IMMUNOASSAYS WITH CHEMICALLY MODIFIED BACTERIOPHAGE.

by

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ABBREVIATIONS

BDB	bis-Diazotized benzidine
BSA	Bovine serum albumin
CGMMV	Cucumber green mottle mosaic virus
CGMMV-P	Protein subunits of CGMMV
DNP	Dinitrophenol
Fab	Fragment of antibody containing combining site
Fc	Crystallizable fragment of antibody
FDNB	1,3 Difluoro-4,6-dinitrobenzene
K value	Pseudo-first order rate constant for the bacteriophage neutralization reaction
NIP	3-Iodo-4 hydroxy-5 nitrophenyl acetic acid chloride
PEG	Polyethylene glycol
pfu	Plaque forming units
P + G buffer	Gelatin containing sodium phosphate buffer
RSA	Rabbit serum albumin
TDIC	Tolylene-2,4-diisocyanate
TMV	Tobacco mosaic virus
TMVP	Protein subunits of TMV
U2-P	Protein subunits of strain U2
Y-TAMV	Tomato atypical mosaic virus (yellow strain)
Y-TAMV-P	Protein subunits of Y-TAMV

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CHAPTER I

INTRODUCTION

The immunospecific inactivation of bacteriophage is one of the most sensitive methods available for the detection of very low concentrations of antibody. By chemically modifying the phage coat-protein, this sensitivity can be extended to antibodies against a wide variety of haptens and proteins. Phage particles that have been modified by attaching some molecule onto their surface are sensitive to antibodies directed against the coupled chemical moiety. Furthermore, the inactivation of the modified phage by antibody can be inhibited by free antigen, and this provides a sensitive assay for small quantities of antigen. Antibody and antigens have been detected at the nanogram level by this technique. The modified phage technique can also be used to distinguish antibodies of different specificities and to discriminate between closely related antigens.

This technique has not yet been applied to the immunochemical study of viral components and the present work represents such an attempt. Tobacco mosaic virus (TMV) was chosen as a model system since it permits the study of numerous immunological phenomena (Rappaport, 1965; van Regenmortel, 1966). A series of preliminary experiments were performed to obtain experience in the methodology of the technique. These included the inactivation of native T4 phage by phage antiserum and anti-

phage IgG, the chemical modification of phage with DNP and the attachment of lysozyme to phage under a variety of conditions.

The success of the covalent binding of protein to phage particles depends on finding conditions under which a proportion of the phage remains viable and, at the same time, can still be neutralized by anti-protein sera. To this end, different proportions of reactants and three different bifunctional reagents were tested. To prevent aggregation of tobacco mosaic virus protein (TMVP) at the high concentration used, the protein was treated with N-bromosuccinimide. TMVP-phage conjugates which were sensitive to antiserum were prepared using bis-diazobenzidine as the bifunctional reagent.

The inactivation of the modified phage was used to show differences in the inactivating abilities of sera raised against TMVP, against protein from related TMV strains and against the assembled capsid of TMV.

The specificity of inactivation was established by inhibiting the phage neutralization by prior incubation of the antiserum with TMVP. This formed the basis of the technique for assaying low concentrations of TMVP as well as for discriminating between different TMV strains. The technique of inhibition of inactivation was also used to show that the NBS treatment of the protein attached to the phage had not significantly altered its antigenic properties. Results obtained with the

modified phage technique were compared with those obtained on the same system by classical serological techniques.

CHAPTER II

REVIEW OF LITERATURE

Antibodies to bacteriophage can be detected by the following serological reactions: precipitation (Hershey, 1943), fixation of complement (Lanni and Lanni, 1953), agglutination of phage-coated bacteria (Burnet, 1933) and a reduction in infectious titre of a phage preparation, i.e. neutralization. The monograph of Adams (1959) contains a review of the work on phage serology prior to 1960 and an appendix of methods used in phage serology. The neutralization of bacteriophage by antibody has been extensively studied and it has proven to be a useful and sensitive means of detecting anti-phage antibodies.

1. BACTERIOPHAGE INACTIVATION

(a) The K Value of an Antiserum

In 1933 Andrewes and Elford summarised the results of their experiments on phage neutralization with their "percentage law". Over a wide range of antiserum dilutions a constant percentage of phage was neutralized in a given time. This law held true for phage concentrations from a few hundred to at least 10^8/ml, and over a range of serum dilutions from undiluted to 10^{-4}. These observations were explained as being the result of a bimolecular reaction

with one of the reactants, the antibody being in excess (pseudo-first order reaction). When fresh phage was added to a completely neutralized preparation (Delbrück, 1945; Kalmanson et al., 1942), the newly added phage was neutralized at the same rate as the original preparation, showing that antibody depletion by the phage was insignificant. (See Fig. 2).

Early observations (e.g. Burnet et al., 1937; Hershey, 1941; Kalmanson et al., 1942) showed that the logarithm of the fraction of surviving phage plotted vs. time gave a straight line until at least 90 - 99% of the phage was inactivated. Some preparations showed this exponential drop in titre to beyond 99% (Burnet et al., 1937; Cann and Clark, 1954 ; Jerne and Avegno, 1956). The first order inactivation kinetics observed in most cases provided a useful measure of phage inactivating capability, or strength of an anti-phage serum.

The equation for the inactivation reaction is :-

$$\frac{dP}{dt} = \frac{KP}{D} \qquad \ldots\ldots\ldots\ldots\ldots\ldots \quad 1.$$

or upon integration :-

$$K = \frac{2,303D}{t} \log_{10} \frac{Po}{P} \qquad \ldots\ldots\ldots \quad 2.$$

where K = the first order rate constant in min^{-1}.

D = the dilution of the inactivating serum.

t = time in minutes.

Po = number of plaque-forming units at time = 0.

P = number of plaque-forming units at time = t.

The unknowns in equation 2 can be determined by experiment (see Adams, 1959; pages 463-464) and the first order rate constant K can be calculated. The rate of inactivation is directly proportional to the serum dilution (Burnet et al., 1937) and K is directly dependent on the amount of precipitating antibody in the serum (Rohrmann and Kreuger, 1970). Thus different bleedings from one animal or different animals will show differing K values when reacted with homologous phage antigen. The rate of inactivation has been shown to be temperature dependent and Q_{10} values (the temperature coefficient for the rate constant) varying from 1,4 to 2,1 have been reported for various phage - antibody systems (Burnet et al., 1937; Kalmanson et al., 1942; Jerne and Skovsted, 1953; Cann and Clark, 1954; Bowman and Patnode, 1964; Dudley et al., 1970).

The ionic strength of the medium in which the inactivation was allowed to take place was found to influence the rate of inactivation. A low salt concentration results in an increased K value (Jerne, 1952; Jerne and Skovsted, 1953; Cann and Clark, 1954). Svehag (1965) made a similar observation in the case of the neutralization of poliovirus. However, within the pH range of 5 - 10, little change in phage neutralization rate was observed (Cann and Clark, 1955).

(b) Deviations From First Order Kinetics

Many workers have observed deviations from ideal first order kinetics of inactivation. These deviations occur as (a) a shoulder at the origin of the kinetic curve suggesting that neutralization of a single phage particle requires more than one antibody molecule to inactivate it. The lag observed before the number of active phage begins to decline would thus be due to the time taken for the accumulation of enough antibody molecules to inactivate each particle (Fig. 1); (b) a decrease in the rate of reaction after a certain percentage of phage has been neutralized, leading in some cases to a persistent fraction which is resistant to further inactivation. This effect is illustrated in Fig. 2.

Kalmanson et al. (1942) found that if two or three antibody molecules were required to inactivate a single phage particle, a theoretical curve based on the Poisson distribution best approximated their experimental results (see Fig. 1). However, it was subsequently found (Sagik, 1954) that host cell components could block the phage's infective process. When these were removed by anti-host antiserum, no shoulder at the origin was seen in the inactivation curve (Cann and Clark, 1954). On the other hand, a lag in neutralization has been observed in the case of a Klebsiella phage (Park, 1956), but no inhibitor was demonstrated in this case. In general, the lack of any marked plateau or shoulder in the kinetic curve of

neutralization indicates that single-hit kinetics are operative in phage neutralization. As Svehag (1968) points out in his review on virus neutralization, it is difficult to study the initial events of the phage - antibody reaction, particularly at 37°C, because of the practical problems presented by the speed of manipulation that would be required.

A decline in rate of inactivation after 90 - 99% of the phage has been inactivated has been observed in many phage - antibody systems (Burnet, 1937; Kalmanson et al., 1942; Cann and Clark, 1954; Jerne and Avegno, 1956; Adams and Wade, 1955; Lafferty, 1963, Bowman and Patnode, 1964; Rolfe and Sinsheimer, 1965; Krummel and Uhr, 1969). Recent work by Hale et al. (1969) on Qβ phage supports the idea (Delbrück, 1945; Cann and Clark, 1954) that if antibody is not depleted during the reaction, and the phage or antibody are not heterogenous, deviations from first order kinetics and the development of a persistent fraction may be due to a secondary phage "heterogeneity", developing as a result of interaction of phage with the antiserum. This is believed to lead to a phage population in which not all particles are equally susceptible to serum because some of the bound antibody molecules prevent further inactivating antibody from attaching to the same particles.

A curvilinear neutralization plot could also result from an antibody population heterogenous in affinity and/or

containing antibody which dissociates readily from the phage. Although most phage - antibody complexes have been shown not to dissociate at a measurable rate (Burnet, 1937; Hershey, 1943; Jerne and Skovsted, 1952; Bowman and Patnode, 1964; Krummel and Uhr, 1969), Jerne and Avegno (1956) found that phage T4 which had reacted with an early serum showed reactivation, due to dissociation of the antibody from the phage. However, a later hyper-immune horse antiserum did not allow reactivation to occur.

In order to prevent the appearance of phage plaques caused by dissociation and reactivation, Jerne and Avegno (1956) developed the "decision" technique of plating. This method gives the phage 10 minutes to "decide" whether they will infect a bacterium, after which they are irreversibly inactivated by the addition of a strong hyper-immune anti-phage serum. This phage antiserum irreversibly inactivates all unabsorbed phage and also prevents reactivation on dilution, or on the assay plate. This technique has proved of great value in later work on the inactivation of phage-hapten conjugates.

(c) <u>The Mechanism of Phage Neutralization</u>

Neutralized phage are not irreversibly destroyed by the action of antibody, but can be reactivated spontaneously (Jerne and Avegno, 1956), by treatment with sonic vibration (Anderson and Doermann, 1952), or by treating the

phage - antibody complex with papain (Kalmanson and Bronfenbrenner, 1943). Following the suggestions made in 1943 by Kalmanson and Bronfenbrenner and by Delbrück (1945), evidence has accumulated which strongly supports the idea that antibody acts by sterically blocking the phage's attachment to the host bacterium. The efficiency of inactivation of phage by fragments of antibody has been found to be directly related to the size of the fragment (Goodman and Donch, 1964, 1965; Stemke and Lennox, 1967). If an antiserum to immunoglobulin (Ig) or Ig fragment raised in another species of animal is added to the reaction mixture, thereby increasing the size of the complex attached to the phage, inactivation is seen to be enhanced (Goodman and Donch, 1964, 1965; Stemke and Lennox, 1967; Krummel and Uhr, 1969). The bivalence of antibody is also an important factor in the stabilization of phage - antibody complexes (Hornick and Karush, 1969). The association constant of bivalent antibody is a few orders of magnitude larger than that of the corresponding monovalent fragment, and marked dissociation of univalent Fab fragments from T4 at 20° and 30°C has been reported (Stemke et al., 1974). From the typical shoulderless inactivation curve, it would appear that a single antibody molecule is sufficient to inactivate a single phage particle. It has therefore been postulated that inactivation occurs by the attachment of an antibody molecule to a "crucial site" on the phage (Jerne and Skovsted, 1953). Although Dudley et al., (1970) suggested that neutralization of phage f2 was due to

conformational change in the architecture of the phage capsid, recent work has shown the existence of a single critical site in this virus (Witte and Slobin, 1972). In 1974 Curtiss and Kreuger also localised such a site in phage MS_2. This work points to a steric mechanism of neutralization, at least in the case of the spherical phages.

In the case of the asymmetrical T-even coliphages which have been most commonly used in chemically modified phage immunoassays, the situation seems to be more complex. Most of the phage neutralizing activity of an antiserum appears to be directed against the tail fibres or base plate (Franklin, 1961; Edgar and Lielausis, 1965; King, 1968). Stemke *et al.* (1974) have suggested that anti-tail fibre antibody causes one or more of the tail fibres to adopt a coiled conformation, in which state the phage is non-infective. In 1975, Conley and Wood found that antibody directed against the phage whiskers in the collar region could inactivate T4, probably by fixing the tail fibres in the retracted or coiled position. This shows that a simple steric blocking of phage attachment is not the only mechanism of inactivation.

(d) Detection of Antibodies by Phage Neutralisation

The extreme sensitivity of bacteriophage to antibody has been exploited in many immunological studies which required

the detection of extremely low concentrations of antibody, e.g. *in vitro* antibody formation (Attardi *et al.*, 1959; Fishman and Adler, 1963; Tien Wen, 1968), the appearance of antibodies after immunization (Uhr and Finkelstein, 1963; Silverstein *et al.*, 1963; Brauer *et al.*, 1963; Sherwin and Rowlands, 1974) and antibody formation at the single cell level (Mäkelä, 1964). In one study (Silverstein *et al.*, 1963) anti- Ø X 174 phage antibody at a concentration of 0,001 µg/ml has been detected. Similar levels of sensitivity have been reported by Sherwin and Rowlands (1974) in the detection of anti-f2 phage antibodies.

2. CHEMICALLY MODIFIED BACTERIOPHAGE

(a) Hapten-Phage Conjugates

(i) Detection and Quantitation of Anti-Hapten Antibody With Hapten-phage Conjugates

In 1966 Mäkelä, recognising the advantages of an immunoassay as sensitive as phage inactivation provided it could be applied to a wide range of antigens, modified the bacteriophage antigenic determinants by attaching the hapten 3-iodo-4-hydroxy-5-nitrophenyl acetic acid chloride (NIP) to "live" T2 and T7 bacteriophage. This chemical modification inactivated ± 90% of the phage preparation. However, the surviving phage particles could be neutralised by anti-NIP as well as anti-phage

antibody, thus providing a sensitive means of detecting anti-NIP antibody.

Mäkelä (1966) assayed the anti-hapten sera by two methods, both of which were widely used in subsequent applications of the modified phage neutralization assay.

The kinetics of inactivation of the modified phage were followed. If a first order reaction appeared to be taking place, K, the rate constant for the neutralization reaction was calculated (Adams, 1959). Mäkelä found that as in the case of non-modified phage, neutralization usually proceeded according to first order kinetics, until 99% of the plaque-forming units (pfu) had been inactivated, after which the reaction slowed down. The degree of chemical modification was found to affect the kinetics as suboptimally coupled preparations showed a marked deviation from first order inactivation kinetics.

Alternately, different concentrations of very dilute (10^{-7} - 10^{-8}) anti-NIP serum were incubated at 37°C with a known number of NIP-phage for 6 hours. Bacteria and soft agar were then added and the mixture was plated. This is known as the "direct plating" technique (Jerne and Avegno, 1956). After incubation, the surviving pfu's were counted and a plot of antibody concentration vs. surviving pfu was made. A reduction in plaque count of $>$ 10% was attributed to serological inactivation.

Mäkelä found that 10^{-5} µg of anti-NIP antibody could be detected by this method, and that NIP-T7 conjugates were slightly less sensitive to the action of anti-NIP serum than NIP-T2 phage.

In the same year Haimovich and Sela (1966) reported the alanylation of coliphage T4. They coupled poly-DL-alanine to the phage, which inactivated 99% of the pfu, and found that the surviving conjugates were sensitive to anti-phage serum and to antibody raised in rabbits against poly-DL-alanyl ribonuclease. They also found that their optimally coupled preparation was as stable as non-modified T4 for up to one year's storage, and that at the end of this time, the modified phage was inactivated by anti-polyalanine antibodies to the same extent as when freshly prepared. Mäkelä (1966) found that spontaneous inactivation of his NIP-T2 conjugates occurred with time but that the sensitivity of the preparation to anti-hapten serum also remained constant. Subsequently other poly-amino-acid modified phage have been reported :- poly-L-tyrosyl T4 (Haimovich and Sela, 1966), poly-D-alanyl (Haimovich and Sela, 1969 a) and poly-L-prolyl T4 (Gurari et al., 1973), as well as a wide variety of low molecular weight haptens (Haimovich and Sela, 1977) such as plant hormones (Fuchs and Fuchs, 1969), penicillin and penicillin derivatives (Shaltiel et al., 1971) and carcinoembryonic antigen (Arnon et al., 1976).

Although the asymmetrical T-even coliphages have been most

commonly used, the RNA-containing male-specific phage f2 (Witte and Slobin, 1972), phage MS_2 (Curtiss and Kreuger, 1974) and the 1-DNA phage, Ø X 174 (Hornick and Karush, 1969) have also been chemically modified.

The sensitivity of the modified phage assay is such that antibody concentrations at the nanogram level are easily detected (Haimovich and Sela, 1966; Mäkelä, 1966, 1967; Haimovich et al., 1967; Carter et al., 1968; Jormalainen et al., 1971; Blakeslee et al., 1973; Sherwin and Rowlands, 1974).

(ii) Methods Used to Enhance the Sensitivity of the Assay

Mäkelä (1966) found that phage preparations coupled with suboptimal quantities of NIP were not inactivated by anti-NIP serum according to first order kinetics. He explained this as due to the heterogeneity of the phage population, because some particles had escaped coupling and hence were insensitive to anti-NIP serum. On the other hand, even optimally coupled phage stocks failed to follow first order kinetics when inactivated with early anti-NIP sera. This, in turn, he explained as being due to antibody heterogeneity. However, the optimally coupled NIP-T2 were sufficiently sensitive to anti-NIP antibody and no modifications of the direct plating technique were necessary to enhance the sensitivity of the antibody assay. Such has not been the case in all

haptenated phage - hapten antibody systems and various workers have used either the "decision technique" (Jerne and Avegno, 1956), or the complex inactivation method (Goodman and Donch, 1965; Krummel and Uhr, 1969) for the plating of the surviving phage.

