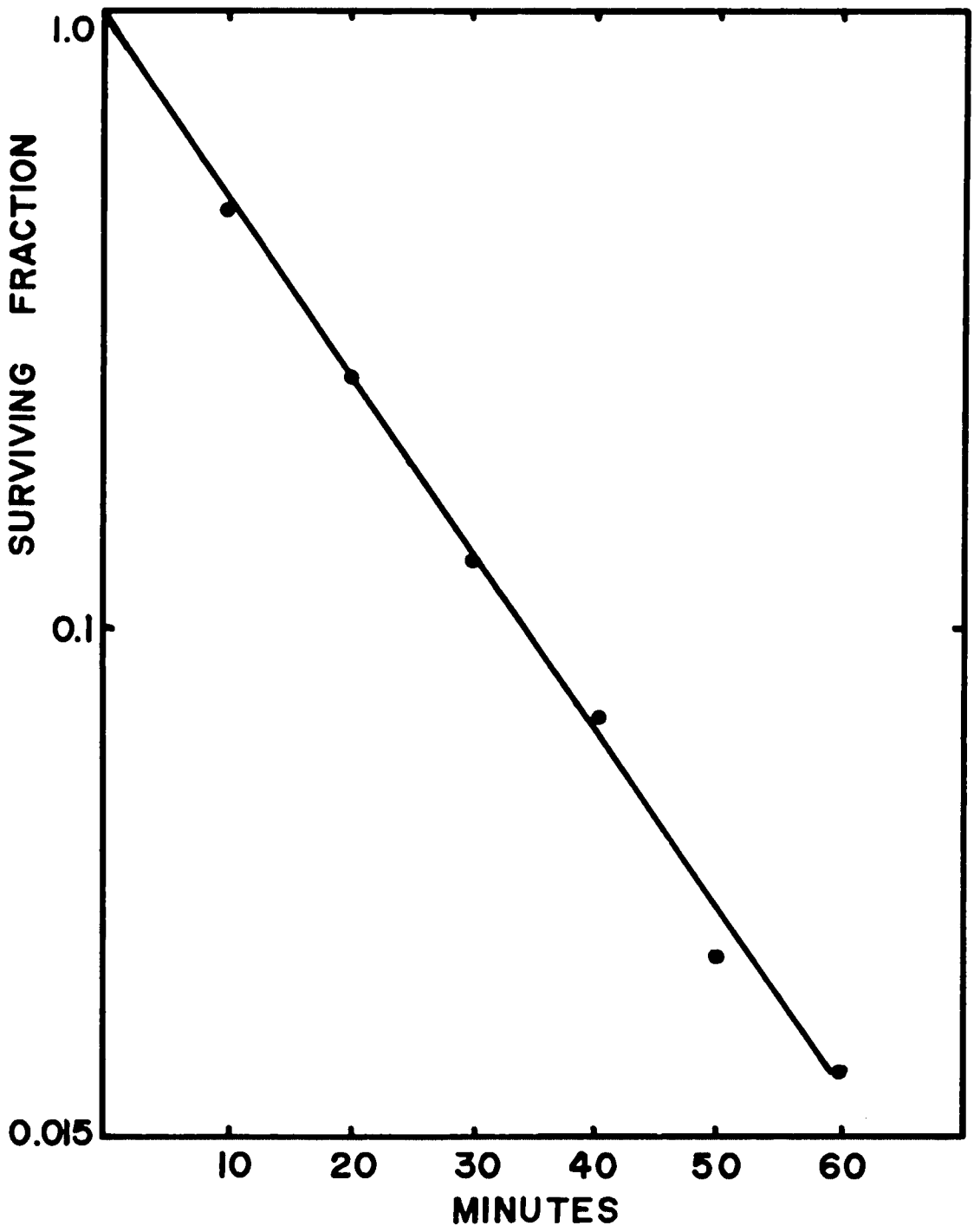

Figure 13. Neutralization of ϕX174b by S24 diluted 6×10^4 times. Incubated at 37 C in broth with 1:100 NRS. P_o was 3.93×10^6 plaque-formers $^{-1}$.

action mixtures, samples were removed, diluted, and assayed for surviving phage. The results are shown in Figure 14. It can be seen that the rate of neutralization of ϕX174b by S24 was proportional to the concentration of serum. The greatest rate of neutralization was obtained with the greatest concentration of S24 (curve D). The lowest rate of neutralization was obtained with the lowest concentration of S24 (curve A). It is also significant that each reaction gave an exponential neutralization curve.

The results of the last experiment permitted the calculation of SNI for S24. A plot of log D (logarithm of the dilution of S24 used) against log P_o/P is given in Figure 15. The slope n of the line was found to be 1.5. The value for SNI was found by extrapolating the curve to the y axis and determining the intercept. This procedure gave a SNI value of 5.84 for S24 after 60 minutes of incubation with ϕX174b at 37 C. The value for SNI determined by the use of formula (6) was found to be 5.90 when n = 1.5.

Determination of Q_{10} for the ϕX174b-S24 system. In this experiment, mixtures of ϕX174b at a P_o of 5.04×10^6 plaque-formers ml^{-1} and S24 diluted 6×10^4 times, were incubated at 0, 10, 22.5, and 37 C. At 10, 20, 40, and 60 minute intervals samples were removed from the mixtures, diluted, and assayed for surviving phage. The results are shown in Figure 16. It can be seen that the rate and extent of neutralization were directly proportional to the temperature of incubation employed. An Arrhenius plot of these data is given in Figure 17. It is seen that there was a linear relationship between the logarithm of the neutralization rate constant, K, and the recipro-

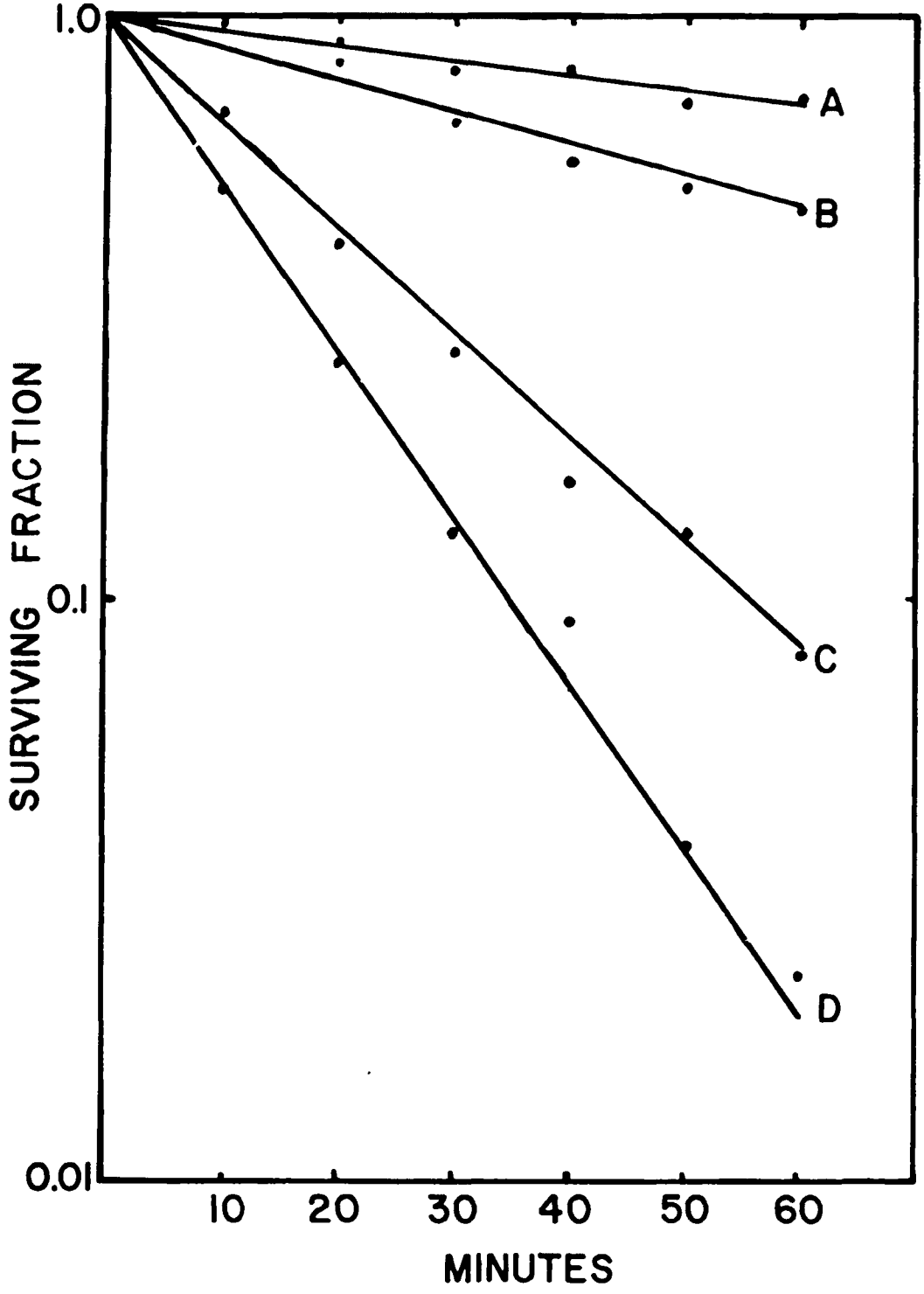

Figure 14. Dependence of the rate of neutralization of ϕX174b on the concentration of S24. Incubated at 37 C in broth with NRS. P_o was 5.36×10^6 plaque-formers ml^{-1}. S24 diluted:

A = 6×10^5 C = 1×10^5
B = 3×10^5 D = 6×10^4

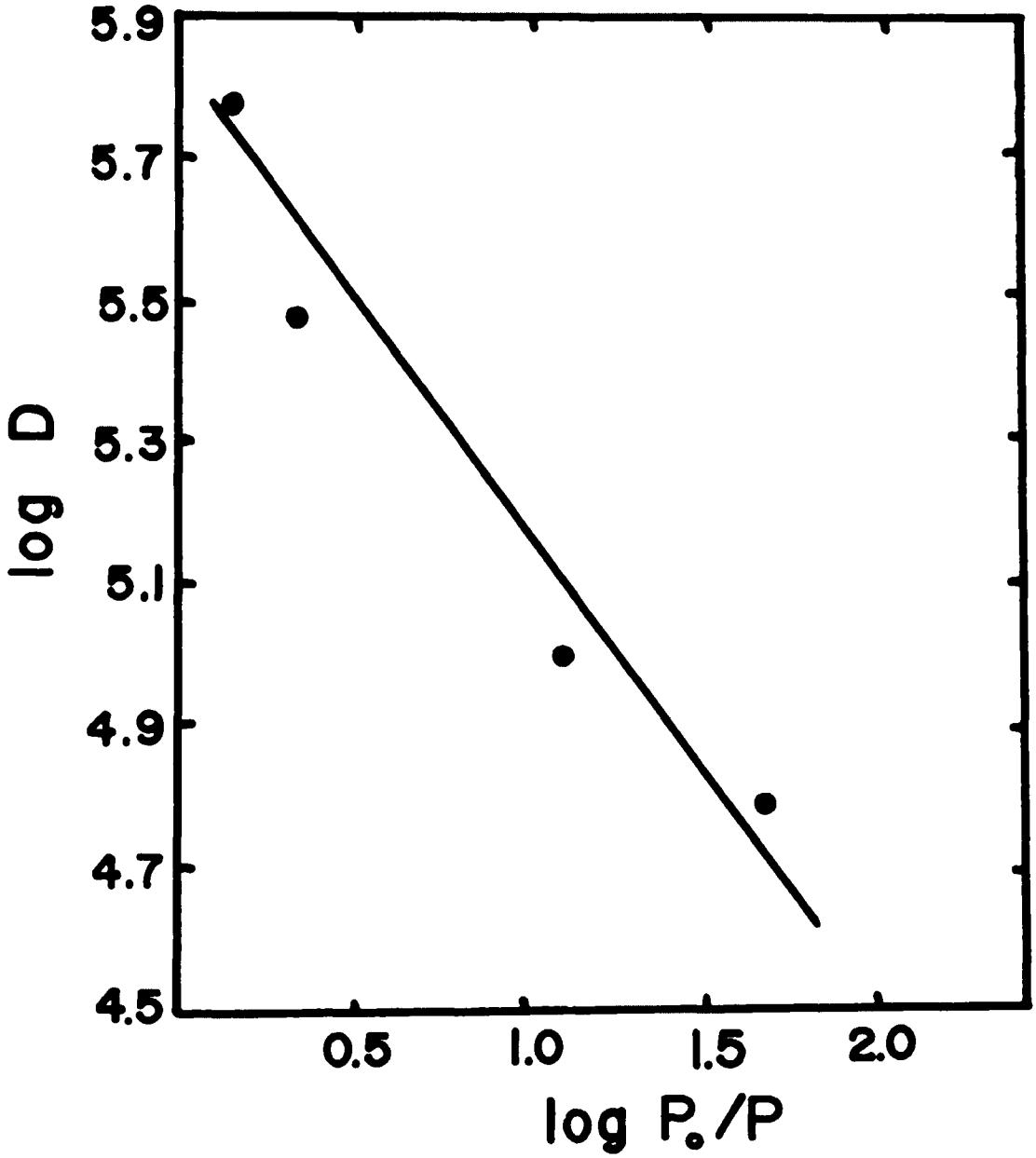

Figure 15. Neutralization of ϕX174b by S24. Incubated at 37 C for 60 minutes in broth with 1:100 NRS. P_0 was 5.36×10^6. D = dilution of S24. P_0/P = ratio of initial to surviving phage.

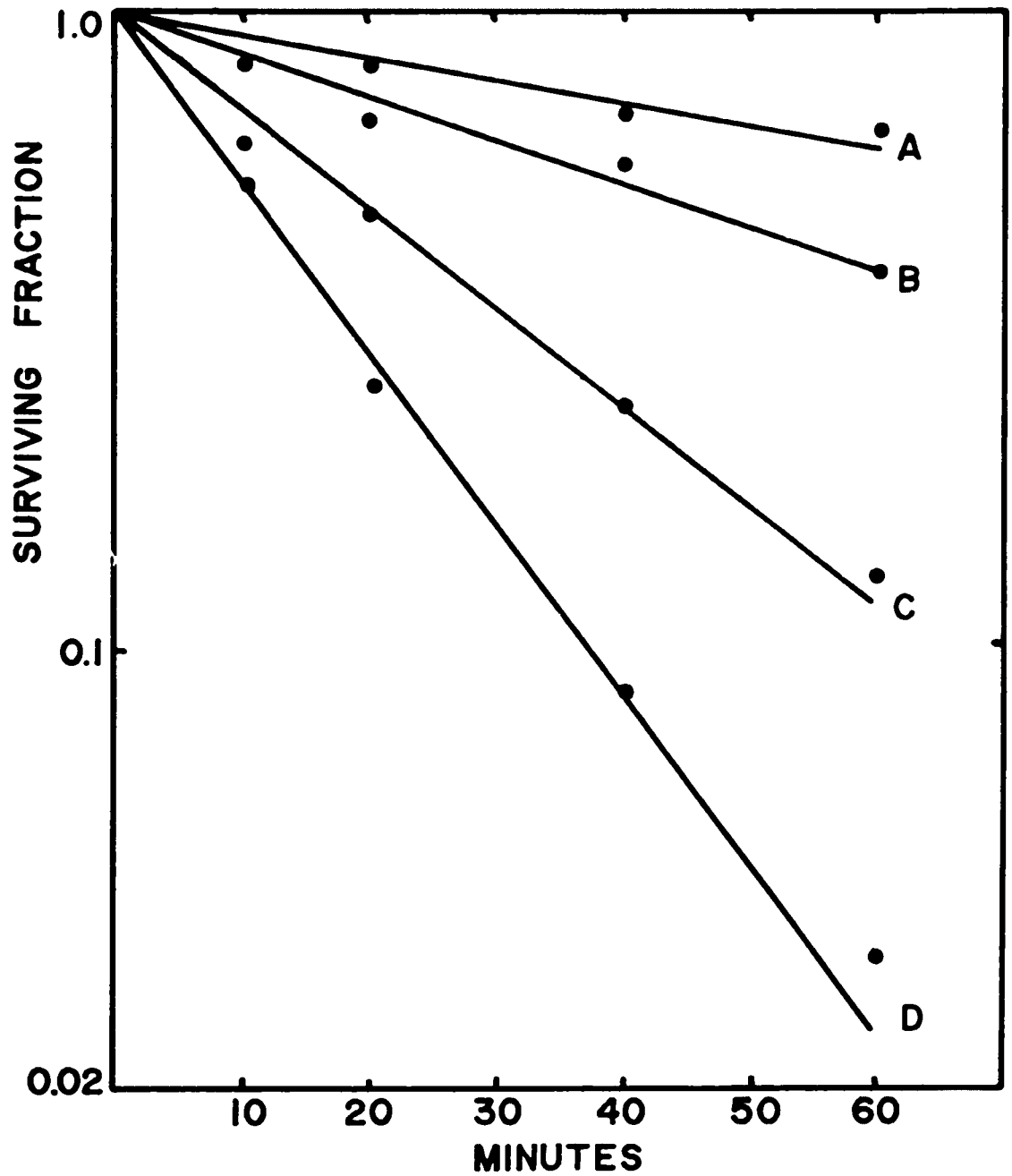

Figure 16. Dependence of the rate of neutralization of ϕX174b on the temperature of incubation. P_o was 5.04×10^6 plaque-formers ml^{-1}. S24 was diluted 6×10^4 times. The temperature of incubation for the mixtures:

A = 0.0 C
B = 10.0 C
C = 22.5 C
D = 37.0 C

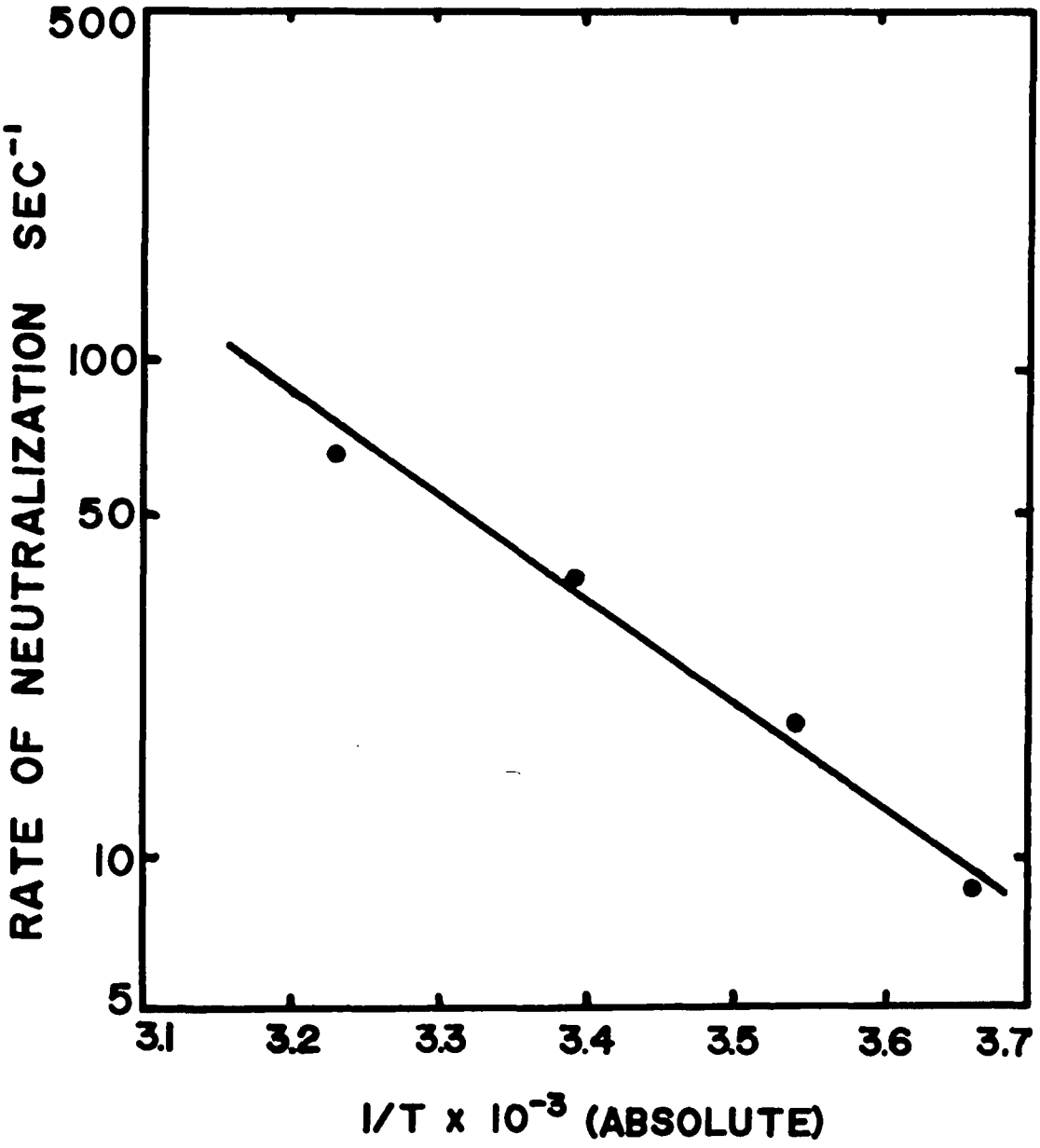

Figure 17. Arrhenius plot showing the dependence of the specific rate constant of neutralization of ϕX174b by S24 on the absolute temperature.

cal of the absolute temperature of incubation employed. The Arrhenius constant was calculated by use of equation (7) and found to be approximately 9,600 calories $mole^{-1}$.

The temperature coefficient, Q_{10}, of the ϕX174b-S24 reaction, calculated by determining the ratio of the rates of neutralization at T^{o} + 10 C and at T^{o} C, was found to be 1.8.

Characterization of the Phage Surviving Neutralization

Inactivation by lupus erythematosus (LE) serum. The possibility that the phage escaping neutralization might be due to a mechanism involving infectious nucleic acid was explored first through the use of anti-DNA antibodies present in serum of patients with LE. The results of a typical experiment, using LE 8, are shown in Figure 18. It can be seen that the rate of neutralization of ϕX174b during the first three hours of neutralization was essentially exponential and that the rate decreased subsequently until, at ten hours, it was approximately zero. At this point, the addition of either broth or S24 to the reaction mixture resulted in a slight increase in the rate and extent of neutralization. The addition of LE 8 resulted in an increase in the rate of neutralization approximately ten times greater than that seen with broth or S24.

The next experiment was designed to determine whether LE serum would have any effect on ϕX174b not previously treated with S24. The P_o of ϕX174b was 5.20×10^{8} plaque-formers ml^{-1}. S24 was used at a dilution (1×10^{4}) which, in preliminary studies, gave a sur-