The "Decision" Technique: A 1/500 serum dilution was required to obtain significant inactivation of the poly-DL-alanyl phage after 15 min prepared by Haimovich and Sela (1966). The conjugates were not inactivated according to first order kinetics and a lengthening of the incubation time could not increase the sensitivity of the system, because of a marked decrease in the rate of inactivation.

As the antibody was not depleted by the reaction, Haimovich and Sela postulated that there was variation in the extent of alanylation of the various individual phage particles. Another possibility was that the anti-polyalanyl antibody had a low affinity for the polyalanyl phage and that as a result of the dissociation of the complexes, reactivation of the phage was taking place. This, in fact, was shown to be the case when the "decision" technique was used : this prevented such reactivation and produced a neutralization plot which was linear. By lengthening the time of phage - antibody contact to 72 hours, 6×10^{-10} g of anti-polyalanyl antibody could be detected. The "decision" technique has been used by several workers to enhance the sensitivity of the

modified bacteriophage assay (Haimovich and Sela, 1969a; Hurwitz et al., 1970; Blank et al., 1972).

Barber and Rittenberg (1969) found that plating by the "decision" technique had no effect on the kinetics of inactivation of trinitrophenyl (TNP) - T2, which was inactivated by anti-TNP in a series of two first order processes. (Zierdt, 1974, found a similar kinetic curve in the case of a Corynebacterium phage). Barber and Rittenberg explained the separate rates as being due either to the position of the hapten relative to the critical site, or to the presence of antibody heterogenous in affinity for the hapten. They also found that anti-hapten antibody could be depleted by a high concentration ($>10^7$ pfu/ml) of haptenated phage, and suggested that in order to maintain a sensitive assay, haptenated phage should be used at low concentrations in kinetic studies.

Andrieu et al., (1974) similarly found that the "decision" technique did not affect the kinetics of inactivation of estradiol-T4 by anti-estradiol serum, but merely reduced the number of surviving pfu throughout the inactivation reaction; the plot they obtained was parallel to that found by direct plating. Thus if dissociation of the phage -antibody complex is not occurring, the"decision" technique is unlikely to greatly improve the sensitivity of an assay for anti-hapten antibody.

The Complex Inactivation Method: In order for a phage-hapten conjugate to be sensitive to anti-hapten antibody, it is necessary for the phage to have its antigenic determinants modified in the region of the "crucial site", i.e. tail, tail fibre or collar region in the case of the T-even phages. When an antibody molecule is bound to the new moiety on the phage, attachment of the phage to its host bacterium is blocked, and the phage is thus neutralised (Sela, 1972; Sela and Haimovich, 1971). In the complex inactivation technique, at the end of the inactivation reaction, excess IgG, or antiserum directed against the rabbit anti-hapten antibodies are added to the reaction mixture, thus increasing the size of the complex on the bacterium and causing more effective steric blocking (Goodman and Donch, 1965). After ± 10 min at 37°C, bacteria and soft agar are added and the whole mixture is plated. In most cases this leads to an increase in sensitivity of the assay for anti-hapten antibody (Carter et al., 1968; Becker et al., 1970; Fuchs et al., 1971; Andrieu et al., 1974). However, Andrieu et al. (1974) found that there was no difference between the direct plating and complex inactivation methods if inactivation of estradiol-T4 by anti-estradiol serum was performed at 0°C, but that when the complex inactivation method was used at 37°C, at pH 5,6 and in low ionic strength buffer (0,005 M instead of 0,05 M phosphate buffer), the rate of inactivation increased tenfold.

A further increase in sensitivity was obtained by Franěk

and Doskočil (1975) by adding polyethylene glycol (PEG) to a final concentration of 8% in the medium in which the inactivation reaction was taking place. They found that when the phage were plated by the complex inactivation method, the presence of PEG enhanced the sensitivity to anti-DNP antibody by 10 - 30 times.

The complex inactivation technique has been used by many workers and has led to an increase in sensitivity greater than that attainable by direct plating or the "decision" technique.

(iii) Preparation of Hapten-phage Conjugates

The methods used in coupling haptens to phage are similar to those employed for coupling such molecules to proteins and for preparing conjugates suitable for eliciting an anti-hapten response in animals.

In the case of phage, only procedures which do not cause total inactivation of the preparation can be used, and since the degree of modification is crucial in determining the capacity of the conjugate to be inactivated by anti-hapten antibody (Mäkelä, 1966), it is necessary to vary the conditions of the reaction until an optimally sensitive conjugate is produced. Appropriate conditions of temperature, pH, time of reaction and concentration of activated hapten must be determined by experiment. The

general procedure involves obtaining the hapten or derivative of the hapten in a form which reacts readily with the phage coat-protein. Dinitrobenzenesulphonic acid for dinitrophenylation of phage (Carter et al., 1968) and oxalazone which couples spontaneously with protein (Jormalainen et al., 1971) have been used. Poly-amino-acid chains are "grown" on the phage by polymerization of the N-carboxy-α-amino acid anhydrides using the phage as multifunctional initiator (Haimovich and Sela, 1966). Coupling reagents, e.g. difluorodinitrobenzene, have been used to produce poly-L-proline T4 (Gurari et al., 1973) and penicilloyl-T4 (Shaltiel et al., 1971) and plant hormones have been coupled to T4 using water-soluble carbodiimides (Fuchs and Fuchs, 1969; Fuchs et al., 1971).

The coupling reaction is stopped by dilution and the unreacted hapten is removed from the conjugates by dialysis. The conjugates are diluted and assayed to determine the percentage of viable phage. These are then tested for their sensitivity to an anti-hapten serum.

(iv) Inhibition of Inactivation of Hapten-phage Conjugates

Mäkelä (1966) inhibited the inactivation of NIP-T2 by anti-NIP serum with several derivatives of NIP, and although he gives no experimental details regarding the time allowed for inhibition, he found that 1 ng/ml of NIP-ε-caproic acid caused measurable inhibition of inactivation. Thus

by inhibiting the inactivation of hapten-coupled bacteriophage, a sensitive assay for small amounts of antigen is obtained. DNP-l-lysine has been detected at a concentration of 10^{-7} to 10^{-8} mM (Carter et al., 1968), plant hormones, giberillic acid and indoleacetic acid at concentrations of 0,1 - 3,0 µM (Fuchs and Fuchs, 1969) and even as little as 2 pg of estradiol have been measured (Andrieu et al., 1974). The method generally used to quantify small amounts of antigen is to preincubate the hapten at various concentrations with anti-hapten serum at a concentration previously shown to inactivate 90 or 99% of the phage. The phage-hapten conjugates are added and the mixture is incubated for a further period of time so that the unreacted antibody inactivates the conjugates to an extent dependent upon the amount of antibody remaining. The percentage of remaining pfu (Blakeslee et al., 1973), or the percentage inhibition of inactivation (calculated as in Haimovich et al., 1970 a) is plotted vs the concentration of inhibiting hapten, thus providing a calibration curve.

The inhibition of inactivation is also used to show that the inactivation of hapten-phage conjugates is indeed caused by specific anti-hapten antibodies and is not due to some non-specific effect, or to naturally occurring anti-phage antibody which may be present in some sera (Haimovich et al., 1970 c; Jerne, 1956). One such instance was reported by Jormalainen and Mäkelä (1971) who

showed that certain haptenated phage inactivators which they detected in sera of non-immunized animals were in fact antibodies specific for the haptens.

(b) Protein-phage Conjugates

(i) Detection and Quantitation of Anti-protein Antibody

Prior to 1969 all modified phage assays involved low molecular weight haptens, e.g. poly-amino acids (Haimovich and Sela, 1966; Haimovich and Sela, 1969 a), penicillin (Haimovich et al., 1967), dinitrophenol (Carter et al., 1968), trinitrophenol (Barber and Rittenberg, 1969) and peptide plant hormones (Fuchs and Fuchs, 1969). In 1969 Haimovich and Sela (1969 b) extended the uses of the technique by attaching to T4 phage the proteins bovine pancreatic ribonuclease, bovine serum albumin and rabbit IgG. In 1970 Haimovich et al. (1970 a) published the details of coupling the proteins RNase, BSA, RSA, IgG, lysozyme and insulin to bacteriophage T4. The phage-protein conjugates which survived the coupling reaction could be inactivated by anti-protein whole serum or by purified anti-protein antibodies, in the same way as hapten-phage conjugates are inactivated by anti-hapten antibody. The concentration of anti-protein antibody (0,2 - 12 ng/ml) that caused 50% inactivation of pfu's after reaction with the protein-phage conjugates for 10 hours at 37°C depended upon the protein attached, the method of plating and the bifunctional reagent used in the coupling process. Thus

Haimovich and his co-workers extended the potential usefulness of the modified phage assay to a vast new range of antigens. Other proteins which have been conjugated to phage to date include papain and chymopapain (Eder and Arnon, 1973), sperm whale myoglobin (Blakeslee et al., 1973), mouse submaxilliary gland nerve growth factor (Oger et al., 1974), Naja naja siamensis toxin (Aharonov et al., 1974), monkey IgG, IgM and Fc, E. coli antigen (Felsenfeld, 1971) and staphylococcal nuclease (Fuchs et al., 1973).

The methods used for the quantitation of anti-protein antibody are the same as those utilized for the assay of anti-hapten antibody, but the methods of plating and preparation can greatly affect the apparent sensitivity of the conjugates to anti-protein antibody.

Plating Methods: After reacting the phage-protein conjugates with anti-protein antibody, Haimovich et al. (1970a) used the three standard plating procedures: direct plating, the "decision" technique and the complex inactivation method. They observed a deviation from first order kinetics with all three methods which they attributed to heterogeneity of the modified phage populations, i.e. to the fact that some phage are not modified, or are only "lightly modified" (Mäkelä, 1966) and are thus resistant to anti-protein antibody. As in the case of haptenated phage, the chemical

modifications obtained with various proteins caused considerable inactivation of the phage. As much as 99.95% of the pfu in the preparation could be inactivated, which according to Haimovich et al. (1970a) is due to direct attachment of protein molecules to the critical site of the phage. The surviving conjugates which are sensitive to anti-protein antibody are assumed to have a protein molecule attached in the vicinity of the critical site. It could be expected, therefore, that the complex inactivation method, by increasing the size of the complex, would help to enhance the inactivation caused by the antibody. Haimovich et al. found that in the case of BSA-T4 and insulin-T4, there was no difference in the extent of inactivation when the complex inactivation or direct plating methods were used. Blakeslee et al. (1973) and Felsenfeld (1971) also found that inactivation of their phage-protein conjugates proceeded satisfactorily by the direct plating technique, but other workers have reported more efficient inactivation when using the complex inactivation method (Eder and Arnon, 1973; Oger et al., 1974; Aharonov et al., 1974). According to Haimovich et al. (1970a), the sensitivity of the phage assay for anti-protein antibody compares favourably with that of the passive haemagglutination technique.

Preparation of Protein-phage Conjugates: An important factor which determines the sensitivity of the test with protein-phage conjugates, is the nature of the bifunctional

reagent used to couple the protein to the phage. Haimovich et al. (1970a) obtained good results with tolylene-2,4,-diisocyanate (TDIC), glutaraldehyde and bis-diazobenzidine (BDB), but were unsuccessful in their attempts to use water-soluble carbodiimide derivatives and 1,3, difluoro-4,6-dinitrobenzene as bifunctional reagents.

Phage-protein conjugates are prepared by adding protein solution to a stock of purified phage of high titre (10^{13}/ml). The bifunctional reagent is added and the coupling reaction is allowed to proceed until stopped by dilution. As only the unreacted coupling reagent is removed by dialysis, the protein-phage conjugates must be separated from the non-coupled protein by differential centrifugation. Similarly to the procedure used with hapten-phage conjugates, the percentage of viable phage and their sensitivity to antibody are then determined.

Tolylene-2,4-diisocyanate (TDIC): Shick and Singer (1961) were the first to use TDIC for preparing conjugates and successfully coupled ferritin to antibody molecules. Diisocyanates are known to react with the free amino groups on proteins (Fraenkel-Conrat et al., 1945) and in 1964 Gyenes and Sehon used TDIC to couple BSA and the water-soluble constituent of ragweed pollen to red blood cells for use in passive hemagglutination experiments.

Haimovich et al. (1970a), using a single-step procedure for

conjugation (Gyenes and Sehon, 1964), added TDIC in dioxane to their protein-phage mixture, thus coupling the protein to the phage via the available amino groups on the protein and the phage particle. They successfully produced conjugates of T4 phage with RNase, BSA, RSA, rabbit IgG, lysozyme and insulin. TDIC has also been used to couple papain and chymopapain (Eder and Arnon, 1973), monkey IgG, IgM and Fc, E. coli antigen (Felsenfeld, 1971) and sperm whale myoglobin (Blakeslee et al., 1974) to bacteriophage T4, but proved unsuccessful in producing phage-staphylococcal nuclease conjugates sensitive to anti-nuclease serum (Fuchs et al., 1973). Haimovich et al. (1970a) found that because of its instability, the coupling efficiency of TDIC varies with different batches.

Bis-diazo-benzidine (BDB): In the presence of nitrous acid, benzidine is converted into the very reactive bifunctional reagent, bis-diazo-benzidine. The diazonium functional groups of the molecule react with tyrosyl, histidyl, amino and carboxyl groups of proteins (Gordon et al., 1958; Nisonoff, 1967) via stable diazo linkages. BDB has been used to couple proteins to erythrocytes for use in passive hemagglutination tests (Pressman et al., 1942; Gordon et al., 1958). Haimovich et al. (1970a) used BDB to couple RNase to T4 phage, but found that their optimally coupled conjugates were less sensitive to the action of anti-ribonuclease serum than were the ribonuclease-

T4 conjugates prepared with glutaraldehyde or TDIC.

Glutaraldehyde: Glutaraldehyde (pentane 1,5 dial) has been used as a bifunctional reagent to couple proteins to enzymes (Avrameas, 1969; Sachs and Winn, 1970), to erythrocytes (Avrameas et al., 1969) and to prepare insoluble protein polymers (Avrameas and Ternynck, 1969). Avrameas and Ternynck (1969) inhibited the insolubilization of proteins with glutaraldehyde by free amino-acids and amino-acid derivatives and found that those possessing a free amino group were effective inhibitors, thus showing that protein-protein coupling with glutaraldehyde occurs via the free amino groups of proteins. The mechanism probably involves the formation of a Schiff base between the aldehydic functions at both ends of the glutaraldehyde molecule, and the free amino groups of the proteins (Sachs and Winn, 1970). However, Avrameas and Ternynck (1969) have suggested that the reaction proceeds further and that some cyclic compounds (absorbing strongly at 280 nm) are formed.

Glutaraldehyde gave good results in the coupling of phage T4 to the proteins ribonuclease (Haimovich et al., 1970a), lysozyme (Haimovich et al., 1970a; Maron and Bonavida, 1971), cobra toxin (Aharonov et al., 1974), mouse submaxilliary gland nerve growth factor (NGF) (Oger et al., 1974), as well as peptide hormones, e.g. angiotensin (Hurwitz et al., 1970) and a synthetic undecapeptide (Arnon et al., 1976). Haimovich et al. (1970a) found that phage conjugates could be

prepared reproducibly using commercial glutaraldehyde which is predominantly polymeric (Sachs and Winn, 1970), at a final concentration of 0,005 to 0,02% v/v. On the other hand, Oger *et al*. (1974) successfully coupled mouse submaxilliary NGF to T4 using *in vacuo* redistilled glutaraldehyde which is monomeric.

(ii) Inhibition of Inactivation of Protein-phage Conjugates

Similarly to the inhibition of inactivation of hapten-phage conjugates by free hapten, inactivation of protein-phage conjugates can be inhibited by free antigen and used as a means of assaying small amounts of protein.

Haimovich *et al*. (1970b) inhibited the inactivation of T4 phage conjugated to RNase, RSA, rabbit IgG, lysozyme and insulin by anti-protein antibody with different concentrations of the respective proteins. They plotted the percentage of inhibition of inactivation vs antigen concentration, thus providing a calibration curve for the quantitation of the various proteins. Using this method for the assay of insulin in human sera, they obtained results which closely agreed with those obtained in radioimmunoassays. When comparing the results of a phage assay for lysozyme present in a variety of biological fluids with the results of an enzymatic assay, Maron and Bonavida (1971) found that the phage assay gave consistently higher values. They assumed that this was due to the fact that the

immunological method detected inactive as well as active forms of the enzyme. Numerous proteins present in biological fluids of animals have been detected by inhibition of inactivation of chemically modified phage, e.g. monkey IgG, IgM and Fc, E. coli antigen in monkey stool filtrates (Felsenfeld, 1971) and mouse submaxilliary NGF in cell culture supernatants (Oger et al., 1974).

In addition to the homologous antigen, the inactivation of protein-phage conjugates can be inhibited by related antigens or fragments of antigen. The extent of inhibition can be used as an indication of the chemical similarity of the inhibitor to the antigenic determinants of the immunogen, the homologous antigen being the most efficient inhibitor. Aharonov et al. (1974) could distinguish poly-DL-alanyl cobra toxin from a non-modified toxin because the modified toxin was a less efficient inhibitor of inactivation of toxin-T4 by anti-toxin antibody. Maron et al. (1972) found that affinity labelled hen egg-white lysozyme inhibited the inactivation of hen egg-white lysozyme-T4 conjugates by anti-lysozyme antibodies to an extent very similar to the non-modified enzyme. On the other hand, in the inhibition of micro-complement fixation, affinity labelled antigen could be distinguished from the native enzyme.

3. SOME APPLICATIONS OF THE MODIFIED PHAGE TECHNIQUE

The detection and quantitation of antibody and antigens by the

modified phage technique has been used in a wide variety of immunological studies. Comprehensive lists of such applications are to be found in reviews by Sela (1972) and by Haimovich and Sela (1977). Applications of the chemically modified phage technique relevant to the present thesis mainly involve studies on the antigenic characteristics of various proteins.