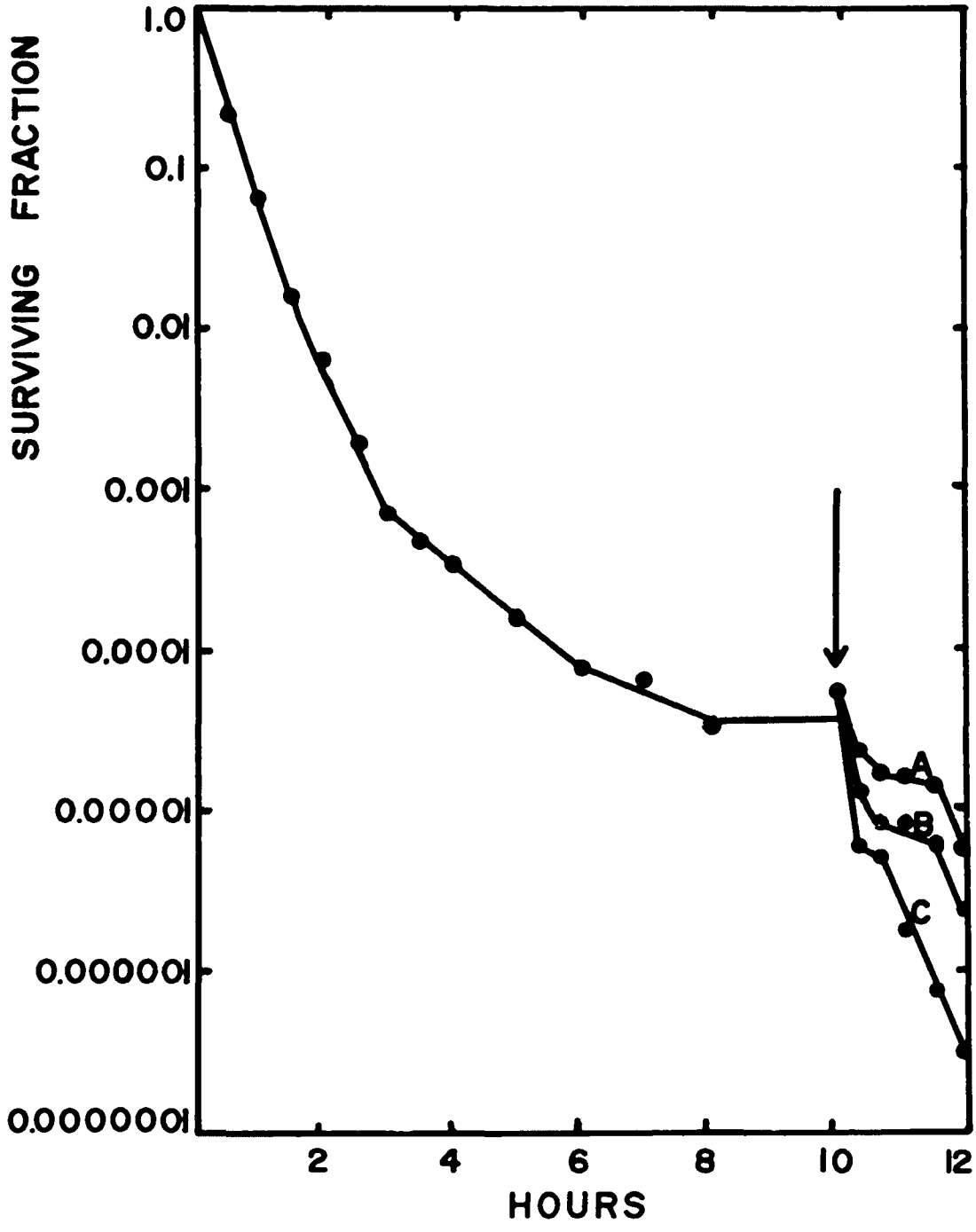

Figure 18. Inactivation of surviving ϕX174b by LE 8. Incubated at 37 C in broth with 1:100 NRS. S24 was used diluted 6 x 10^4 times and P_o was 3.28 x 10^7 plaque-formers ml^{-1}. After ten hours of incubation (arrow), 0.9 ml samples of the reaction mixture received 0.1 ml broth (curve A); 0.1 ml S24 diluted 6 x 10^3 times (curve B); and 0.1 ml LE 8 (curve C).

viving fraction of approximately 1.22×10^{-5} after four hours of incubation at 37 C. At the end of four hours of incubation, a control suspension containing no S24 was diluted 10^5 times so that its concentration of phage would be approximately the same as that in the reaction mixture containing S24. To 0.9 ml of the control phage suspension was added 0.1 ml of broth, to another 0.9 ml portion of the control suspension was added 0.1 ml of IE 8. Two 0.9 ml portions of the phage-serum reaction mixture were treated in exactly the same manner. The results presented in Figure 19 show that IE 8 affected ϕX174b only if the latter was first pre-treated with S24.

All of the IE sera listed in Table 1 were tested for their ability to inactivate ϕX174b previously treated with S24. Only IE 2, 8, 9, 11, and 12 were found to be effective. Ten sera from normal persons and hospitalized individuals who did not have IE were found to be ineffective.

Effect of DNAase. DNAase in final concentrations of 0.25, 2.5, and 25 micrograms ml^{-1} had no effect on the infectivity of either untreated phage or of phage pre-treated with S24.

Ability to infect E. coli K12S protoplasts. Further investigation into the possibility that the phage surviving neutralization might be due to a mechanism implicating infectious nucleic acid involved the use of protoplasts. The experiment was performed three times with essentially the same results. In a typical experiment surviving phage was obtained from a reaction mixture in which P_o was 3.02×10^7 plaque-formers ml^{-1} and S24 was diluted 4.5×10^4 times. After five hours of incubation at 37 C the concentration of surviving

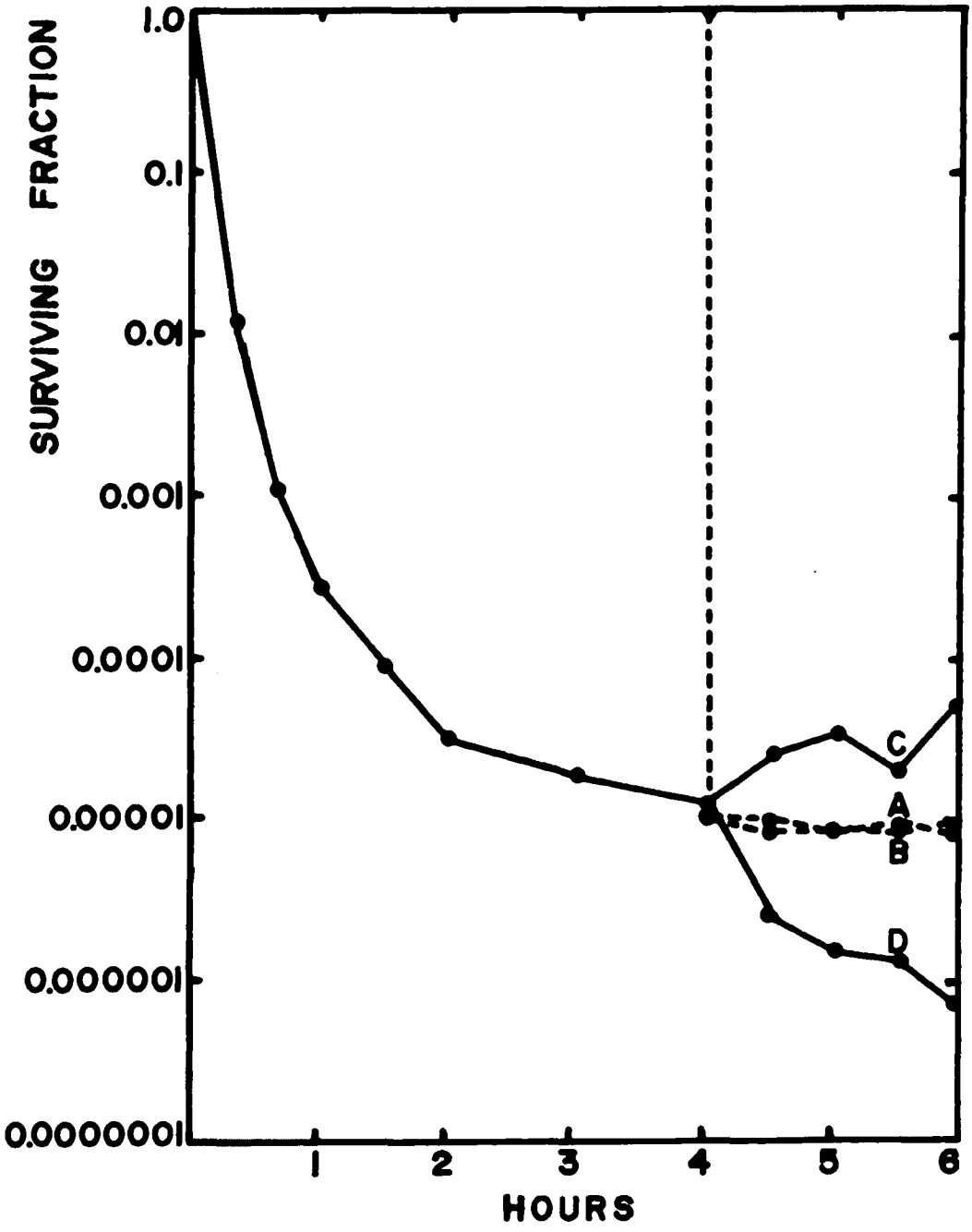

Figure 19. Inactivation of surviving ϕX174b by IE 8. The mixtures were incubated at 37 C in broth with 1:100 NRS. S24 was used diluted 1 x 10^4 times and P_o was 5.20 x 10^8 plaque-formers ml^{-1}. After four hours of incubation the control mixture (without S24) was diluted 1:10^5, as shown by the vertical broken line, and 0.9 ml samples received 0.1 ml broth (curve A) and 0.1 ml IE 8 (curve B). After four hours of incubation of the ϕX174b-S24 mixture (solid line), 0.9 ml samples received 0.1 ml broth (curve C) and 0.1 ml IE 8 (curve D).

phage was 2.30×10^3 plaque-formers ml^{-1}. A control reaction mixture, in which P_o was also 3.02×10^7 plaque-formers ml^{-1}, contained no S24. After five hours of incubation its titer dropped to 5.40×10^6 plaque-formers ml^{-1}. This mixture was then diluted 1:70 to give a concentration of phage which approximated that contained in the mixture containing S24. Each of these mixtures were then mixed 1:2 with E. coli K12S protoplasts and incubated at 37 C. Further experimental details, and the results, are shown in Figure 20. It can be seen that after ten minutes of incubation Adsorption Mixture A contained 0.4×10^3 infected protoplasts ml^{-1} and that after 80 minutes of incubation Growth Mixture A contained 2.97×10^5 plaque-formers ml^{-1}. This represented an average yield of approximately 750 plaque-formers per infected protoplast. On the other hand, Adsorption Mixture B contained no infected protoplasts after ten minutes incubation and Growth Mixture B contained only 2.68×10^4 plaque-formers ml^{-1}. This represented no increase in the concentration of phage and hence no infection of the protoplasts.