As well as being used in an assay for lysozyme (Haimovich et el., 1970a; Maron and Bonavida, 1971), lysozyme-T4 conjugates have been used to help elucidate the antigenic structure of the lysozyme molecule. Maron et al. (1970) compared several lysozymes from various sources for their capacity to inhibit the reaction of anti-hen egg-white lysozyme antibodies with lysozyme-T4 and with the "loop" peptide of lysozyme conjugated to T4 phage. Human lysozyme inhibited the lysozyme-T4/anti-lysozyme, but not the loop-T4/anti-lysozyme systems, so they concluded that despite many differences in amino-acid sequence, the hen egg-white and human lysozymes had common antigenic determinants which were not situated in the loop peptide region.

In 1971 Maron et al., using lysozyme-T4 and loop peptide-T4, and antibodies specific for the loop region of the lysozyme molecule found that unfolded "loop" peptide was a far less efficient inhibitor than native "loop" peptide; This confirmed the role of conformation of this peptide in its antigenic specificity.

Using immunoabsorbents, Eder and Arnon (1973) separated antibodies produced against the cross-reacting enzymes papain and chymopapain into common (cross-reacting) and non-common (specific) fractions. Using papain-T4 and chymopapain-T4 conjugates they showed that inactivation only occurred with the total and common antibody fractions but not with the specific non-common antibodies. In precipitation and antigen-binding capacities against papain and chymopapain, the common antibody fractions appeared to be identical, but in the inactivation of protein-phage conjugates and difference was discernible. Eder and Arnon explained this as being due to the greater sensitivity of the modified phage technique for distinguishing between antibody species of very similar specificity. In addition, the inhibition of hen egg-white lysozyme-T4 by antibodies directed against the whole enzyme and active site-specific antibodies was used to differentiate between lysozyme from various sources. The degree of inhibition of inactivation was found to be correlated with differences in the amino acid sequences of the whole proteins and of the enzyme active site (Maron et al., 1972).

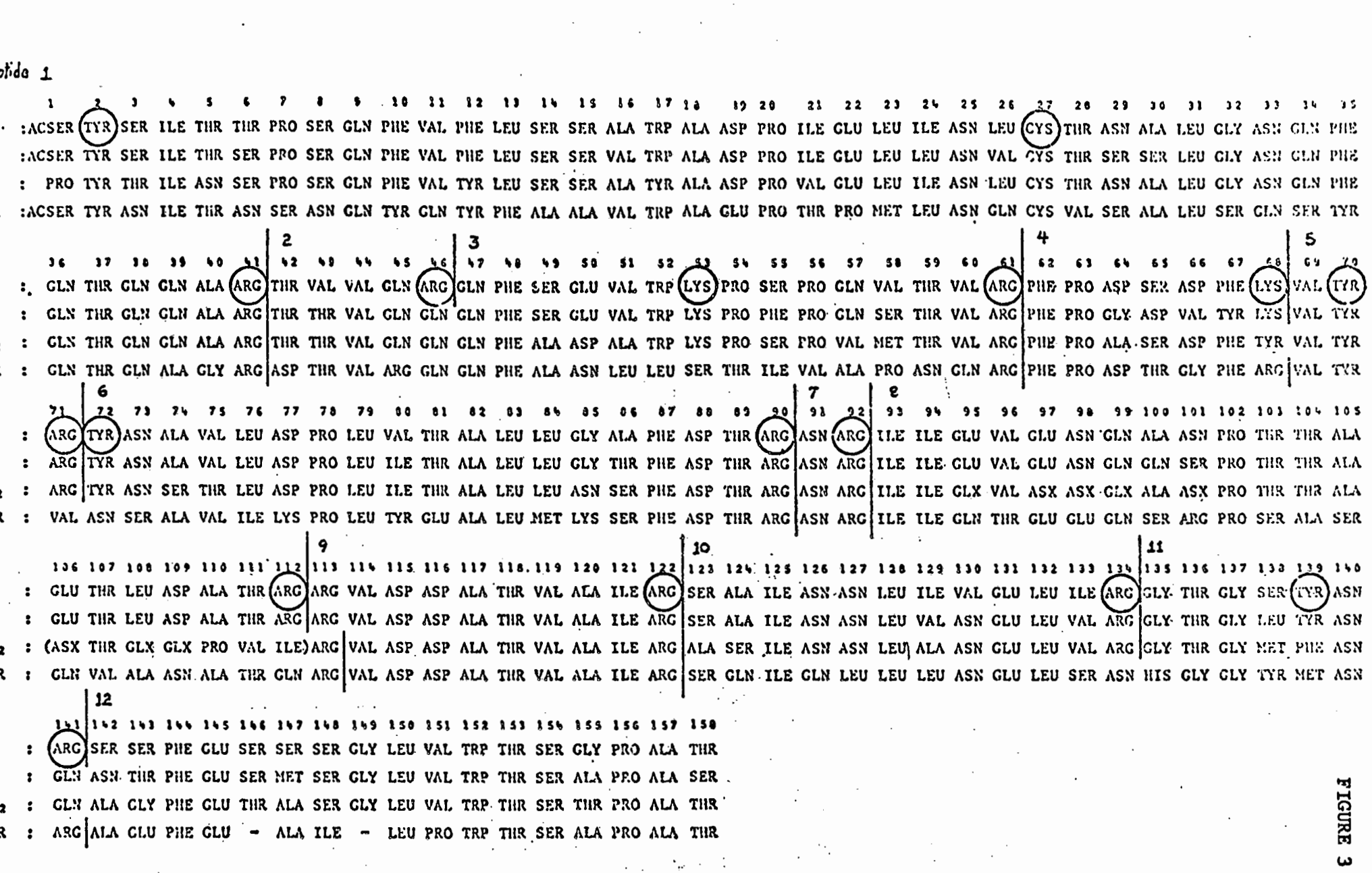
Fuchs et al. (1973) conjugated the enzyme staphylococcal nuclease to T4, and produced protein-phage conjugates sensitive to anti-nuclease serum. Nuclease at a concentration of 10^{-9} M caused detectable inhibition of inactivation. The enzyme was digested by trypsin to yield three fragments, P_1, P_2, and P_3. Individually fragments P_2 and P_3 caused inhibition of inactivation of the nuclease conjugates by an anti-nuclease serum,

although much less efficiently (100 to 1000 - fold) than the native enzyme. Fragments P_2 and P_3 associated reversibly to form a complex (nuclease-T), which produced an inhibition curve similar to that produced by inhibition with native enzyme. Synthetic analogs of P_2 did not inhibit inactivation unless complemented with fragment P_3. The modified phage technique in this situation provided a sensitive means of discriminating between the immunological activity of the various fragments.

4. IMMUNOCHEMISTRY OF TOBACCO MOSAIC VIRUS

The serological properties of tobacco mosaic virus (TMV) have been the subject of much study and extensive reviews on the subject are available (Rappaport, 1965; Wetter, 1965; van Regenmortel, 1966). In this thesis the applicability of the modified bacteriophage technique to the study of the serological properties of the TMV protein subunit (TMVP) will be investigated.

TMVP is a useful subject for immunochemical studies, as its amino acid sequence, as well as that of a number of related strains and mutants is known (Hennig and Wittmann, 1972; Fraenkel-Conrat, 1974). The protein has a molecular weight of 17500 daltons and consists of a polypeptide chain of 158 amino acids. The amino acid sequence of the vulgare strain, as well as that of some related strains is shown in Fig. 3. Features of the wild-type protein are the absence of hystidyl and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: GLN THR GLN GLN ALA ARG THR VAL VAL GLN ARG GLN PHE SER GLU VAL TRP LYS PRO SER PRO GLN VAL THR VAL ARG PHE PRO ASP SER ASP PHE LYS VAL TYR
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: GLN THR GLN ALA GLY ARG ASP THR VAL ARG GLN GLN PHE ALA ASN LEU LEU SER THR ILE VAL ALA PRO ASN GLN ARG PHE PRO ASP THR GLY PHE ARG VAL TYR
6 7 8
71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105
: ARG TYR ASN ALA VAL LEU ASP PRO LEU VAL THR ALA LEU LEU GLY ALA PHE ASP THR ARG ASN ARG ILE ILE GLU VAL GLU ASN GLN ALA ASN PRO THR THR ALA
: ARG TYR ASN ALA VAL LEU ASP PRO LEU ILE THR ALA LEU LEU GLY THR PHE ASP THR ARG ASN ARG ILE ILE GLU VAL GLU ASN GLN GLN SER PRO THR THR ALA
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106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140
: GLU THR LEU ASP ALA THR ARG ARG VAL ASP ASP ALA THR VAL ALA ILE ARG SER ALA ILE ASN ASN LEU ILE VAL GLU LEU ILE ARG GLY THR GLY SER TYR ASN
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: GLN VAL ALA ASN ALA THR GLN ARG VAL ASP ASP ALA THR VAL ALA ILE ARG SER GLN ILE GLN LEU LEU LEU ASN GLU LEU SER ASN HIS GLY GLY TYR MET ASN
12
141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158
: ARG SER SER PHE GLU SER SER SER GLY LEU VAL TRP THR SER GLY PRO ALA THR
: GLN ASN THR PHE GLU SER MET SER GLY LEU VAL TRP THR SER ALA PRO ALA SER
: GLN ALA GLY PHE GLU THR ALA SER GLY LEU VAL TRP THR SER THR PRO ALA THR
: ARG ALA GLU PHE GLU - ALA ILE - LEU PRO TRP THR SER ALA PRO ALA THR

FIGURE 3

methionine residues and the presence of an acetylated N-terminal threonine. Trypsin cleaves the molecule into 12 peptides which can be separated by gel chromatography and electrophoresis (Fraenkel-Conrat, 1965). The sequences comprising these peptides are shown in Fig. 3. Durham and Butler (1975) proposed a model for the way in which the polypeptide chain is arranged to form the TMV subunit. They based their model on information derived from sequence data, X-ray crystallography and chemical and immunological reactivity of specific residues. Fig. 4 shows a later model proposed by Champness et al. (1976) based on similar criteria.

In 1963 Anderer and Handschuh found that in the intact virus none of the lysyl or tyrosyl residues could be dinitrophenylated. This showed that these residues were probably not on the surface of the virus. In the separated protein, however, reaction proceeded normally (Fraenkel-Conrat, 1965) indicating that the reactivity of certain residues depended upon the disaggregation of the virus into protein subunits.

The immunological reactivity of TMVP is also a function of the quaternary structure of the protein, and differences between serological specificity of the virus and the disassembled protein have been known for many years. In the assembled virus, antigenic determinants are found which do not occur on the separated subunit. These new determinants (neotopes) may arise as the result of conformational changes in the protein molecule when it assumes its quaternary structure. Also,

antigenic determinants present in the protein may be masked by adjacent subunits in the assembled virus. These hidden determinants (cryptotopes) cannot react with their antibodies when the subunits are assembled into a virus particle (Jeener, et al. 1954; Starlinger, 1955; Aach, 1959; Takahashi and Gold, 1960, van Regenmortel, 1966, 1967). However, van Regenmortel points out in his review (1966) that not all workers agree regarding the existence of cryptotopes and neotopes, and that this is probably due to the difficulty of preventing aggregation and disaggregation when performing serological tests with viral antigens.

The immunological activity of TMVP has been found to be limited to certain regions of the peptide chain. A variety of immunological techniques have been applied to isolated peptide preparations and to a number of chemically induced mutants, and have led to the elucidation of the antigenic structure of the molecule. Anderer (1963a) found that 7 out of 20 peptides obtained by different enzymatic degradation procedures, could inhibit precipitation of TMV with an anti-TMV serum. The 12th tryptic peptide, which had previously been shown to play a role in virus antigenicity (Harris and Knight, 1955), also showed inhibitory activity.

Anderer, using TMV serum, could detect no inhibitory activity with the 8th tryptic peptide (residues 93 - 112) but Benjamini et al. (1964) found that this peptide could inhibit the fixation of complement with an anti-TMVP antiserum. This suggests that the determinant contained in this peptide is hidden in the

intact virus. Benjamini and his co-workers confirmed the immunological activity of peptide 8 in a protein/anti-protein system by means of a direct binding radioimmunoassay (Benjamini et al., 1965). Another approach which was also used consisted in determining the binding properties of synthetic analogs to the peptide. These were found to cross-react with the native peptide (Young et al., 1967). Complement fixation could also be inhibited with a mixture of the peptides 2 - 12, showing that some immunological activity still remained even though a considerable part of the tertiary structure of the determinant must have been lost on isolation (Benjamini et al., 1964). Results of passive haemagglutination experiments, however, have shown that some determinants may be conformation-dependent. TMV antibody was found to possess specificity for the TMV surface structure in the region of the terminal hexapeptide (Anderer et al., 1971).

A further valuable source of information on the antigenic structure of TMVP has been obtained from work with a variety of mutants of TMV having known amino acid replacements. Using an antiserum directed against TMV vulgare, von Sengbusch (1965) used the quantitative precipitin test to compare the abilities of numerous mutants to react with the antiserum. He found that amino acid exchanges in the regions corresponding to tryptic peptides 4, 8, 11 and 12 could be distinguished. Van Regenmortel (1967) used double diffusion in gel to distinguish mutant virus from the wild-type. A difference in immunological reactivity was discernible by the formation of spurs. The contribution of specific amino acids to the antigenicity of

of the molecule established in this way agreed closely with the results of von Sengbusch (1965), although an exchange in peptide 8 could not be detected. There have been other discrepancies in the localisation of immunological activity: peptides in position 18 - 23 and 123 - 134 were also found by Anderer (1963a) to inhibit precipitation of TMV with virus antiserum, but van Regenmortel (1967) could not distinguish mutants with amino acid replacements in these regions. He postulated that some of the inhibitory effects observed in Anderer's system may have been non-specific. It is thus apparent that it is important to try to apply as many serological techniques to the problem as is possible, rather than to depend entirely on the results of one method.

The need for basing conclusions on many individual determinations is also apparent in the study of antigenic relationships between strains of TMV. Van Regenmortel in 1967 attempted to establish the nature and strength of the serological relationship between different TMV strains and to correlate the biochemical and serological criteria of virus classification, using precipitation in liquid and gel and intragel cross-adsorption. He concluded that there were no valid quantitative criteria for distinguishing between various degrees of serological correspondence and suggested that the use of serology for defining degrees of relationship between virus strains should be abandoned. A few years later he studied the serological relationship between 6 strains of TMV with antisera from 40 rabbits bled at regular intervals over a period of 8 months. When the results were averaged and analysed statis-

tically he found, in contrast to his earlier findings, a good correlation between apparent serological relatedness and the extent of sequence homology in the coat-protein of different strains (van Regenmortel, 1975). These results emphasized the danger of basing conclusions on too few bleedings from too few animals, and pointed out the variations which can be expected in different sera.

From the foregoing it is clear that no single technique can be applied to the study of TMV serology. In view of the tendency of the protein to aggregate, a system which can be used at very low antigen concentration would be especially useful. This thesis investigates the use of the chemically modified phage technique in the elucidation of the immunological properties of TMVP.

CHAPTER III

MATERIALS AND METHODS

1. BUFFERS

Barbital Acetate Buffer pH 8,6 (For Electrophoresis of IgG)

5,4 g Sodium barbital.

4,3 g Sodium acetate.

58,2 ml of 0,1 M HCl

Make up to 1000 ml with distilled water.

Phosphate + Gelatin Buffer pH 6,8 (P + G Buffer pH 6,8

Solution A - 0,5M Na_2HPO_4 102 ml

Solution B - 0,5M NaH_2PO_4 98 ml

Solutions A and B are mixed and diluted to 2000 ml. Adjust pH. Add 20 µg/ml gelatin. Warm buffer to dissolve gelatin.

Phosphate + Gelatin Buffer pH 7,5 (P + G Buffer pH 7,5)

Solution A^1 - 0,2M Na_2HPO_4 420 ml

Solution B^1 - 0,2M NaH_2PO_4 80 ml

Solutions A^1 and B^1 diluted to 2000 ml. Adjust pH. Add 20 µg/ml gelatin. Warm buffer to dissolve gelatin.

2. GROWTH MEDIA

T-Phage Nutrient Agar

Agar	10g
Tryptone Broth	10g
NaCl	8g

Distilled Water to 1000 ml.

The above solution was autoclaved at 120 psi for 15 minutes to sterilize and melt the agar. Approximately 30 ml of the molten agar was poured into sterile plastic petri-dishes and allowed to solidify. Before use the plates were dried by overnight incubation at 37°C.

Soft (0,7%) Agar

Prepared in the same way as above but contained 7g of Agar per 1000 ml. 2,5 ml were placed into Wasserman tubes and autoclaved to sterilize.

3. BIFUNCTIONAL REAGENTS

Glutaraldehyde

Glutaraldehyde used in the coupling experiments was EM grade obtained from TAAB laboratories, Reading. Samples of this glutaraldehyde were distilled under vacuum for use in some experiments by the method described by Vogel, (1959).

Vacuum Distillation of Glutaraldehyde

Glutaraldehyde was distilled under a vacuum of 10 mm of mercury at 80°C using a vacuum distillation apparatus. Nitrogen was bled via a fine capillary tube into the distilling vessel to prevent "bumping". The concentration of glutaraldehyde in the distillate was determined by the method of Frigerio and Shaw (1969). Sodium bisulphite (0,25 M solution) was added to 0,5 ml of a 1/100 dilution of distillate and the excess bisulphite was then titrated with standardised 0,1 M iodine. The distillate was calculated to contain 40% w/v glutaraldehyde which was probably mainly monomeric. The distilled glutaraldehyde stock was sealed in glass ampoules under nitrogen and stored at 4°C.

Tolylene-2,4-diisocyanate (TDIC)

TDIC (technical grade) was obtained from Fluka (Switzerland). It was diluted in absolute dioxane (technical grade - Merck, Darmstadt) for use in coupling experiments.

Bis-diazotized benzidine (BDB)

BDB was prepared according to the method described by Herbert (1973). To 0,23g benzidine (Merck, Darmstadt) dissolved in 45 ml of 0,2 N HCl and cooled in an ice bucket was added with constant stirring 0,175g of $NaNo_2$ in 5 ml of cold water. The mixture was left in ice for 30 minutes and stirred gently every

5 minutes for a few seconds. This stock was diluted 1/15 in phosphate buffer and was used immediately.

4. PROTEINS

Lysozyme

The enzyme (source not specified) was obtained in crystalline form from E. Merck (Darmstadt). It was dissolved in P + G buffer pH 6,8 for use.

Tobacco Mosaic Virus Protein (TMVP)

TMVP was prepared from the virus by the cold acetic acid method (Fraenkel-Conrat, 1957) or by the ethanolamine method described by Durham (1972). The proteins of some strains and mutants of TMV were supplied by Dr. M.B.von Wechmar. All proteins were freeze-dried after preparation and were stored at $4^{o}C$ with the exception of that of mutant 371 protein which was used immediately without freeze-drying.

Freeze-dried protein was usually dissolved in cold water and buffer was then added to the required strength. As preparations varied in their solubility, any insoluble material was removed by centrifuging at 5000 r.p.m. for 10 minutes in a Sorvall SS34 rotor. As much of the precipitate as possible

was redissolved in the supernatant and this was centrifuged again to clarify the solution, which was then poured off for use. The concentration of TMVP was determined spectrophotometrically using an extinction coefficient of $E^{0,1\%}_{280}$ of 1,27. In some experiments it was necessary to remove aggregates. This was done by centrifuging the preparation in a Beckman 50 Ti rotor at 40 000 r.p.m. for 2 h at 4°C.

5. PREPARATION OF ANTISERA

Antisera were produced in rabbits and were labelled by a series of letters which refer to the animal and by Roman numerals which refer to the particular bleeding, e.g. JOR IX. The various anti-TMV and TMVP sera were obtained from the stocks of the Department of Microbiology.

Anti-phage Serum

Anti-T4 sera were prepared by injecting three rabbits with purified T4 at a titre of approximately 10^{10}/ml (Adams, 1959). The first injection was made intramuscularly with 1 ml of Freund's incomplete adjuvant. Subsequent injections of T4 in P + G buffer pH 6,8 were made 9 and 25 days after the first injection. Approximately 5 ml of blood was collected from the ear vein of each rabbit after 9 days (bleeding I); 60 days (bleeding II) and 76 days (bleeding III). Serum from each bleeding of the three rabbits was pooled.

Approximately 25 - 30 ml of blood were collected at each bleeding and allowed to clot in a glass beaker. The serum was drawn off, the red blood cells were removed by centrifugation for 10 minutes at 10 000 r.p.m. in a Sorvall SS 34 rotor and the sera were stored frozen at -20°C.

Anti-lysozyme

One rabbit was injected intramuscularly with 5 mg lysozyme in saline and incomplete Freund's adjuvant at approximately weekly intervals. The animal was bleed weekly.

Anti-TMVP

Rabbits were injected intramuscularly with 1 ml protein at a concentration of 2,5 mg/ml in water emulsified with an equal quantity of incomplete Freund's adjuvant. The animals were bled weekly from the ear vein.

Normal Rabbit Serum (NRS)

Normal rabbit serum was tested for anti-T4 activity by incubating 0,1 ml of undiluted serum for 60 minutes with ± 500 bacteriophage T4 or chemically modified phage. The number of surviving phage was compared with an identical sample which was plated without having come into contact with the serum.

6. PREPARATION AND ASSAY OF BACTERIOPHAGE

The bacteriophage used was a rapid lysis mutant (T4r) obtained from Prof. D. Woods, Rhodes University, Grahamstown. This phage does not show the phenomenon of lysis inhibition and forms clear and well defined plaques on a lawn of *E. coli* B host bacteria. *E. coli* B 10243 was obtained from The National Culture of Industrial Bacteria, Edinburgh, and was grown in tryptone broth and on T-phage nutrient agar for purposes of phage propagation and assay.

Although strict sterile technique was not adhered to during the assay and propagation, all glassware was sterilized and reasonable precautions were taken to prevent contamination. As the inoculum was large, any contaminants were effectively competed against by the *E. coli* B, and if the plates were examined within 18 hours of inoculation, no problems with extraneous contaminants were encountered.

(a) Culture of Host Bacteria

A stock culture of *E. coli* B was kept at room temperature on soft nutrient agar in a sealed McCartney bottle. Inoculations were made into test tubes containing 20 ml of sterile tryptone broth and incubated overnight at 37°C without aeration. This overnight culture was used for propagation and assay of T4.

(b) Growth of Bacteriophage

Bacteriophage preparations were obtained by the general procedure for lysis in a solid medium described by Adams (1959). Usually 30 to 40 plates were prepared at a time.

For each plate approximately 10^5 phage were added to 2,5 ml of melted soft (0,7%) agar at 46°C containing 0,2 ml of an overnight culture of E. coli B host bacteria. This mixture was poured onto petri dishes containing about 30 ml of solidified T-phage nutrient agar and allowed to harden. The plates were incubated overnight at 37°C. After incubation 5 ml of 0,05 M phosphate buffer pH 6,8 containing 20 µg/ml gelatin (P + G buffer) were pipetted onto the confluent layer of phage plaques on each plate and the soft agar layer scraped from the plates into plastic Sorvall centrifuge bottles. The agar was shaken with buffer and allowed to stand for approximately 30 minutes.

The agar and some of the bacterial debris was removed by spinning in a Sorvall GSA rotor at 8 000 r.p.m. for 10 minutes. The supernatant was then centrifuged at 10 000 r.p.m. for 10 minutes in the Sorvall SS-34 rotor for final clarification. Phage was sedimented from the supernatant by centrifugation at 20 000 r.p.m. for 45 minutes in polycarbonate tubes in a Beckman type 30 rotor. The pellet was resuspended in 2 ml of P + G buffer per tube by allowing it to stand at 4°C with occasional shaking. Further purification was then carried out.

(c) Purification of Phage

Phage preparations were purified by sucrose density-gradient centrifugation, followed by dialysis against 0,05M phosphate buffer pH 6,8. Sucrose gradients (5 - 40% in 0,05M phosphate buffer) were prepared with an LKB density-gradient layering apparatus. The resuspended phage pellet (2 ml) was layered onto the gradient and centrifuged for 25 minutes at 20 000 r.p.m. in a Beckman type SW 27 swing-out rotor.

The virus band was clearly visible and was located approximately halfway down the tube. It was withdrawn by means of a bent Pasteur pipette and the sucrose dialysed out overnight against 0,05M phosphate buffer pH 6,8. An ultraviolet absorbance scan of the preparation showed a peak at 260 nm.

The phage preparation was concentrated by spinning the purified preparation in a Beckman type 50 Ti fixed-angle rotor at 20 000 r.p.m. for 45 minutes, and resuspending the pellet in approximately 0,4 ml of buffer. Preparations with a titre of 10^{12} to 2 x 10^{12} pfu/ml were obtained.

(d) Assay of Phage

Plaque forming units (pfu) were assayed by the double agar layer method (Adams, 1959). Total numbers of phage were determined from the ultraviolet absorbance at 260 nm.

Double Agar Layer Assay

Samples of 2,5 ml of nutrient agar containing 0,7% Difco agar ("soft agar") were melted in Wasserman tubes in a boiling water bath. The molten agar was cooled in a 46°C waterbath and 0,2 ml of an overnight culture of E. coli B host bacteria were added. Aliquots of dilutions of the phage preparations to be assayed were added (in some experiments the agar plus bacteria was added to phage) and the mixture poured over solidified T-phage nutrient agar plates. The plates were incubated overnight at 37°C and the resulting plaques in the lawn of bacteria were counted. From the number of plaques per plate and the dilution and volume of the phage added, the titre in pfu per ml was calculated - each plaque representing one viable phage particle.

Determination of Phage Concentration by Absorbance at 260 nm.

The concentration of high titre phage ($> 10^{10}$ per ml) could be determined by the ultraviolet absorbance at 260 nm in a 1 cm path length quartz cell of a Unicam SP 1700 ultraviolet spectrophotometer. Bacteriophage T4 in 0,05M phosphate buffer pH 6,8 at a titre of 1×10^{11} pfu/ml were found to have an absorbance at 260 nm in a 1 cm cell of 1,0 (Haimovich et al., 1970a). Samples of higher titre preparations were diluted until the absorbance was within the working limits of the spectrophotometer and the original

concentration was calculated from the dilution factor and the absorbance.

7. DETERMINATION OF THE K VALUE OF ANTI-PHAGE SERA

The kinetics of inactivation were studied by withdrawing aliquots from a phage/anti-phage mixture and diluting into buffer to greatly reduce the chance of further phage-antibody contact. The surviving phage were assayed by the double agar layer method and K, the rate constant for the reaction was determined. K is a useful measure of the strength of an anti-phage serum. The procedure described by Adams (1959) was generally used but in one experiment, anti-phage activity of an early serum was determined by a variation of this method described by Clowes and Hayes (1968).

Phages were assayed and diluted to a titre of approximately 10^7 per ml in P + G buffer pH 6,8. At time = 0, 0,1 ml of a phage preparation ($\pm$ 10^6 particles was added to 0,9 ml of a 10^{-2} or 10^{-3} dilution of anti-phage serum. The phage-antiserum mixture was kept at 37°C in a waterbath and at various consecutive time intervals 0,1 ml was withdrawn and added to 9,9 ml of P + G buffer pH 6,8. From this hundredfold dilution a further 0,1 ml was withdrawn and added to 2,5 ml of molten (46°C) soft agar containing 0,2 ml of an overnight culture of *E. coli* B. This mixture was immediately poured onto a T-phage nutrient agar plate and incubated overnight at 37°C. The number of survivors at each time interval was counted and a

plot of the fraction of surviving phage (P/Po) vs. time (t) was made on semi-logarithmic co-ordinates. From the slope of the curve the K value was determined using the formula for a first order reaction.

$$K = 2{,}303 \frac{D}{t} \log_{10} \frac{Po}{P} \qquad \text{equation (1)}$$

where Po = number of phage at t = 0

P = number of phage at t min.

D = Dilution of serum in phage/serum mixture

K = rate constant (min^{-1}).

If the inactivation reaction was obeying first order kinetics the plot of log P/Po vs. time was linear. The slope (m) of this plot was obtained by the sum of least squares method using the experimentally determined values of P/Po and t.

Equation 1 was rearranged :

$$K = -2{,}303 \; D \times m \qquad \text{equation (2)}$$

where $m = \frac{\log P/Po}{t}$ i.e. slope of the inactivation curve.

The relevant values of m and D were substituted into equation (2) and the first order rate constant for the inactivation reaction by the serum (K value) was calculated.

8. PREPARATION OF IMMUNOGLOBULIN G FROM AN ANTI-T4 SERUM

Immunoglobulin G (IgG) was prepared from anti-T4 serum II by the rivanol/ammonium sulphate method described by Heide and Schwick (1973). Using 0,1 M NaOH the pH of the undiluted serum was adjusted to pH 8. For each 1 ml of alkaline serum was added 3,5 ml of 0,4% rivanol (trade mark of Hoechst for 2-ethoxy-6-9-diaminoacridine lactate). A precipitate formed which was removed by spinning at 8 000 r.p.m. for 10 minutes in a Sorvall SS34 rotor. The supernatant fluid was decanted and the precipitate was redissolved in 5 ml of water. Rivanol (5 ml of a 0,4% solution) was added and after another spin at 8 000 r.p.m., the supernatants were pooled. To remove the rivanol, saturated KBr was added dropwise and the precipitate of rivanol-Br was removed by spinning at 12 000 r.p.m. for 30 minutes. The IgG was precipitated by adding to the supernatant 2/3 of its volume of saturated $(NH_4)_2\ SO_4$. The precipitate was removed by centrifugation at 10 000 r.p.m. for 15 minutes. The precipitation and centrifugation were repeated twice and the final pellet was resuspended in 3 ml of distilled water. The IgG preparation was then dialysed against saline and concentrated to about 2,5 ml by placing the dialysis tube into polyethylene glycol (molecular weight 20 000) until the volume was sufficiently reduced. The IgG concentration was determined spectrophotometrically using $E^{0,1\%}_{280} = 1,43$.

Electrophoresis of IgG on Cellulose Acetate.

Immunoglobulin G which had been prepared by the rivanol method was electrophoresed on cellulose acetate to determine its

homogeneity (Köhn, 1968).

A sample of the preparation (2,9 mg/ml) was applied to a cellulose acetate strip with a Beckman sample applicator. The sample was electrophoresed in a Shandon Universal Electrophoresis apparatus using 0,05 M barbital acetate buffer pH 8,6, at 160 V (constant voltage) for 120 minutes. The strips were stained in 0,002% nigrosine in 2% acetic acid for 4 hours and rinsed in distilled water.

9. PREPARATION OF N-BROMOSUCCINIMIDE MODIFIED TOBACCO MOSAIC VIRUS PROTEIN.

The method described by Ohno et al. (1972) was utilised for the NBS-modification of TMVP (vulgare strain). An amount of 100 mg of lyophilized TMVP was added to 16 ml of cold (4°C) distilled water. The pH of the preparation was adjusted to 8,0 with 0,1 M NaOH. After all the protein had dissolved, 4 ml of 0,05 M phosphate buffer pH 7,5 was added. The preparation was kept at 4°C throughout the procedure. To the protein suspension was added 1,3 ml of a fresh NBS solution (3 mg/ml) and the preparation was stirred gently for 30 minutes. To terminate the reaction, 1,3 ml of 2% $Na_2S_2O_3$ was added. This was followed by overnight dialysis at 4°C against 2 changes of 5 l of water. After dialysis the preparation was centrifuged in a Beckman 50 Ti rotor at 50 000 r.p.m. for 60 minutes to remove any aggregated protein.

An ultraviolet scan of the modified protein showed a characteristic shoulder from 310 to 318 nm (see Fig. 15; Chapter IV). NBS-modified protein was concentrated at a pressure of 40 psi in an Amicon ultrafiltration apparatus using a PM10 membrane. The concentration of NBS-TMVP was determined by the absorbance at 281 nm using an $E^{0,1\%}_{281}$ of 1,13 (Inoue *et al.*, 1974).

10. ULTRACENTRIFUGAL ANALYSIS OF TMVP

The sedimentation coefficient (S) of TMVP preparations was determined by sedimentation velocity experiments in a Beckman model E analytical ultracentrifuge (Chervenka, 1969). The AnD rotor with a standard 12 mm single sector centrepiece was used. The sample was spun at 56 000 r.p.m. and photographs were taken at various time intervals with the schlieren optical system. The logarithm of the distance of the peak from the axis of rotation at various time intervals was plotted vs. time and the S value was calculated from the resulting graph.

11. PREPARATION OF CHEMICALLY MODIFIED BACTERIOPHAGE

(a) Preparation of DNP-T4 Conjugates

Dinitrobenzenesulphonic acid reacts with free amino groups

(predominantly the ϵ-NH_2 groups of lysine residues) on the coat protein of phage to form dinitrophenyl (DNP)-protein conjugates (Eisen, 1964). Under favourable conditions of reaction some phage-DNP conjugates remain viable, the coat protein antigenicity being modified by the presence of DNP groups. The resulting DNP-phage can be inactivated by antibodies specific for the DNP group (Carter et al., 1968).

DNP-T4 conjugates were prepared by the method described by Eisen (1964) for the coupling of the DNP group to proteins. The sodium salt of dinitrobenzenesulphonic acid (DNB-sulphonic acid) was obtained from Eastman Chemicals (Rochester, N.Y.) and was twice recrystallized from ethanol before use as follows:

Fifteen grams of DNB-sulphonic acid were dissolved in 950 ml of absolute ethanol by stirring vigorously and heating to 70°C in a waterbath. About 3 g of powdered charcoal were added to the hot solution which was filtered under vacuum through three layers of Whatman's No. 1 filter paper into a Buchner flask. The filtrate was cooled by immersing the flask in water and the fine yellow crystals of DNB-sulphonic acid which formed were separated from the mother liquor by filtration. These were redissolved in 500 ml of hot ethanol and recrystallized as above, but without the addition of charcoal. The crystals were collected and dried on a piece of filter paper at 60°C.

Bacteriophage T4 (10 ml at 1,5 x 10^{10}/ml) in P + G buffer pH 6,8 was pelleted by spinning at 20 000 r.p.m. in a Beckman 50 Ti rotor for 45 minutes. After resuspending the phage pellet in 10 ml of 0,3M sodium carbonate buffer pH 9,5, containing 50 mg/ml of dissolved recrystallized DNB-sulphonic acid, the preparation was transferred to a small conical flask. The flask was enclosed in aluminium foil to exclude light and was left at room temperature for 24 hours with gentle stirring. After reaction, the DNP-T4 conjugates were dialysed against 2 l of 0,05M phosphate buffer pH 6,8 for five days at 4°C with four changes of buffer to remove unreacted DNB-sulphonate. The dialysis also removes 2,4 dinitrophenol which forms as a by-product by the hydrolysis of DNB-sulphonic acid. The phage conjugates were then assayed to determine the percentage of phage which had survived the coupling reaction, and were used in subsequent inactivation experiments with anti-DNP serum.

(b) Preparation of Protein-phage Conjugates

Protein-phage conjugates were prepared by modification of the methods described by Haimovich et al. (1970a). The exact procedure which was followed depended upon the particular protein and the bifunctional reagent which was used (see Results, Chapter IV). Protein (5 - 20 mg/ml) and T4 phage 10^{12} - 10^{13}/ml were mixed, the bifunctional reagent was added and after the time allowed for the

coupling reaction, 5 ml of P + G buffer were added to stop the reaction by dilution. The diluted preparation was then dialysed overnight against 6 l of phosphate buffer (gelatin omitted). Because only the low molecular weight bifunctional reagent was removed by dialysis, the protein-phage conjugates were separated from the unreacted protein by centrifuging the solution in a Beckman 50 Ti rotor at 20 000 r.p.m. for 45 minutes. The supernatant fluid was poured off and the phage pellet was washed (without resuspending) with 2 ml of P + G buffer. The pellet was then resuspended in 5 ml of buffer and the above procedure was repeated. Any precipitate was removed by spinning at 5 000 r.p.m. for 10 - 15 minutes. The uv absorbance of the final supernatant was determined and the number of phage was calculated using E_{260} of 1,0 for 1×10^{11} particles (Haimovich *et al*, 1970a).