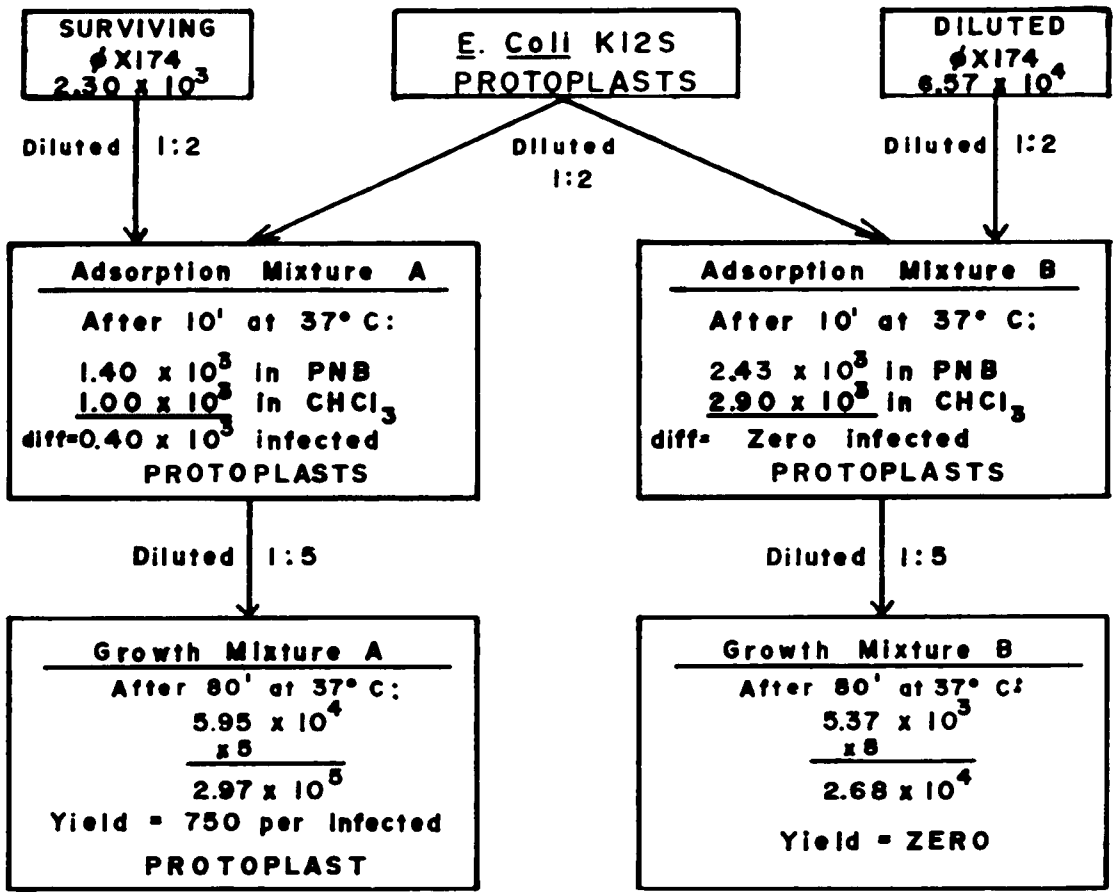

Figure 20. Infection of E. coli K12S protoplasts with surviving phage. PNB = protoplast nutrient broth with 2.0 per cent BSA. $CHCl_3$ = chloroform water.

CHAPTER IV

DISCUSSION

Of the three phage preparations described in the present investigation, the greatest yields were obtained with ϕX174d and ϕX174a. In agreement with the report of Soller (1961), the ϕX174d virions were found to be stable during dialysis against carbowax 4,000. Only about a one per cent recovery was obtained with the phage (ϕX174b) concentrated by equilibrium density gradient ultracentrifugation. This could be due to the fact that more centrifugations and washings were employed in this technique than in the procedures used for ϕX174d and ϕX174a. Sinsheimer (1959a) reported a recovery of about two per cent in one of his ϕX174 preparations after density gradient ultracentrifugation. Rueckert et al. (1962) purified ϕX174 phage by a modification of Sinsheimer's method and recovered less than two per cent of the initial virus. Thus the percentage recovery of the ϕX174b preparation does not appear to differ significantly from that reported in the literature.

Studies by Sinsheimer (1959a) of the sedimentation characteristics of ϕX174 showed that one of his phage preparations was composed of 114 S and 70 S particles prior to equilibrium density gradient ultracentrifugation. The latter technique separated the two types of

particles, permitting the phage fraction (114 S virions) to be isolated. In analytical ultracentrifugation sedimentation studies of the latter fraction, a single peak was obtained which was composed of the 114 S particles. Sinsheimer showed that the 70 S particles were DNA-free capsids antigenically identical to the 114 S virions. Rueckert et al. (1962) also found that the 114 S and 70 S particles of ϕX174 could be separated by equilibrium density gradient ultracentrifugation. The sedimentation patterns obtained for ϕX174a and ϕX174b in the present study were thus in agreement with those reported by other workers. As will be discussed later in greater detail, a phage preparation containing both 114 S and 70 S particles would probably give rise to non-exponential neutralization curves due to empty capsids reacting with antibody molecules. Such reactions would not be detected by differences in plaque-forming titers since neither the empty capsid nor the empty capsid-antibody complex would give rise to plaque formation.

In general, phages are stable in the lysates in which they are produced after removal of inactivating agents such as bacterial receptor substances, provided suitable electrolytes are present. After purification, phages are usually stable in neutral, aqueous solutions of 0.1 M sodium chloride and 10^{-3} M magnesium sulfate. Frequently, if the phage concentration is low (1×10^{9} plaque-formers ml^{-1} or less), the addition of a small amount of gelatin to the phage suspension prevents decreases in its infective titer. Although the studies reported herein on the stability of ϕX174 virions under various conditions of incubation were not comprehensive nor performed in great detail, they

did show that the phage was stable to continued incubation at 37 C provided 1:100 normal rabbit serum (NRS) was added to the medium. Apparently the small concentration of NRS used in these experiments replaced the requirement for gelatin mentioned above. The requirement for gelatin or NRS may not be specific, but may represent a general requirement for protein. This possibility was not investigated. When approximately 10^7 plaque-formers ml^{-1} or less were incubated in broth with 1:100 NRS for up to eight hours at 37 C no inactivation occurred. On the other hand, if the temperature of incubation was raised to 44 C, an appreciable inactivation was observed in two hours. Since definitive studies were not done on the kinetics of inactivation of ϕX174 during incubation at 37 C and 44 C, no data on the temperature coefficient of inactivation are available.

Purified ϕX174 is a very good antigen. Rabbits receiving ϕX174d responded by producing high levels of antibody. Since the first serum samples were not obtained until the fifth day after injection it is not known how soon the first antibodies would have been detectable. By the fifth day the K values of the two rabbit sera were 31 and 126 $minute^{-1}$. Obviously antibody production must have begun much earlier. Although precise measurements of the rate of ϕX174 antibody production in rabbits were not made, the greatest K values detectable after the primary immunization were found on the seventh day after injection. The rate of antibody production must have decreased greatly or completely by the ninth day, since at this time a significant decrease in the K values was observed. After the second injection of phage, a heightened antibody response was noted. By the eighth day the K values

of the sera were approximately 3,000 minute^{-1}. Uhr et al. (1962a) found that the initial rise in serum antibody levels after injection of guinea pigs with ϕX174 appeared to be exponential for four or five days, followed by a much slower rise for at least one week. In addition, they found that the kinetics of ϕX174 antibody production during the secondary response was very similar to that during the primary response. Their primary response sera had K values of about 10 minute^{-1} while their secondary response sera had K values of approximately 1,000 minute^{-1}. Their results also showed that in guinea pigs injected with ϕX174 over a 10,000-fold concentration range both the amount of antibody produced and the duration of antibody production were related directly to the concentration of phage used in the inoculum. They interpreted these findings as indicating that high concentrations of phage stimulated more cells to produce antibody. They further suggested that the quantitative differences between the primary and secondary antibody responses were attributable to the number of cells stimulated to produce antibody.

In the initial studies on neutralization of ϕX174d by S24, it was found that the mathematical expression for determining the fraction of phage surviving neutralization, when K was known, did not hold with every serum concentration/P_o ratio used. The results of two studies (Figures 3 and 4) revealed at least two instances of experimental deviations from the mathematical expression. One deviation was that seen in the reaction mixture containing S24 diluted 2.8 x 10^4 times and a P_o of 1.22 x 10^8 plaque-formers ml^{-1} (curve D in Figure 3) showing 10^{-2} survivors after 60 minutes of incubation. The surviving

fraction calculated mathematically was approximately 10^{-3}. The second instance was that illustrated by the non-exponential neutralization reactions observed in Figures 3 and 4 when the P_o was higher than about 10^5 plaque-formers ml^{-1}. The results shown in Figure 3 differ from those in Figure 4 largely in the frequency with which samples were removed from the reaction mixtures. The fact that the deviations mentioned above occurred in both experiments suggested that they were not artifacts related to the frequency of sampling. Further studies of the non-exponential neutralization curves seen with high P_o revealed that they were also not artifacts produced by sampling variables since they occurred even when the number of replicate platings on each sample was doubled (Figure 5).

According to the percentage law (Andrewes and Elford, 1933a), the fraction of P_o surviving neutralization should be independent of the initial phage concentration over a wide range of the latter. In the three experiments just discussed, and illustrated in Figures 3, 4, and 5, it appeared that the range of P_o covered by the percentage law was rather narrowly limited. In fact, it appeared that the upper limit of P_o covered by the percentage law was between 10^5 and 10^7 plaque-formers ml^{-1}. In addition, it appeared that the mathematical expression for first-order neutralization kinetics did not apply to the ϕX174-anti-ϕX174 system. Two factors which have been established as responsible for breakdown of the percentage law are the use of low concentrations of antibody and the use of high P_o (Burnet et al., 1937; Hershey et al., 1943). The results given in Figures 6, 7, and 8 tend to support the concept that the breakdown of the

percentage law in the early phases of the present study was related to the serum/P_0 ratio. In each of these experiments approximately the same range of P_0, but different concentrations of S24 were used, i.e. the serum was diluted 2.80×10^4 times (Figure 6), 2.80×10^3 times (Figure 7), and 2.80×10^5 times (Figure 8). When S24 was used at a dilution of 2.80×10^4 times, two levels of P_0 appeared to obey the percentage law (curves C and D, Figure 6). When the concentration of serum used was increased tenfold (Figure 7) three levels of P_0 appeared to obey the percentage law (curves B, C, and D). However, when the concentration of serum used was decreased tenfold (Figure 8) the percentage law could not be demonstrated. It is apparent from these results that a certain minimal serum/P_0 ratio is necessary for demonstration of the percentage law. Unless it is known definitely that the serum/P_0 ratio being used gives percentage law neutralization, completely erroneous estimates of the rate and extent of neutralization may be obtained.

Jerne and Avegno (1956) demonstrated that phage T4 was reversibly neutralized by primary-response serum but irreversibly neutralized by secondary-response serum. The reversibility of the former system was demonstrated by showing an increase in the fraction of P_0 surviving neutralization when the phage-antiphage mixture was diluted. In the present investigation, diluting a partially neutralized suspension of ϕX174d 1:50 in broth containing 1:100 NRS did not result in an increase in the fraction of P_0 surviving neutralization on subsequent incubation. It thus seems possible to conclude that neutralization of ϕX174 by secondary response serum is irreversible

and, therefore, that reversible neutralization cannot account for non-exponential neutralization rates. These results are in agreement with those of Jerne and Avegno (1956). Also, Hershey (1943) found no evidence of reversible neutralization with phage T2.

It is known that different strains of host bacteria may give different estimates of the fraction of P_0 surviving neutralization. Kalmanson and Bronfenbrenner (1942) analyzed this host effect with a strain of T2 that plated on certain strains of Shigella dysenteriae with very nearly the same efficiency of plating as on E. coli. However, when the kinetics of neutralization was studied, it was found that the rate of neutralization appeared to be greater when S. dysenteriae was used. This was interpreted to mean that a certain fraction of the initial phage suspension lost its ability to produce plaques on Shigella but retained its infectivity for E. coli. In other words, the reaction with antiserum had apparently altered the host range. The effect was thought to be due either to differences in phage receptor sites on the different bacterial hosts or to a heterogeneity inherent in individual phage particles. The results of the present studies suggest that the reaction between ϕX174 and antiserum does not result in an alteration of the host range of ϕX174 with respect to E. coli strains C and H.

In most of the preliminary studies on neutralization of ϕX174 by S24 serum the fraction of P_0 surviving neutralization was about 0.1 after ten minutes of incubation. It seemed possible that, in order to characterize more quantitatively the rate of neutralization, it might be desirable to decrease the rate such that about 60 rather than

ten minutes would be required to attain a phage survivor fraction of 0.1. This was accomplished by using S24 diluted 6 x 10^4, rather than 2.8 x 10^4 times. In the first experiment, using lot I phage, ϕX174d, it was found that four different serum concentration/P_o ratios, all of which were smaller than had been used previously, gave almost identical phage survivor fractions throughout the entire course of incubation (Figure 11). The P_o ranged from 6.14 x 10^5 up to 6.14 x 10^8 plaque-formers ml^{-1}. These results suggested that the percentage law was operating in the ϕX174-anti-ϕX174 system. However, the kinetics of the reactions were still not free of complications, since the neutralization curves were not exponential.

Since neither preparation had been subjected to equilibrium density gradient centrifugation it seems reasonable to assume that ϕX174d, like ϕX174a, was composed of at least two types of phage particles. ϕX174a was inhomogeneous with respect to its sedimentation characteristics and gave two peaks in the analytical ultracentrifuge. These peaks were interpreted to represent particles having different sedimentation constants. One peak corresponded with virions having a sedimentation constant of 114 S and the other peak corresponded with particles having a sedimentation constant of 76 S. Essentially the same findings were observed by Sinsheimer (1959a) with one of his phage preparations. He showed that the 114 S virions, but not the 70 S particles were infectious. Further investigations by Sinsheimer revealed that the 70 S particles were empty capsids devoid of DNA. On the other hand, the infectious 114 S particles contained their full complement of DNA. The possibility that the ϕX174d preparation also

consisted of infectious and non-infectious particles permits a reasonable explanation for the curvilinear neutralization curves in Figure 11. As estimated from the size of the peaks in analytical ultracentrifugal studies on ϕX174a, the ratio of 114 S to 76 S particles was assumed to be approximately 1.0. Therefore, the neutralization reaction mixtures contained approximately equal amounts of 114 S virions and 76 S particles. During the neutralization reaction both types of phage particles reacted with antibody. When the 114 S particles reacted with antibody they were inactivated and at any given time the amount of free residual antibody would be inversely proportional to the amount of phage inactivated. When the 76 S particles reacted with antibody they also bound antibody molecules and removed them from the system. It would not be obvious immediately that antibody was being removed from the system by the 76 S particles, since there is no quantitative assay for them, either free or bound to antibody. Hence the decrease in concentration of antibody in the reaction mixture would appear to be much greater than if only one type of phage particle had been present originally. It is also possible that empty capsids have more sites available with which antibody may react than do intact virions and this would add to the complication. In addition, it is not known whether fragmented capsomeres were present in the ϕX174d preparation. If fragmented capsomeres were present neutralization would be complicated further. In such case, the minimum serum concentration/P_0 ratio necessary for exponential neutralization may be reached and passed in neutralization experiments with a preparation such as ϕX174d. The net result would be that the original antibody

concentration would decrease at a rate much greater than could be accounted for simply by the interaction of antibody molecules and 114 S virions.

For these reasons it seemed desirable to prepare a more highly purified lot of ϕX174. This phage, ϕX174b, was used in a neutralization experiment and the percentage law was readily demonstrated with P_o ranging from 4.08×10^5 up to 4.08×10^8 plaque-formers ml^{-1}. In addition, the curvilinear nature of the curves was abolished. The elimination of the curvilinear aspect of the neutralization curves through the use of highly purified phage does not appear to have been reported previously in the literature.

Under suitable conditions the percentage law could be demonstrated with either density gradient or non-density gradient purified phage. However, with the latter the reactions were complicated by the curvilinear characteristic of the neutralization curves. These findings were interpreted to mean that the percentage law is not restricted to phage-antibody systems which follow only first-order kinetics and support work of Burnet et al. (1937) showing that the percentage law applied to coliphages in serological group 3 even though neutralization deviated markedly from a first-order rate reaction.

The percentage law has been demonstrated with phages C13, C16, and C36 whose purification procedures did not include equilibrium density gradient centrifugation (Andrewes and Elford, 1933a; Burnet et al., 1937). In the work of Andrewes and Elford it is not clear whether their neutralization reactions would be curvilinear. The percentage law has also been demonstrated with poliomyelitis virus (Mandel,

1960) and with WEE virus (Dulbecco et al., 1956). Careful examination of the kinetic curve of neutralization of WEE by horse antiserum showed that after five to ten minutes of incubation, when the fraction of WEE surviving neutralization was between 10^{-1} and 10^{-2}, the curve began to exhibit curvilinearity. It is of interest that the virus was obtained from supernatant fluids of cultures of infected chicken embryo monolayers, stored in the deep-freeze, and not subjected to purification (Dulbecco and Vogt, 1954).