12. THE "DECISION" TECHNIQUE

The "decision" method of plating was used to limit the time available for dissociation of phage - antibody complexes during the assay of neutralized phage. If dissociation occurs it leads to a departure of linearity in the plot of the logarithm of surviving fraction against time. This leads to a reduction in sensitivity of the phage assay for low concentrations of anti-phage or anti-hapten antibodies. The method followed was first described by Jerne and Avegno in 1956.

In modified phage inactivation experiments, the phage/serum mixture was mixed with 0,2 ml of an overnight culture of E. coli B. This mixture was left at 37°C for 10 minutes with frequent gentle shaking during which time the surviving phage particles "decided" whether or not they would adsorb to, and infect a bacterium. After this "decision period", 0,1 ml of 10^{-2} dilution of anti-T4 serum (anti-T4 III) was added to inactivate in an irreversible manner any unadsorbed phage particles. This dilution of antiserum was sufficient to inactivate > 99% of phage in 5 minutes. It is known that adsorbed phage is not affected by antiserum (Delbrück, 1945). After 5 minutes at 37°C, 2,5 ml of soft agar was added and the whole mixture was poured onto a T-phage nutrient agar plate, allowed to solidify and incubated overnight at 37°C. The surviving pfu's were then counted.

If a phage preparation which had not reacted with antibody was assayed, the titre obtained by the "decision" technique was found to be about 80% of that obtained by a standard double agar layer method of assay. This is in accordance with the figure obtained by Jerne and Avegno (1956).

13. THE COMPLEX INACTIVATION METHOD

Plating of modified phage by the complex inactivation method was similar to the normal direct plating method, except that at the end of the time allowed for reaction with the inactivating

serum, 0,05 ml of a 1/50 dilution of goat anti-rabbit IgG serum was added to the reaction mixture. Incubation was continued for a further 10 minutes when 2,5 ml of soft agar and 0,1 ml of host bacteria were added. The whole mixture was then poured over the assay plate (Haimovich et al., 1970a).

14. INHIBITION OF INACTIVATION OF TMVP-PHAGE CONJUGATES

The inhibition of inactivation was performed at the dilution of inactivating serum which caused 80 - 85% inactivation of the modified phage, i.e. a 16 x 10^{-5} dilution of JOR XIV anti-TMVP serum or FRE XXXI anti-U2-P diluted 10^{-3}.

In all the inhibition experiments 0,2 ml of the diluted serum was allowed to react with 0,1 ml of the antigen for 20 h at 4°C. This was followed by a further 2 h incubation at 37°C after which 0,1 ml of 40% PEG 6000 and 400 - 600 pfu of TMVP-phage conjugates were added in 0,1 ml of P + G buffer pH 7,5. Inactivation was carried out as usual for 5 h at 37°C. The mixture was then plated by the complex inactivation method. As controls, 0,1 ml of buffer instead of inhibitory antigen and 0,2 ml of normal rabbit serum (10^{-2} dilution) instead of the inactivating serum were added to two of the reaction tubes. All results shown are the average of duplicate determinations.

CHAPTER IV

RESULTS

1. REACTION OF BACTERIOPHAGE T4 WITH ITS ANTISERUM

An exponential decrease in pfu with time is observed when anti-phage sera are allowed to react with the homologous phage. Due to antibody excess the reaction is pseudo-first order. The first order rate constant for the reaction, or K value, is a useful measure of the inactivating strength of an antiserum. It can be used for calculating the extent to which a serum must be diluted to inactivate a desired fraction of phage in a given time (Adams, 1959). In the following section two methods for determining K values of anti-T4 sera will be illustrated: The simplified method described by Clowes and Hayes (1968) where serum at various dilutions is reacted with phage for 6 minutes, and the method described by Adams (1959) in which the kinetics of inactivation are followed.

Since anti-T4 sera were required for subsequent use in the "decision" technique experiments it was necessary to determine their capacities to inactivate phage. In addition, a study of the kinetics of phage inactivation was useful in providing experience in the methodology of phage inactivation experiments.

(a) Inactivation of Phage with Early Anti-T4 Serum

Sera from the bleedings of three rabbits were pooled and

the approximate K value of the first bleeding (9 days after injection) was determined by the method described by Clowes and Hayes (1968) - a threefold dilution series (1/1 to 1/243) of anti-T4 I was made and incubated with phage for 6 minutes at 37°C. The reaction was stopped by dilution. The phage which had been reacted with the undiluted serum were found on assay to have been reduced in titre by approximately 99%. The final dilution of serum in this reaction tube was 10^{-1} and the phage titre was reduced from 1,08 x 10^4 pfu/plate to 1,55 x 10^2 pfu/plate. By substituting into equation 2 (Chapter III), the K value was calculated to be 7,1.

(b) Kinetics of Inactivation of Phage with Late Anti-T4 Sera

The K values of later serum bleedings were determined by the method described by Adams (1959) at two different dilutions. Anti-T4 II (bled 60 days after injection) at a dilution of 10^{-2} was reacted with 9 x 10^6 phage pfu at 37°C. Aliquots were withdrawn at various times and diluted by a factor of 100 to greatly reduce the chance of further phage - antibody contact, thereby effectively stopping the reaction.

The decrease in the surviving fraction (P/Po) with time was exponential until $>$ 99% of the pfu were inactivated (Fig. 5, Table 1 a, b). The 10^{-3} dilution caused an exponential decrease in phage titre of up to 99,99% inactivation

(Fig. 5) and no persistent fraction was observed. The first order rate constants for both serum dilutions were calculated, giving values of 168 for the 10^{-2} and 228 for the 10^{-3} dilution of serum. The slopes of the curves for the 10^{-2} and 10^{-3} dilutions were respectively -0,73 and -0,099 and are thus approximately proportional to dilution. A K value of 659 for the third bleeding (obtained 76 days after injection) was determined by the same method (see Fig. 6, Table 2). These anti-T4 sera were used in subsequent "decision" technique experiments.

The phage inactivating ability of the sera were seen to increase as immunization proceeded. The later sera had increasingly higher K values, which reflects an increase in antibody titre and possibly higher affinity of the antibody for the phage.

Table 1 (a)

Time (min.)	Plaques per Plate		Average per Plate	P/Po
2½	80	44	62	0,0068
5	2	2	2	0,00022
7½	0	0	0	-
10	0	0	0	-

Table 1 (b)

Time (min.)	Plaques per Plate		Average per Plate	P/Po
2½	TNC		-	-
5	TNC		-	-
7½	1500		-	-
10	960	864	912	0,1013
15	240	241	240,5	0,0267
20	64	80	72	0,0080
30	8	12	10	0,0011
40	0	0	0	-

TNC = too numerous to count

K value determinations of anti-T4 II serum. The antiserum was allowed to react at initial dilutions of 10^{-2} (1a) and 10^{-3} (1b) with bacteriophage at 9×10^{7} pfu/ml. The reaction was stopped by dilution at the times shown and the survivors were assayed by the double agar layer technique. The P/Po values shown here were plotted vs. time (see Fig. 5).

Table 2

Time (min.)	Plaques per Plate		Average per Plate	P/Po
0	1500		1500	1,0
2½	158	154	156	0,1040
5	10	14	12	0,0080
7½	-	0	0	-
10	-	0	0	-
15	-	0	0	-

K value determination of anti-T4 III serum. The serum was allowed to react at a dilution of 10^{-3} with T4 bacteriophage. The P/Po values shown here plotted vs. time are illustrated in Fig. 6.

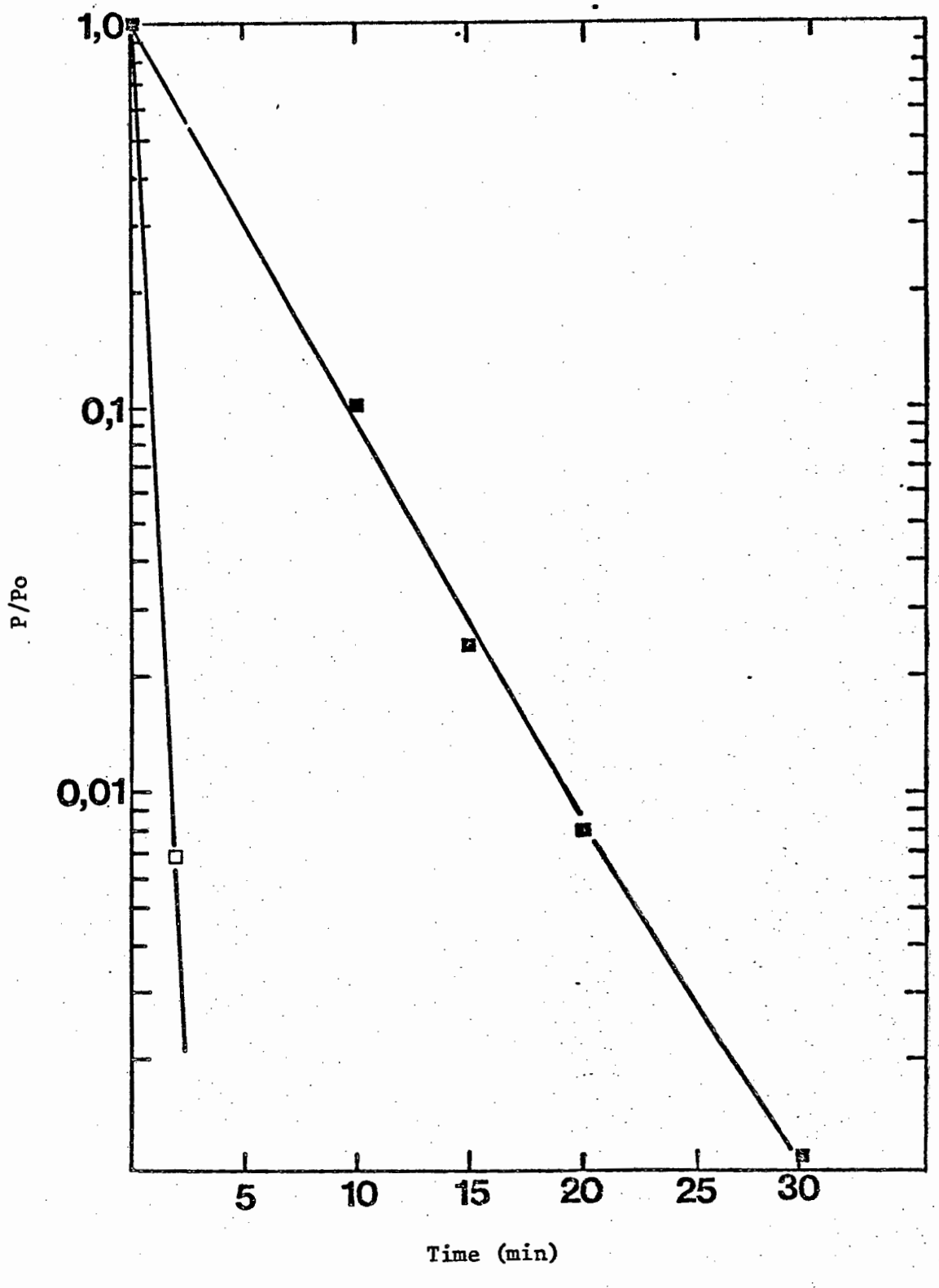

Fig. 5:

Kinetics of inactivation of bacteriophage T4 by anti-T4 serum II at dilutions of

■ 10^{-3}

□ 10^{-2}

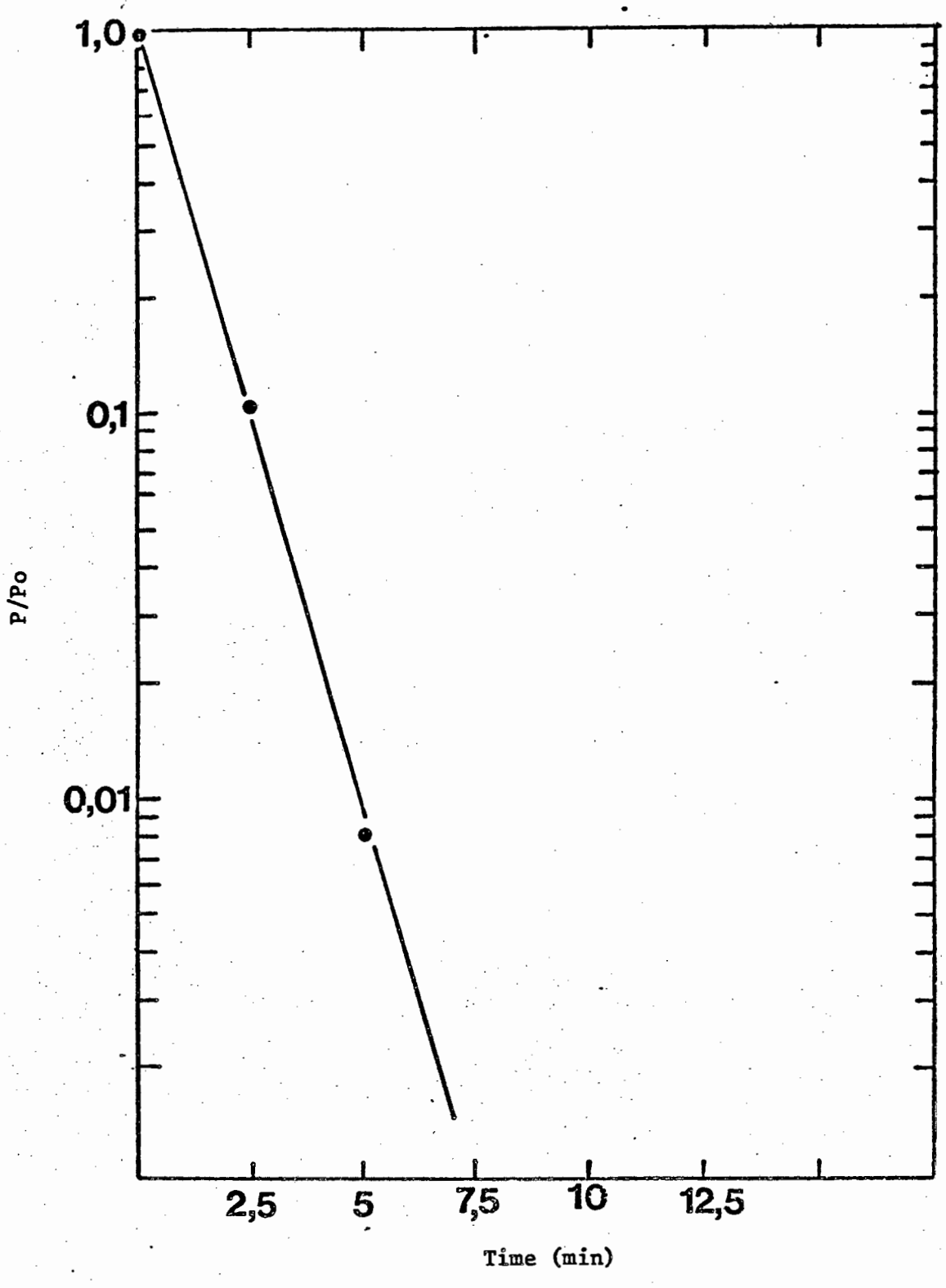

Fig. 6:
Kinetics of inactivation of bacteriophage T4 by anti-T4 serum III at a dilution of 10^{-3}

(c) Inactivation of Phage by the IgG Fraction of Antiserum

(i) Preparation of IgG

The IgG fraction of anti-T4 serum II was isolated from the serum by the rivanol method. The final purified preparation contained 2,87 mg/ml IgG as determined spectrophotometrically and was analysed by electrophoresis on cellulose acetate strips to determine whether it was free of contaminating proteins. In Fig. 7 a comparison between whole anti-T4 serum and the isolated IgG fraction shows that the isolated IgG is free of other serum proteins.

(ii) Kinetics of Inactivation

The IgG preparation was diluted 1/200 in P + G buffer (i.e. to a concentration of 14,4 μg/ml). Using 0,9 ml of this preparation and 0,1 ml of a suspension of T4 bacteriophage at 1,5 x 10^7 pfu/ml, the kinetics of inactivation of T4 were followed over 30 minutes. As found in the case of inactivation by whole serum, the reaction followed first order kinetics until $>$ 90% of the phage was inactivated (Fig. 8). The first order rate constant for the reaction was calculated to be 73,5 min^{-1} using a value of 1/200 for "D".

(iii) Inactivation of Phage for a Fixed Period of Time

Samples of a phage preparation diluted to 10^4 pfu/ml were added to 0,05 ml of varying dilutions of the IgG preparation

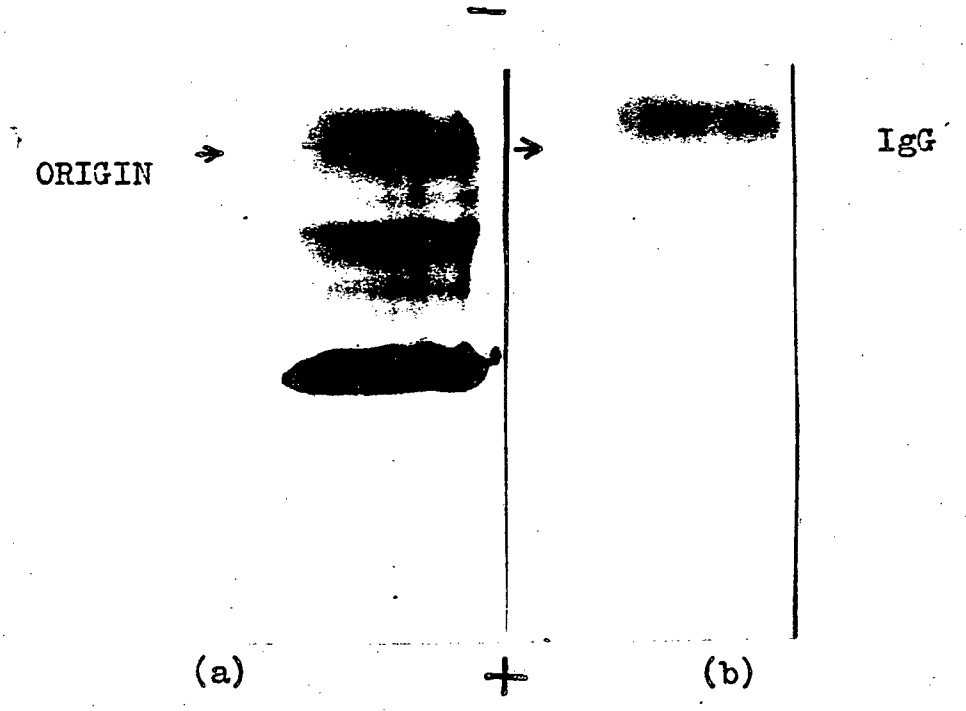

Fig. 7

Electrophoretic patterns on cellulose acetate strips of :

(a) whole anti-T4 serum II,

(b) IgG preparation isolated from anti-T4 serum II.