The rate of neutralization in the ϕX174b-S24 system was shown to be dependent on the concentration of serum used. Thus, in addition to obeying the percentage law, the system agreed with the integrated form of the first-order rate equation (2) over a tenfold range of serum concentration. Burnet et al. (1937) found that the kinetics of neutralization of phage C16 agreed satisfactorily with this equation at serum dilutions of from 1:30 to 1:3,000. Hershey et al. (1943) found that the inactivation of phage T2 by antiserum followed first-order kinetics, the K value being independent of D, t, and P_o, up to a P_o of about 10^9 plaque-formers ml^{-1}. Above this P_o level, aggregation of the phage by antiserum resulted in an increased K value.

As given by Bradish et al. (1962), the logarithmic form of the equation for determining the activity of an undiluted serum, equation (6) above, was used to find log S_o (SNI) for S24. A value of 5.9 was obtained. A second estimate of SNI for S24 was obtained by plotting the residual infectivity of a neutralization mixture against dilution of antiserum. By the latter technique a value of 5.84 was obtained. It can be seen that the two values agreed very closely. Bradish et

al. (1962) used foot-and-mouth disease virus in their serological system and obtained values for SNI of between 5.0 and 7.0. A rearrangement of the logarithmic form of the percentage law equation, given as equation (4) above, describes the fraction of P_o surviving neutralization after a given period of incubation in terms of the original serum concentration, S_o. The term on the right-hand side of the equation, $K \times S_o/D$, shows the dependence of the percentage law upon the initial concentration of antiserum. The neutralization curve in a double logarithmic plot is linear, as defined by the percentage law equation (6), only when antiserum is present in adequate excess and when the depression of infectivity is about two log units. In curves obtained from double logarithmic plots, departures of neutralization reactions from the percentage law would be noticeable by deviations from a straight line. Curvatures of this kind would be expected with high dilutions of antiserum or with high P_o.

Further studies of the ϕX174b-S24 system showed clearly that the neutralization rate constant, K, was temperature dependent. An Arrhenius plot of the data (Figure 17) yielded a straight line relationship. This suggests that the ϕX174b-S24 system followed the Arrhenius equation (7) over a temperature range of 0 C to 37 C. Since no breaks or bends were observed in the curve, it can be concluded that neither of the components of the neutralization reaction mixture underwent significant physical or chemical change. In enzyme reactions, breaks in the curve are interpreted to represent a shift in the configuration of the enzyme molecule (Sizer, 1943).

With most chemical reactions, an increase in temperature leads

to an increase in rate, and thus an increase in the specific rate constant. With many reactions, including the heat denaturation of proteins and enzyme catalyzed reactions, the rate may double or even triple for every 10 C rise in temperature. In other words the Q_{10} for the reaction may be 2.0, 3.0, or more. It appears that the Q_{10} for the neutralization of bacteriophages and animal viruses is somewhat smaller. In the present study the Q_{10} for the ϕX174-S24 system was calculated to be 1.8. This value is only slightly larger than the Q_{10} value (1.4) estimated for the T2 phage-antiphage system (Cann and Clark, 1954; 1956). The tryptone broth medium employed for the determination of the Q_{10} for the ϕX174-S24 neutralization contained 4×10^{-2} M potassium chloride, 5×10^{-4} M sodium chloride, and 10^{-3} M magnesium sulfate. Although Adams (1959) attempted to relate low Q_{10} values to the use of concentrations of sodium chloride lower than 0.15 M in neutralization reactions, Cann and Clark (1954; 1956) showed that this relationship was not real. The present studies on the ϕX174-S24 system show that Q_{10} values higher than 1.4 can be attained in neutralization experiments utilizing various salts at concentrations lower than 0.15 M.

A Q_{10} value of 1.4 represents a small energy of activation and suggests a diffusion controlled reaction (Cann and Clark, 1954) yet, as shown with the T2-anti-T2 system (Cann and Clark, 1954) and the WEE-anti-WEE system (Dulbecco et al., 1956), the temperature dependence of the rate of neutralization still obeys the Arrhenius equation. The energy of activation in these systems must be the energy of activation for the viscous flow of the solvent. This probably means that in

diffusion controlled reactions the dependence of the specific rate constants on temperature is due to the increased frequency of encounter between phage and antibody molecules in the less viscous solvent at the higher temperature (Talmage and Cann, 1961). The energy of activation obtained for the ϕX174b-S24 system (9,600 calories $mole^{-1}$) was larger than the energies of activation that have been reported for the T2 system. It seems logical to conclude, therefore, that inactivation of ϕX174 by S24 was dependent on a mechanism requiring more energy than that required simply for the energy of activation for the viscous flow of the solvent. The nature of this mechanism is unknown.

The relationship between Q_{10} values and the type of bonding mechanisms involved in phage-antiphage reactions is largely unknown (Talmage and Cann, 1961). It is completely unknown in the ϕX174-S24 system. Bever (1963) has pointed out the similarity between the Arrhenius constant for the ϕX174-S24 system and that for the enzymatic transfer of two moles of phosphate from adenosine triphosphate (ATP) to an acceptor which results in formation of low-energy phosphate ester compounds. For example, in the transfer of one phosphate group from ATP to glucose, yielding adenosine diphosphate and glucose-6-phosphate, the free energy change of the reaction would indicate a net change of approximately 4,500 calories $mole^{-1}$. Similarly, the utilization of ATP for the transfer of acyl groups to form ester linkages as in the synthesis of acetyl choline results in a net change of -4,000 to -5,000 calories per mole of ATP utilized. He has suggested that the transfer of phosphate groups could be involved in the inactivation of

one virion of ϕX174. However, this explanation does not provide a clue as to whether the phage or the antibody is the phosphate donor or acceptor.

Antibodies to DNA have been produced in experimental animals and are known to occur naturally. Experimentally produced antiserum to disrupted T4 bacteriophage was shown by Levine et al. (1960) to contain antibodies to DNA. They showed that bacteriophage T4 DNA which had been thermally denatured, presumably in the single stranded form, was a much more effective antigen than native DNA. Butler et al. (1962) found that a rabbit antiserum to purinoyl-protein conjugates fixed complement in the presence of native DNA from ϕX174 and heat denatured DNA from Escherichia coli, Bacillus subtilis, and Hemophilus influenzae.

In the case of naturally occurring antibodies to DNA, one of the most intensively studied phenomenon has been the apparent occurrence, in serum of patients with lupus erythematosus (LE), of antibodies to nuclei, nucleoprotein, or DNA. Stollar and Levine (1961), using quantitative complement fixation, described a reaction between DNA and the serum of a patient with disseminated lupus erythematosus. They found that thermally denatured DNA was a more reactive antigen than native DNA. In addition, their LE serum detected serological differences in denatured DNA preparations from calf thymus, Clostridium kluyveri, Streptococcus salivarius, and Staphylococcus aureus. However, the differences could not be correlated with phylogenetic relationships among the species studied or with the variations in the guanine + cytosine or adenine + thymine content of the DNA.

Stollar et al. (1961) showed by complement fixation tests that antibodies reacting with denatured DNA could be found in 11 of 37 samples of LE serum and in none of 124 sera of patients with other diseases and of normal persons. The results of their original experiments led them to the hypothesis that sera of patients with systemic lupus erythematosus contained antibodies which reacted more effectively with denatured than with native DNA. They tested an LE serum with boiled and unboiled samples of DNA from ϕX174. They found that both antigens fixed identical and relatively large amounts of complement. Similar results were obtained with another LE serum which failed to react with any other native DNA sample tested. Since the DNA of ϕX174 is known to be single stranded (Sinsheimer, 1959a), and one LE serum reacted only with denatured DNA and with ϕX174 DNA, they concluded that the anti-DNA antibodies in LE serum reacted more effectively with single stranded DNA.

The results of studies by Barbu et al. (1960) showed that DNA extracted from ϕX174 gave a precipitin reaction with LE sera. They also found that complement was fixed when various LE sera were tested with ϕX174 DNA.

Butler et al. (1962) showed that DNA-induced transformation to streptomycin resistance in pneumococci was inhibited by anti-purinoyl antibody. They also stated that studies were in progress on the possible effects of the latter antibody on cellular infection and neoplasia induced by DNA-containing viruses.

In the ϕX174b-S24 system a need existed for an agent, such as anti-DNA antibody, capable of inhibiting ϕX174 DNA. This need was

recognized after the following hypothesis was constructed concerning the nature of the phage escaping neutralization. It was assumed that, as a consequence of the reaction between ϕX174b and S24, a fraction of the phage was completely inactivated and that the remaining fraction was infectious. The change in the physical state of the individual infectious particles was assumed to be due to either (1) an alteration of the capsid so that a part of the DNA was exposed or (2) complete liberation of the DNA from the capsid, presumably as free INA. Further, the exposed or free DNA would be infectious for E. coli by a mechanism similar to that involved in the transfer of DNA in transformation. Since the INA would be resistant to inactivation by S24 during its passage from the capsid to the host bacterium, it would be expected to escape neutralization. Preliminary attempts to detect free DNA in terminal ϕX174-S24 reaction mixtures involved the use of pancreatic DNAase. The results were completely negative. This may be explained by the fact that, as shown by Fiers and Sinsheimer (1962), DNA of ϕX174 probably exists in a ring form. They found that ϕX174 DNA not previously treated with pancreatic DNAase was about 17 per cent hydrolyzed by treatment with E. coli phosphodiesterase. On the other hand, ϕX174 DNA treated first with pancreatic DNAase and then by E. coli phosphodiesterase was about 85 per cent hydrolyzed.

Subsequent studies were directed toward determining whether LE serum would inactivate the surviving phage. Several LE sera were found to have this ability. One in particular, LE 8, almost completely neutralized the infectivity of a phage suspension which, after reacting with S24, had several thousand plaque-formers ml^{-1}. Phage

not previously treated with S24 was not affected by LE serum. These results suggest that some of the phage surviving neutralization by S24 could have been physically altered to the extent that its DNA was exposed, and now sensitive to anti-DNA antibodies. The results of experiments by Butler et al. (1962) on inhibition of transformation in pneumococci by antipurinoyl antibody would seem to support this concept.

Further support for the suggestion that INA might be involved in the phenomenon under investigation was gained by testing the surviving phage for their ability to infect protoplasts of a bacterial strain resistant to the intact phage. Previous studies have shown that INA from various sources will infect cells normally resistant to intact virus. In particular, infectious RNA isolated from poliovirus was found by Holland et al. (1959) to infect non-primate cells ordinarily insusceptible to infection by poliovirus. The same workers also showed that species of animals insusceptible to poliovirus, the mouse, rabbit, guinea pig, chick, and hamster, yielded intact virus 18 hours after they had been inoculated intracerebrally with infectious RNA. Guthrie and Sinsheimer (1960) demonstrated that protoplasts of E. coli K12S were readily infected by ϕX174 DNA isolated chemically, but not by whole virions of ϕX174. Similarly, Sekiguchi et al. (1960) infected protoplasts of E. coli B with ϕX174 DNA but not with whole virus. The results obtained in the present investigation showed clearly that a fraction of ϕX174 treated with S24 had the ability to infect E. coli K12S protoplasts. The protoplasts were insusceptible to infection with ϕX174 which had not been

treated previously with S24. It can be concluded that, during treatment with S24, a fraction of the phage surviving neutralization acquired the ability to infect protoplasts of a strain of a bacterium normally resistant to ϕX174. These results, and those obtained with the LE sera, give two lines of evidence in support of the hypothesis that a change in the physical state of a part of the phage occurred during neutralization.

Dulbecco et al. (1956) reported that excess antibody failed to reduce below a certain minimum the infectivity titer of either WEE or poliovirus. The residual infectivity of up to one per cent was interpreted as a distinct population of non-neutralizable virus particles, the "persistent fraction." Fazekas de St. Groth et al. (1958) and Fazekas de St. Groth and Reid (1958) re-analyzed the data of Dulbecco et al. and considered the "persistent fraction" to represent the terminal equilibrium established by the reversible reaction between antibody and homogeneous virus population. Herriott's (1961) interpretation of the same results centered around the concept of a liberation of INA during neutralization. This idea provides a biological mechanism to account for the virus escaping neutralization known as the "persistent fraction."

The neutralization of infectivity of foot-and-mouth disease virus by guinea pig, rabbit, and bovine antiserum was studied by Bradish et al. (1962). The virus escaping neutralization was assayed in unweaned mice and on monolayers of pig kidney cells. Their results indicated that the virus survivors in equilibrated virus-antiserum mixtures were not free infective particles but complexes of virus and antibody.

These complexes were simultaneously infective and capable of neutralizing infectivity. The infective virus in the complex resisted neutralization by additional specific antiserum. The authors drew attention to the similarity between this residual infectivity and Dulbecco's "persistent fraction." The resistant complexes showed distinct differences in infectivity for different host systems, a finding which would be inconsistent with the presence of free virus particles. They believed the effect was related to the distinct responses of the cells of each host system to the potential infectivity of the virus-antibody complex. Here, the status of the residual infectivity contributed by complexes would be distinct from that of the infectivity of the initial free virus particles. It would have been interesting if the authors had tried to determine if part of this residual infectivity could have been accounted for by a mechanism involving INA.

Rustigan (1962) recently described a persistent infection by measles virus in the presence of excess antibody. He found that measles virus continued to be infectious for normal HeLa cell cultures after 39 passages in the presence of measles antibody contained in human gamma globulin. Examination of measles virus-infected HeLa cells by electron microscopy has revealed two particulate structures, one of which appears to be the fully developed mature virus and the other, immature virus or viral nucleic acid (Tawara et al., 1961).

It seems possible that INA, either free or in damaged capsids, might be responsible for viral infectivity in the presence of anti-

body. The ϕX174-S24 system is, so far as is known, the first *in vitro* phage-antiphage system in which (1) the host range of the phage was increased by treating it with specific antibody and (2) the phage escaping neutralization by specific antiserum was inactivated by serum from patients with IE. The various properties of INA may explain a number of conditions presently considered to be anomalous. For example, the release of small amounts of INA from virus-infected tissues could lead to a low level of infection which might help explain persistent immunity. If the nucleic acid was protected from serum nucleases by an inert envelope, viremias, with or without antibodies, would be possible.

CHAPTER V

SUMMARY

Three lots of purified and concentrated bacteriophage ϕX174 were prepared by different techniques. Lot I (ϕX174d) was concentrated by dialysis against carbowax 4,000. Further concentration and purification were performed by differential centrifugation. Lot II phage (ϕX174a) was purified and concentrated by ammonium sulfate precipitation, followed by differential centrifugation. Lot III phage (ϕX174b) was treated exactly as the lot II phage except that it was further purified by rubidium chloride equilibrium density gradient ultracentrifugation. Analytical ultracentrifugation studies revealed that the lot II phage was composed of 114 S and 76 S particles and that the lot III phage contained only 114 S virions.

ϕX174 virions were stable in broth containing 1:100 normal rabbit serum (NRS) at 4 C and 37 C but not at 44 C.

The antibody levels of sera from rabbits injected with ϕX174d showed a typical primary response. The levels increased more rapidly and to a greater extent following the second injection of phage. Serum obtained from one rabbit on the sixth day after the second injection of phage was designated S24 and was used for all subsequent neutralization studies. Serum from rabbits injected with filtrate

material from a broth culture of the host organism, Escherichia coli strain C, failed to neutralize phage.

In neutralization reactions with S24 diluted 2.8×10^4 times and ϕX174d at an initial phage concentration (P_o) of between 10^8 and 10^5 plaque-formers ml^{-1}, theoretical survivor fractions were not obtained with every serum concentration/P_o ratio. This could not be attributed to the frequency of sampling during the incubation period, experimental error arising during sampling, reversible neutralization, nor the strain of host bacterium used for assay of surviving phage.

With P_o between 6×10^8 and 6×10^5 plaque-formers ml^{-1}, neutralization of ϕX174d by S24 diluted 6×10^4 times obeyed the percentage law, but the neutralization curves were not exponential. When ϕX174b (lot III phage) was used in neutralization experiments with S24 diluted 6×10^4 times, the percentage law was obeyed when P_o was between 4.08×10^8 and 4.08×10^5 plaque-formers ml^{-1}. In addition, all of the neutralization curves were exponential.

The specific rate constant of the ϕX174-S24 system was dependent on the concentration of serum used.

The serum neutralization index (SNI) of S24 was found to be approximately 5.9.

The Arrhenius constant for the ϕX174-S24 system was approximately 9,600 calories $mole^{-1}$. This corresponds to a Q_{10} of about 1.8.

After four to ten hours of incubation, essentially all of the surviving phage in the ϕX174-S24 system was sensitive to further inactivation by several sera from patients with lupus erythematosus (LE). Phage not previously incubated with S24 was resistant to LE serum.

DNAase had no effect on the infectivity of either untreated phage or of phage pre-treated with S24.

The surviving phage in the ϕX174b-S24 system acquired the ability to infect protoplasts of E. coli K12S, which are normally resistant to infection by intact ϕX174. ϕX174 not pre-treated with S24 was unable to infect the protoplasts.

The results suggest that the phage escaping neutralization might be due to a mechanism involving infectious nucleic acid.

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