Note the absence of other serum proteins in the purified preparation.

in the range 2,9 mg/ml to 2,3 ug/ml. The mixture was incubated at 37°C for 2 h. At the end of this time the phage were assayed by the direct plating method: approximately 2,5 ml soft agar and 0,1 ml of an overnight culture of E. coli B were added and the whole mixture was poured onto a tryptone agar plate. After overnight incubation at 37°C, the plaques were counted. If no inactivation had occurred, ± 500 plaques/plate were expected, as was found on the control plates in the absence of IgG. The results presented in Fig. 9 show that 46 ng of IgG caused 50% inactivation, illustrating the extreme sensitivity of the phage to antibody. As it is unlikely that more than 10% of the IgG would be T4 phage antibody, the threshold of sensitivity is probably of the order of 4,6 ng of antibody.

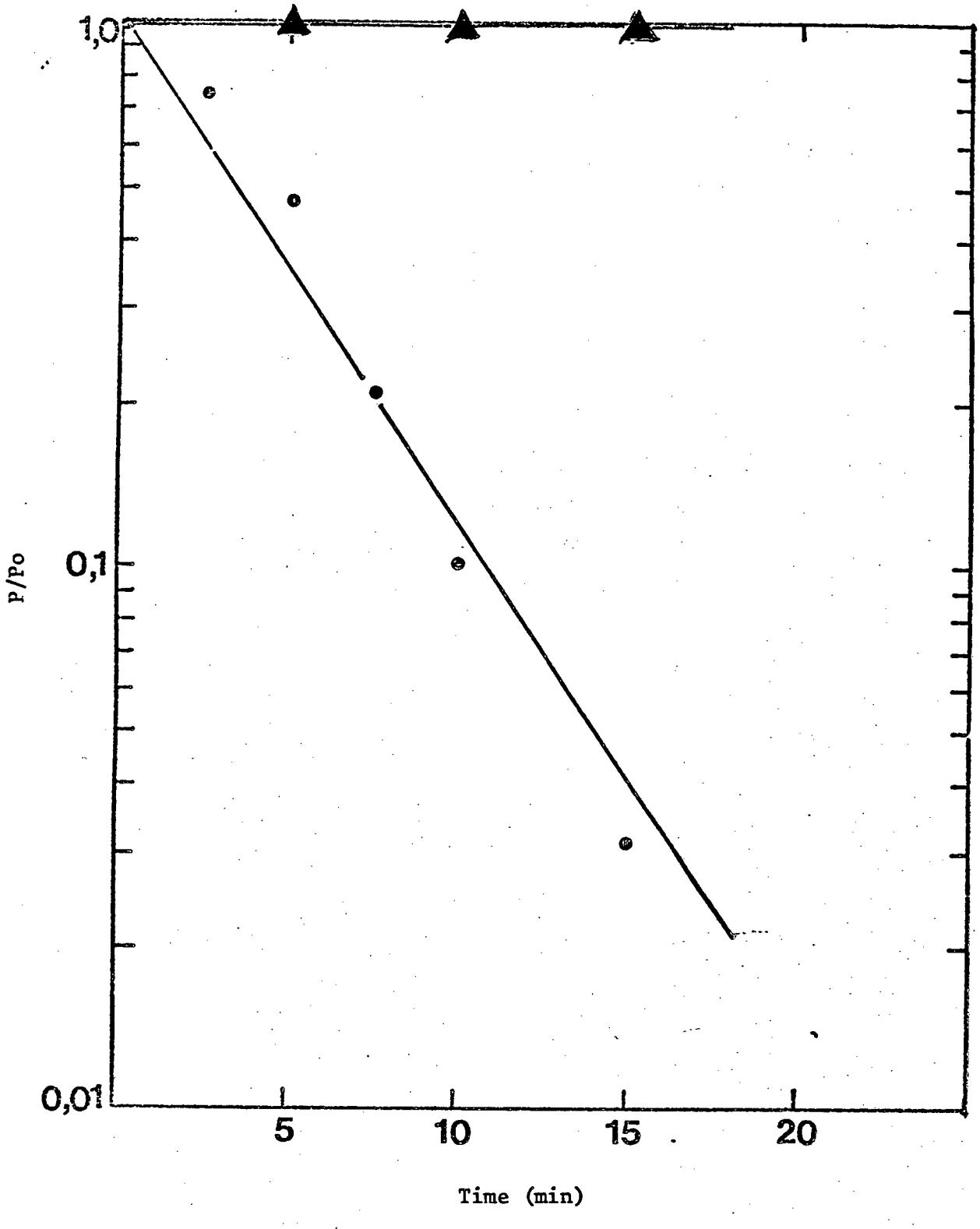

Fig. 8:

● Kinetics of inactivation of bacteriophage T4 by IgG prepared from an anti-T4 serum. The concentration of the IgG was 14,4 µg/ml.

▲ Results of a control experiment in which P + G buffer pH 6,8 was substituted for IgG.

Phage were assayed by the direct plating method.

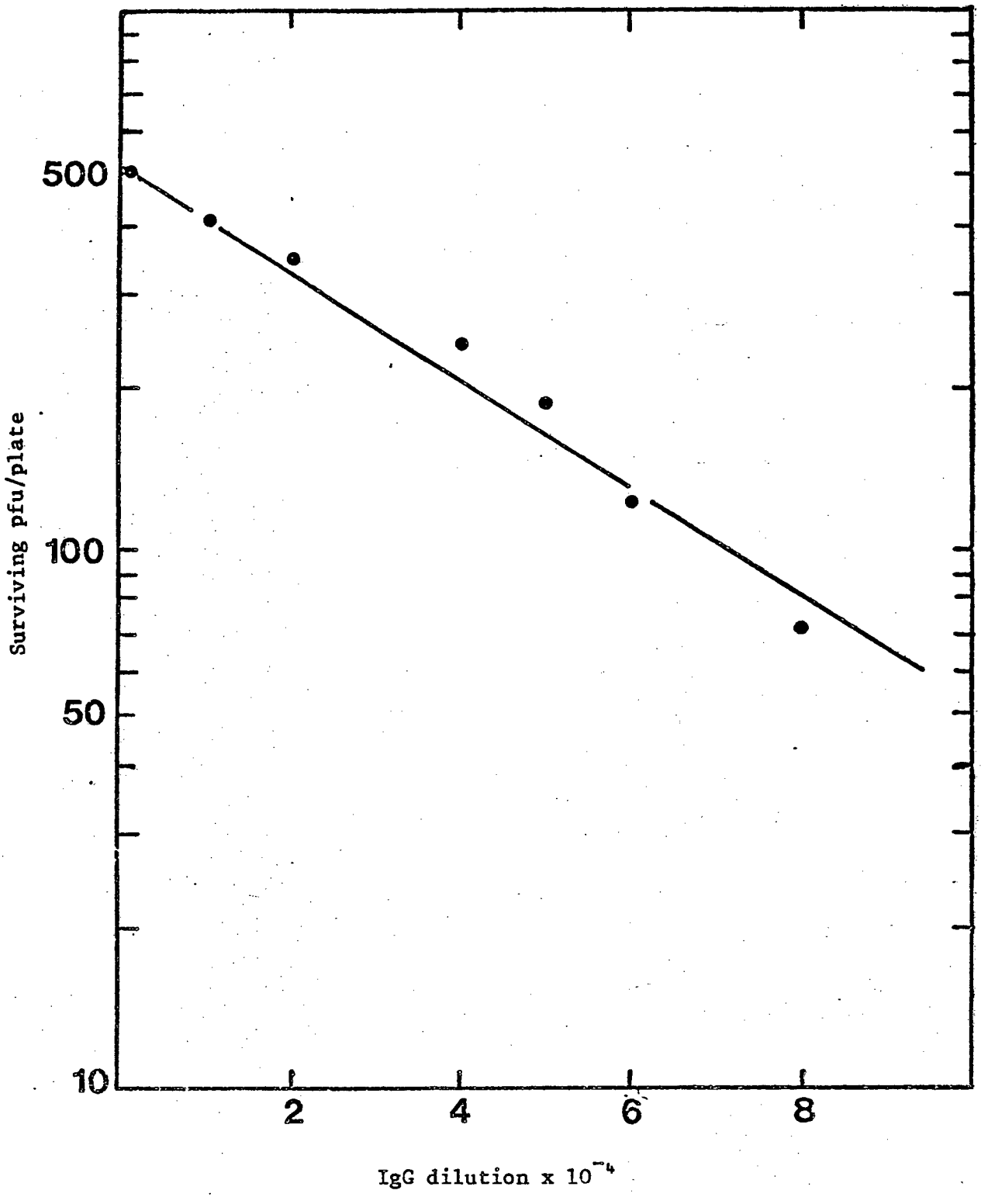

Fig. 9:

Inactivation of bacteriophage T4 by IgG prepared from anti-T4 Serum II. The concentration of the undiluted IgG was 2,87 mg/ml. Inactivation was for 5 h at 37°C. Surviving phage was assayed by the direct plating method.

2. REACTION OF DNP-MODIFIED BACTERIOPHAGE T4 WITH ANTI-DNP SERUM

(a) Preparation of DNP-modified Phage

Dinitrophenol-modified T4 phage was prepared by reacting the phage ($\pm 10^{10}$ pfu/ml) with dinitrobenzenesulphonic acid. The hapten coupling caused a decrease in infective titre of the phage preparation from $3,75 \times 10^{10}$/ml to $1,49 \times 10^{9}$/ml - a reduction of 96,03%. The surviving phage was shown to be sensitive to anti-DNP antibodies in subsequent inactivation experiments. Specificity of inactivation was shown by inhibiting the inactivation with free hapten.

(b) Kinetics of Inactivation

The neutralization of the DNP-modified phage was studied by following the kinetics of inactivation. Rabbit antiserum prepared against DNP-coupled rabbit serum albumin (anti-DNP serum), obtained from Miles Yeda (Rehovoth), was diluted and reacted with DNP-modified phage T4. Surviving pfu's at various times were assayed by the direct plating method and by the "decision" technique of Jerne and Avegno (1956).

Assay by the Direct Plating Method: Anti-DNP serum (containing 1,2 mg/ml antibody - according to the supplier's specifications) was diluted 10^{-2}, 10^{-3}, and 10^{-4} in P + G buffer pH 6,8. The kinetics of inactivation of DNP-phage with each antiserum dilution were determined. From each dilution, duplicate samples of 0,1 ml were removed and plated.

The average number of surviving pfu's at each time interval was calculated. To ascertain the number of hapten-phage conjugates added at time = 0, a value of 0,1 ml of control DNP-T4 was diluted in P + G buffer to the same extent as the phage treated with antiserum. The starting concentration of DNP-phage added to the 10^{-2}, 10^{-3} and 10^{-4} dilutions of anti-DNP serum was $1,49 \times 10^{7}$ pfu/ml. If no inactivation had occurred, 1490 plaques would be expected on the assay plates and this is the figure considered to be "Po", i.e. the plaques at time = 0. Fig. 10 shows the ratio P/Po plotted on a semi-log scale vs. time of sample withdrawal. If the neutralization reaction is obeying first order kinetics, the plot of log P/Po should be a straight line (Adams, 1959).

In the case of the experiment performed at 10^{-2} serum dilution, it is possible that the inactivation of DNP-T4 by anti-DNP serum proceeded according to first order kinetics, until approximately 90% of the hapten-phage conjugates was inactivated, but due to practical difficulties, no point before 1 min was available. At the lower serum concentrations (10^{-3} and 10^{-4} dilutions), the neutralization reaction is seen to deviate considerably from ideal first order kinetics, as shown by a marked decrease in rate of inactivation after $<$ 90% of the conjugates are neutralized. Although the kinetics of inactivation were not first order, the rate of reaction depended on the concentration of antibody present, as shown by the fact that

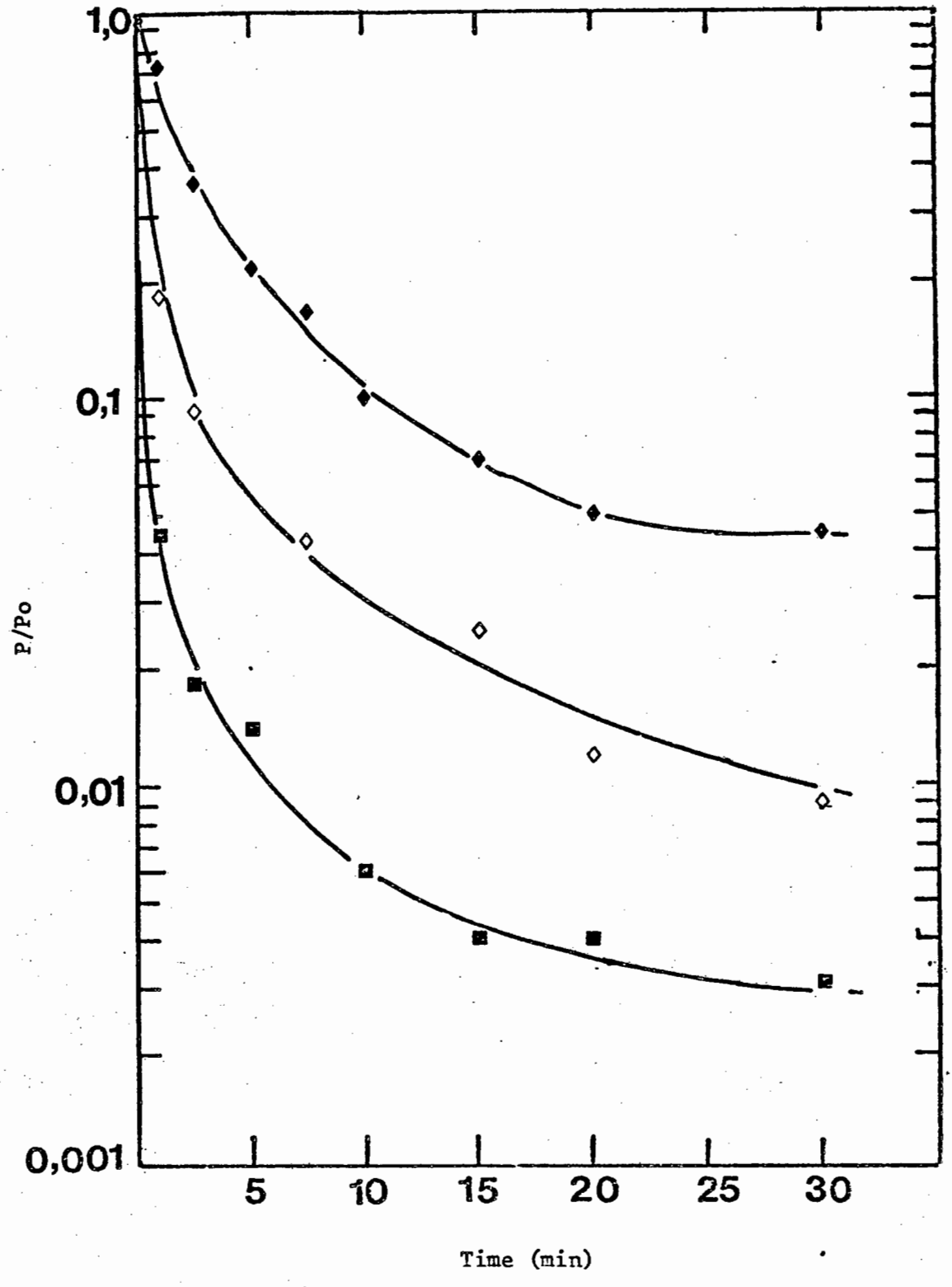

Fig. 10

Kinetics of inactivation of DNP-phage conjugates by anti-DNP serum (1,2 mg/ml antibody) at serum dilution of 10^{-4};◆ 10^{-3};◇ 10^{-2}.■ Assays were made by the direct plating method.

the 10^{-2} neutralization plot is much steeper than the plot for the 10^{-4} dilution.

In all three experiments, a "persistent fraction" capable of forming plaques after 40 minutes of contact with the anti-DNP serum was present, due to the fall off in rate of inactivation with time. In addition, many of the plaques on the assay plates were smaller than the usual T4r plaque - probably due to dissociation of the phage-antibody complexes on the assay plates (Jerne and Avegno, 1956).

If the neutralization of DNP-T4 were to be used for detecting low concentrations of anti-DNP, the sensitivity of the assay would suffer as a result of the presence of a persistent fraction. In the following experiment, an attempt was made to eliminate the decrease in inactivation rate with time, by assaying survivors by the "decision" technique.

Assay by the "Decision" Technique: Dilutions of 10^{-3} and 10^{-4} of anti-DNP serum in P + G buffer were used. Instead of being plated directly, the 0,1 ml sample from the dilution tube was added to 0,1 ml of an overnight culture of *E.coli* B and left for 10 minutes. At the end of this "decision" period, 2,5 ml of molten soft agar was added and the whole mixture was poured over a tryptone agar plate. When DNP-T4 phage which had not been in contact with anti-DNP serum were plated in this way, 1093

plaques (Po) were counted, instead of the 1490 obtained by direct plating - a reduction of 27%.

Fig. 11 shows the kinetic curves of inactivation for the two anti-DNP serum dilutions. The curves for these "decision" experiments should be compared with those obtained in the series of direct plating kinetic studies (Fig. 10). The "decision" technique has resulted in far steeper inactivation curves which follow first order kinetics over a wider range - to at least 99% in the case of the 10^{-3} dilution.

In the direct plating experiment the rate of inactivation was beginning to decrease at this dilution. No persistent survivors were found after 7½ minutes in the case of the 10^{-3} dilution and after 15 minutes with the 10^{-4} dilution. After these times, however, the rate of inactivation had already begun to decrease. Because of the steeper inactivation curves obtained by the "decision" technique, this method of plating was used for the preparation of a graph in which the antibody at different concentrations was incubated with DNP-T4 for a fixed period of time.

(c) Inactivation of DNP-T4 Phage for a Fixed Period of Time

The effect of different concentrations of anti-DNP antibody on the inactivation of DNP-T4 was studied by incubating hapten-phage conjugates with varying dilutions of

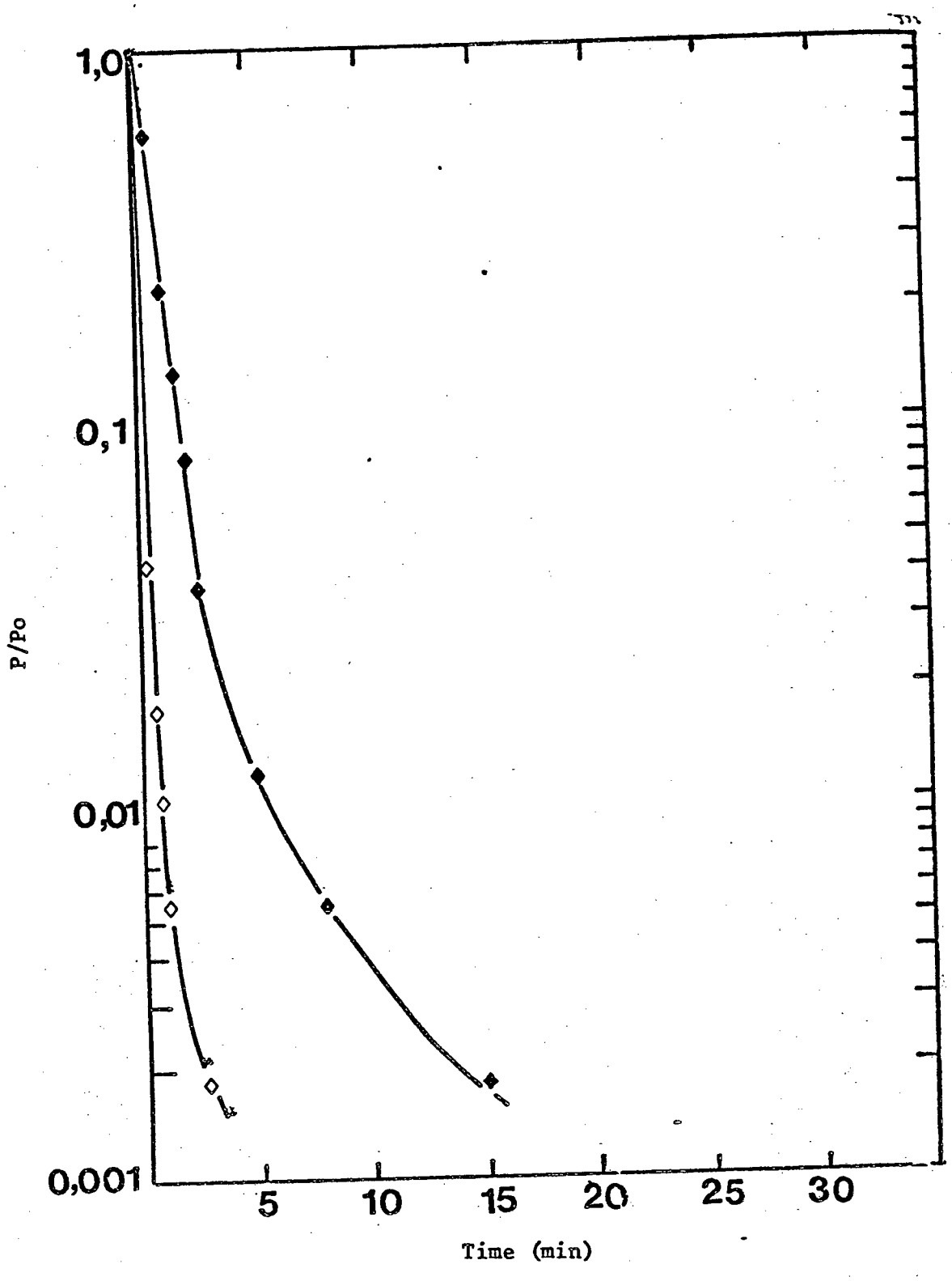

Fig. 11

Kinetics of inactivation of DNP-phage conjugates by anti-DNP-RSA serum containing 1,2 mg/ml antibody. Dilutions of 10^{-4};◆ 10^{-3}◇ Surviving phage were assayed by the "decision" technique.

anti-DNP serum. Since first order inactivation kinetics were observed when survivors were plated by the "decision" technique, this method was used to assay the survivors in this experiment. Anti-DNP serum was serially diluted in tenfold steps and 0,05 ml of each dilution was added to 0,05 ml of DNP-phage previously diluted to contain about 10^4 pfu/ml. As a control, 0,05 ml of DNP-T4 was incubated separately with 0,05 ml of buffer and 0,05 ml of normal rabbit serum.

The surviving DNP-phage were assayed by the "decision" technique as follows: After 60 minutes at 37°C each reaction tube received 0,1 ml of an overnight culture of *E. coli* B, and 10 minutes later, 0,1 ml of 10^{-2} dilution of anti-T4 serum III. Five minutes after this, 2,5 ml of molten soft agar was added and the entire mixture was plated. The experiment was done in duplicate and the points plotted in Fig. 12 were calculated from the average number of plaques from the duplicate platings. The average number of plaques (567) on the control plates was taken to represent no inactivation (100% survivors). The plot in Fig. 12 shows that fifty percent inactivation was brought about by a $6,5 \times 10^{-5}$ dilution of anti-DNP serum. Since the undiluted serum contained 1,2 mg/ml, the concentration of antibody at this dilution was $7,8 \times 10^{-5}$ mg/ml. As 0,05 ml were added, 50% inactivation of DNP-phage was caused by 3,9 ng of anti-DNP antibody.

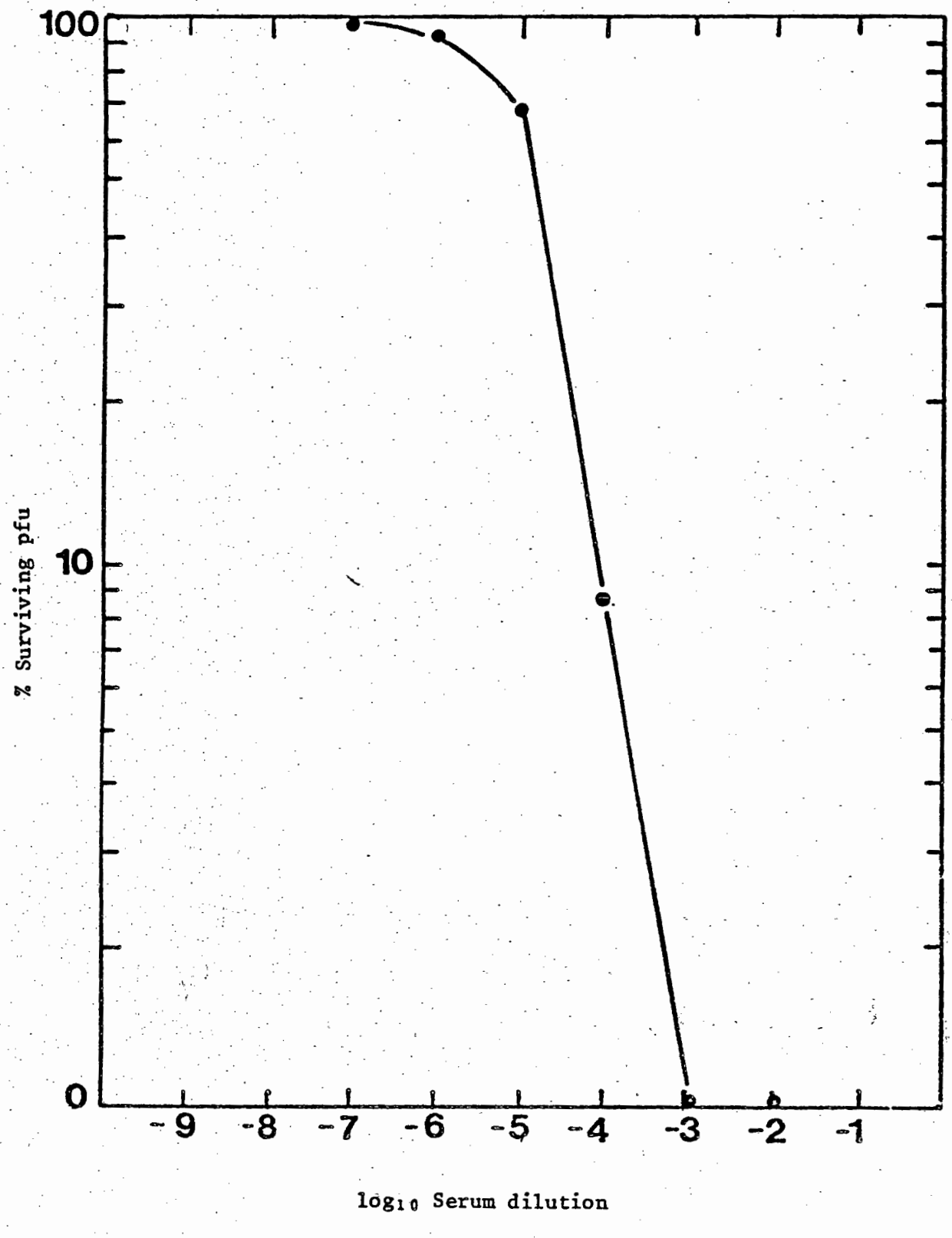

Fig. 12

Inactivation of DNP-phage by anti-DNP serum. The undiluted serum contained 1,2 mg/ml antibody. Inactivation was for 60 minutes at 37°C. Assays were made by the "decision" technique.

(d) Inhibition of Inactivation of DNP-phage with Free Hapten

To establish the specificity of the inactivation reaction, and at the same time to demonstrate that closely related antigens can be differentiated by inhibiting the inactivation of haptenated phage, 2,4 dinitrophenol (DNP) and 2,4,6 trinitrophenol (TNP) were each tested for their ability to inhibit the inactivation of DNP-phage by DNP antiserum. The haptens were dissolved in P + G buffer to a concentration of 1 mM. Serial dilutions were made, and 0,01 ml of each dilution was added to 0,05 ml of anti-DNP serum which had been diluted 10^{-4} in P + G buffer. After 30 minutes at 37°C, 0,04 ml of DNP-T4 conjugates (± 300 pfu) were added to each sample and inactivation was allowed to proceed for 60 minutes at 37°C, after which time the entire mixture was assayed by the "decision" technique. The reduction in the extent of inactivation of the conjugates by the anti-DNP serum was proportional to the concentration of hapten added. Fig. 13 shows the extent of inhibition of inactivation caused by DNP and the cross-reacting TNP hapten. In the absence of inhibitor, the plaque count of the DNP-phage was reduced by the anti-DNP serum (10^{-4} dilution) from 331 to 10 pfu (average of duplicate platings). Normal rabbit serum caused no inactivation of DNP-phage and non-coupled T4 phage was not inactivated by anti-DNP serum.

After pre-incubating the antiserum with 0,1 mM DNP, no reduction in the plaque count was obtained. TNP at the same

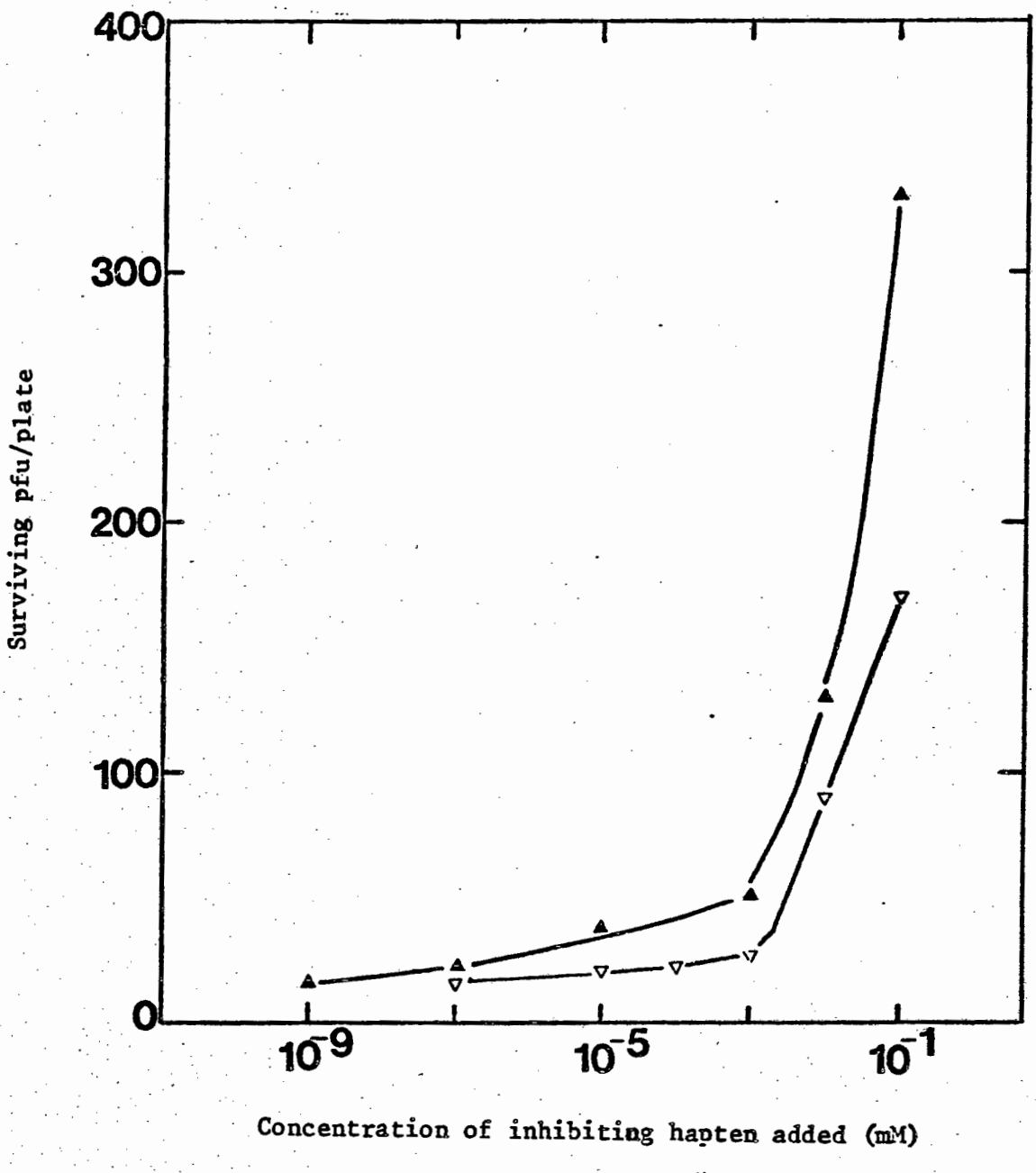

Fig. 13

Inhibition of inactivation of DNP-T4 conjugates by free hapten:-

▲ DNP

▽ TNP

concentration reduced the count to 170 plaques per plate, showing that TNP reacted less well with the DNP antibodies than the homologous DNP hapten.

In contrast to non-modified phage inactivation, the inactivation of the DNP-phage did not follow first order kinetics beyond 90% inactivation. The "decision" technique resulted in first order kinetics over a wider range of neutralization and it allowed the detection of antibody at the nanogram level. By inhibiting the inactivation, it was possible to detect low concentrations of free homologous hapten and, at the same time, distinguish between DNP and the cross-reacting TNP hapten.

3. REACTION OF LYSOZYME-MODIFIED T4 PHAGE WITH LYSOZYME ANTISERUM

The following experiments were performed with the object of preparing protein-phage conjugates which would be sensitive to anti-protein antibodies. Lysozyme-T4 conjugates have been obtained previously (Haimovich *et al.*, 1970a), and it was envisaged that information on the various factors affecting the preparation of protein-phage conjugates could be gained by using lysozyme in a series of phage coupling experiments. Glutaraldehyde was chosen as the bifunctional reagent since Haimovich *et al.* (1970a) recommended its use in view of its stability, efficiency, and ability to give reproducible results.

As a preliminary investigation on the factors affecting the sensitivity of protein-phage conjugates, a variety of conditions for preparing lysozyme-phage conjugates were tested. The effects of such factors as the temperature at which coupling is carried out, the purity of the glutaraldehyde, and variations in the relative amounts of reactants on the sensitivity of the conjugates to antiserum were determined. Glutaraldehyde was either freshly vacuum distilled or used straight from the supplier's bottle. P + G buffer pH 6,8 was the common diluent in these experiments.

The antisera used were prepared by injection of a single rabbit with lysozyme. It was bled at various times after injection. Different bleedings of the serum were used.

(a) Preparation of Lysozyme-phage Conjugates

(i) Coupling of Lysozyme to Phage Using Distilled Glutaraldehyde

In a preliminary experiment, glutaraldehyde distilled under vacuum, lysozyme and bacteriophage T4 suspension (absorbance at 260 nm = 100, equivalent to 10^{13} particles/ml) were mixed in the amounts shown in Table 3. After 60 minutes at 23°C the reaction was stopped by diluting the mixture to 5 ml with P + G buffer, pH 6,8. After overnight dialysis against buffer (gelatin omitted), the protein-phage conjugates were separated from the unreacted protein by differential centrifugation.

Table 3:

Preparation of lysozyme-phage conjugates. The table shows the amount of reagents added to 0,1 ml of T4 phage (10^{12} phage particles) and the % pfu which survived the conjugation reaction.

Sample No.	Lysozyme Concentration (0,1 ml)	% Vacuum distilled glutaraldehyde (0,025 ml)	Phage particles in supernatant (per ml) (a)	Surviving pfu (per ml) (b)	% surviving pfu
1	40 mg/ml	0,4	$1,2 \times 10^{9}$	$1,1 \times 10^{5}$	0,009
2	40	0,2	$3,1 \times 10^{9}$	$6,4 \times 10^{6}$	0,2
3	40	0,1	$1,7 \times 10^{10}$	$1,6 \times 10^{8}$	0,9
4	10	0,4	$6,8 \times 10^{9}$	$1,9 \times 10^{8}$	2,8
5	10	0,2	$3,4 \times 10^{9}$	$1,3 \times 10^{9}$	38,0
6	10	0,1	$1,1 \times 10^{9}$	$1,8 \times 10^{8}$	15,9

(a) Determined spectrophotometrically. If no loss had occurred during preparation, a concentration of 2×10^{11} would be expected.

(b) Determined by dilution and double agar layer assay.

After a final low speed (5 000 r.p.m. for 15 minutes) centrifugation, the absorbance of the supernatant fluids was determined at 260 nm and the concentration of phage particles was calculated. During the final centrifugation a pellet formed which probably consisted of phage-protein aggregates. The concentration of phage in the final supernatant was therefore reduced accordingly. In addition, some phage were lost due to the difficulty of recovering all of the preparation after dialysis. An initial volume of 0,1 ml of phage suspension containing 10^{12} particles was used for coupling. The final lysozyme-phage preparation contained 1,2 x 10^{9} to 1,7 x 10^{10} particles/ml. If no loss of phage had occurred during preparation, a total of 10^{12} particles in 5 ml, i.e. 2 x 10^{11} phage/ml would be expected. Thus 92 to 99% of the phage were physically lost during the preparation of the conjugates. After coupling, the number of viable phage was also considerably reduced, as shown in Table 3. There was no direct correlation between the concentration of protein or glutaraldehyde used and the percentage of phage which survived coupling.

The surviving pfu were tested for their sensitivity to lysozyme antiserum. A 0,1 ml sample containing about 200 pfu was incubated with 0,1 ml of a 10^{-2} dilution of antiserum. The serum, JAM II was previously shown not to neutralize native T4 phage at this dilution. The results summarized in Table 4 show that sample 4 was the most sensitive to inactivation by lysozyme antiserum.

Table 4:

Effect of anti-lysozyme serum (JAM II, diluted to 10^{-2}) on lysozyme-phage conjugates. Incubation was for 60 minutes at 37°C. The survivors were assayed by the complex inactivation method. Each result shown is the average of duplicate determinations.

Sample No.	Pfu/ml surviving incubation with		% serological inactivation
	0,1 ml buffer	0,1 ml serum	
3	$1{,}65 \times 10^8$	$1{,}54 \times 10^8$	7
4	$1{,}94 \times 10^8$	$4{,}95 \times 10^7$	75
5	$1{,}30 \times 10^9$	$1{,}09 \times 10^9$	17

The amounts of reactants used for the preparation of sample 4 were applied in the subsequent experiments.

(ii) Influence of Temperature and Purity of Glutaraldehyde on Conjugation of Lysozyme to Phage

TMVP is known to polymerize readily at room temperature, but not at 4°C. It would be desirable to coat phage with low molecular weight monomers of the virus protein, as large aggregates could spontaneously inactivate the phage without the need for antibody. The following experiments with lysozyme were performed to determine whether glutaraldehyde could be used as a bifunctional reagent at this temperature. The effect of the purity of the glutaraldehyde on conjugation was also studied.

A volume of 0,1 ml of lysozyme at 10 mg/ml, 0,025 ml of 0,4% glutaraldehyde, and 0,1 ml of T4 (10^{12} phage particles) were mixed and left at either 23°C or 4°C for 60 minutes. The lysozyme-phage conjugates were recovered and the percent surviving pfu was calculated. These conjugates were tested for their sensitivity to an anti-lysozyme serum (JAM III). A summary of the results is shown in Table 5.

There was again no correlation between the % pfu which survived conjugation and the conditions of reaction. The percentage serological inactivation for the various samples is also not strictly comparable as samples 7 and 8 were incubated with anti-serum for 120 minutes while inactivation was allowed to proceed for only 60 minutes in the case of samples 9 and 10. The results show that glutaraldehyde functions efficiently at 4°C and that there is no advantage to be gained from using distilled reagent.

Table 5:

Preparation of lysozyme-phage conjugates at 23° and 4°C using distilled and non-distilled glutaraldehyde. After reaction with lysozyme antiserum the surviving phage were assayed by the complex inactivation method.

Sample No.	Temperature (°C)	Glutaraldehyde	% Pfu surviving coupling	% Pfu inactivated by anti-lysozyme serum
7	23	distilled	7	52
8	4	distilled	66	29
9	23	non-distilled	57	37
10	4	non-distilled	27	94

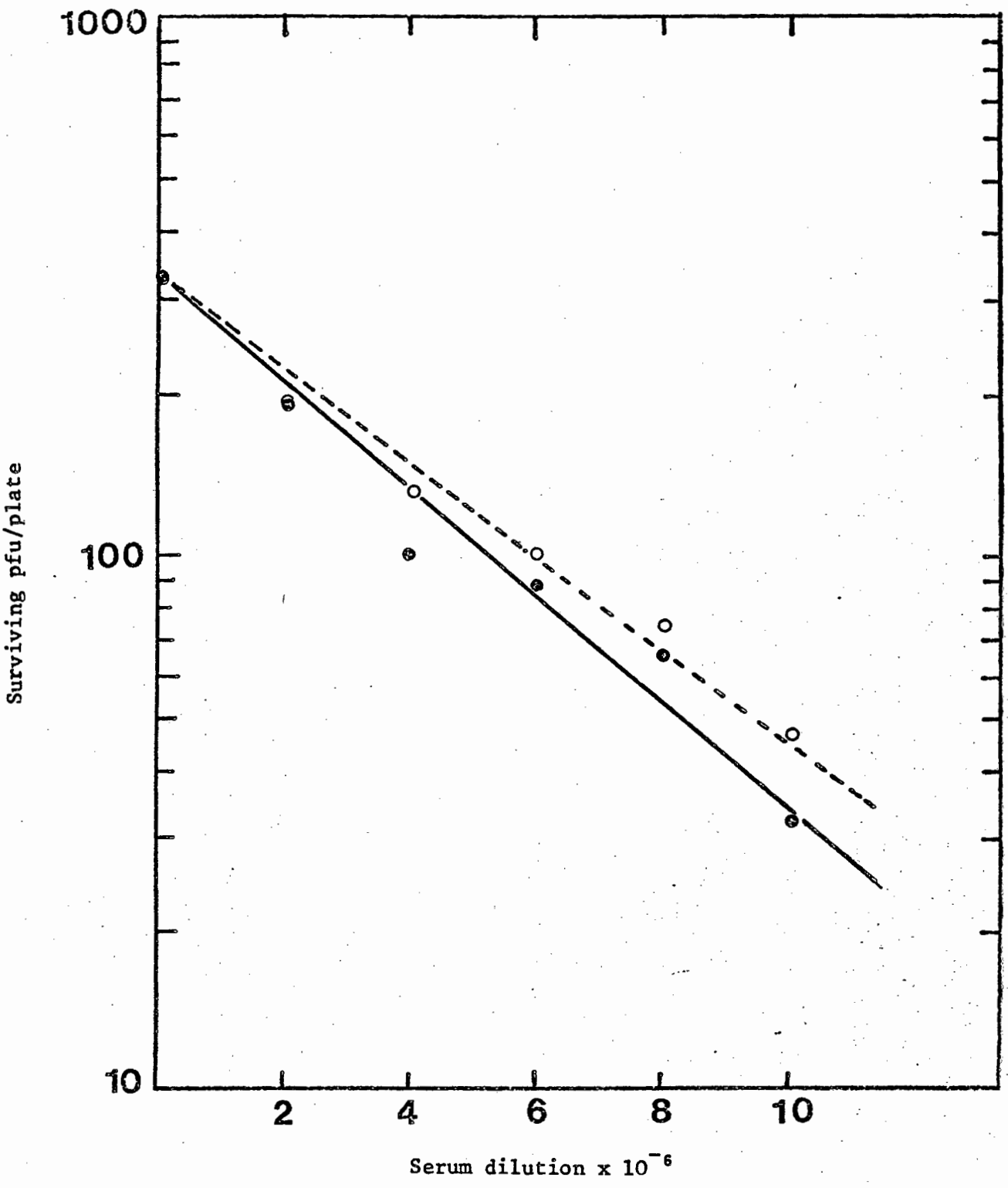

Fig. 14:

Inactivation of lysozyme-phage conjugates (Preparation 11) by lysozyme anti-serum. (JAM IV) Inactivation was for 2 h at 37°C. Surviving phage were assayed by:

● Complex inactivation method.

○ Direct plating.

(b) Inactivation of Lysozyme-phage Conjugates with Anti-lysozyme Serum.

The lysozyme-phage conjugates (Sample 11) used in this experiment were prepared at 4°C using non-distilled glutaraldehyde. Lysozyme (0,1 ml at 10 mg/ml) was mixed with 0,1 ml of T4 phage (10^{12} particles) in P + G buffer pH 6,8 and 0,02 ml of 0,5% glutaraldehyde was added. After 60 minutes at 4°C followed by overnight dialysis, the conjugates were separated from the unreacted protein.

Dilutions of antiserum JAM IV (0,2 ml) and lysozyme-phage conjugates (0,2 ml corresponding to about 300 pfu) were mixed and incubated for 2 h at 37°C. The extent of inactivation was determined by direct plating assays and the complex inactivation method. For the latter method, 0,05 ml of a 1/50 dilution of goat anti-rabbit serum was added to the reaction tubes and after 10 minutes incubation, molten soft agar and bacteria were added. As seen in Fig. 14 the complex inactivation method and the direct plating assays gave similar numbers of survivors at each dilution. In addition, it was found that the lysozyme-phage conjugates were not inactivated by incubation in P + G buffer at pH 6,8 for 2 hours, and that no significant drop in titre occurred over a period of 4 weeks if they were stored at 4°C in buffer.

(c) Inhibition of Inactivation of Lysozyme-phage Conjugates

The specificity of the inactivation of lysozyme-modified

phage was shown by inhibiting the neutralization reaction with lysozyme. An anti-lysozyme serum (JAM II) at a dilution of 10^{-4} decreased the number of viable conjugates from 640 to 380 in 2 hours at 37°C. This inactivation was inhibited as follows: Lysozyme (0,025 ml) at concentrations of 0,1 and 0,01 mg/ml was added to 0,075 ml of the 10^{-4} antiserum dilution and incubated at 4°C for 24 hours. After incubation with 0,1 mg/ml lysozyme, the antiserum caused no inactivation of the phage conjugates. The addition of 0,01 mg/ml lysozyme inhibited the inactivation slightly less as the pfu were decreased from 640 to 522 per plate. As the antiserum also caused no inactivation of non-coupled phage, it may be concluded that the inactivation of lysozyme-modified phage was specific and was caused by anti-lysozyme antibodies.

To summarize, the results show that lysozyme-phage conjugates can be prepared with glutaraldehyde as a bifunctional reagent, and that the most sensitive conjugates are obtained with non-distilled glutaraldehyde which is known to consist mainly of polymers (Sachs and Winn, 1970). The graph describing the logarithm of % survivors as a function of antibody concentration was linear, with the complex inactivation method showing very little difference compared with direct plating. The inactivation could be inhibited by lysozyme which shows that the inactivation was caused by anti-lysozyme antibodies. The success of conjugation at 4°C demonstrated that it is feasible to perform the coupling reaction at this temperature,

an important consideration for the subsequent work with TMVP, since this protein tends to polymerize at room temperature.

4. REACTION OF TMVP-MODIFIED T4 BACTERIOPHAGE WITH ANTI-TMVP SERUM

The serology of TMV and its subunits has been extensively studied. Methods such as radioimmunoassay (Benjamini et al., 1968), passive haemagglutination (Anderer et al., 1971) and precipitation in gel and liquid (Van Regenmortel, 1967) have been used. There are numerous strains and mutants of the virus which may be serologically distinguished (Hennig and Wittman, 1972) and several of these are readily available. As the amino acid sequence is known, the TMV subunit is a useful subject for immunochemical studies. In this section attempts to exploit the extreme sensitivity of the modified phage technique will be described. By inhibiting the serological inactivation of the TMVP-phage conjugates, it was possible to differentiate proteins prepared from various TMV strains, as well as to demonstrate immunological activity in tryptic peptides prepared from the protein.

(a) Reaction of TMVP with N-bromosuccinimide (NBS)

NBS-TMVP was prepared as described in Chapter III.

Treatment of TMVP with n-bromosuccinimide (NBS) converts the tyrosine residue at position 139 to dibromotyrosine,

causing a shoulder in the TMVP ultraviolet absorbance spectrum at 310 - 315 nm (Ohno et al., 1972). Figs. 15 (a) and (b) show the u.v. absorbance spectra obtained with NBS-TMVP and untreated protein. The shoulder visible at 305 - 318 nm with the NBS-TMVP is absent in the untreated preparation. The $E^{0,1\%}_{281}$ of NBS-TMVP was reported by Inoue et al. (1974) to be 1,13 and this value was used in spectrophotometric determinations of the protein concentration.

At pH 7,2 and at concentrations greater than 5 mg/ml, normal TMVP sediments as a mixture of 4S (A-protein), 8S (aggregated subunits) and 20S (discs) components.

At higher concentrations the proportion of aggregated protein is increased (Durham and Klug, 1971). The NBS treatment was used to help prevent polymerization of the viral subunits into discs or other high molecular weight aggregates, so that when the protein was coupled to phage, the phage particle would be coated with only smaller components, preferably monomers. Large discs and other aggregates could cause spontaneous inactivation of the particle by occluding the "critical site". The sedimentation behaviour of the NBS-protein in conditions similar to those which were to be used in phage coupling experiments was examined by ultracentrifugation in a Beckman Model E analytical ultracentrifuge. At 20°C and in 0,05 M phosphate buffer pH 7,5, NBS-TMVP sedimented as a single component, seen as a single sharp peak in Fig. 16. The sedimentation

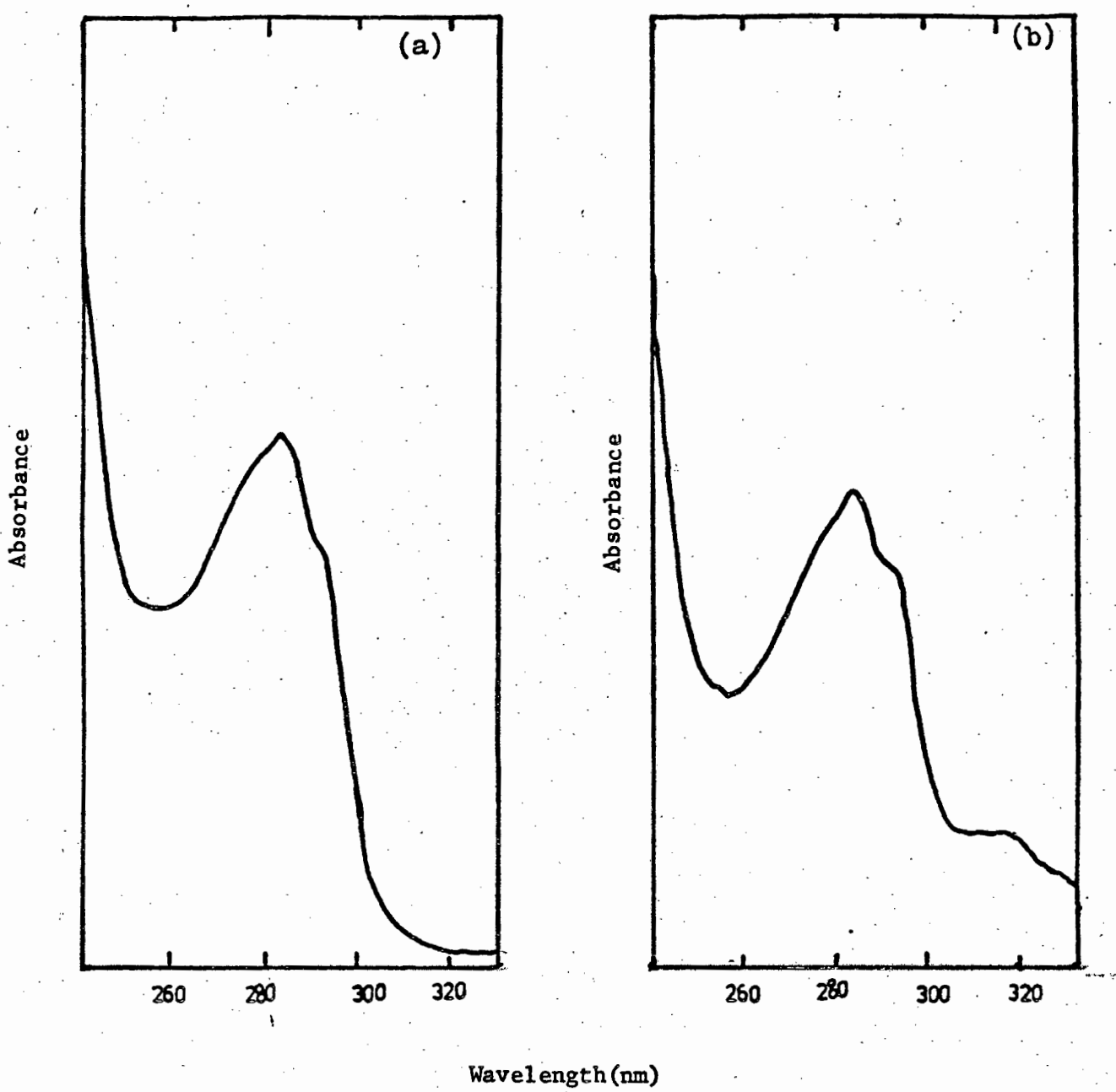

Fig. 15:

Ultraviolet absorbance spectra of (a) TMVP vulgare, (b) TMVP vulgare treated with n-bromosuccinimide (NBS-TMVP) in 0,05 phosphate buffer pH 7,5.

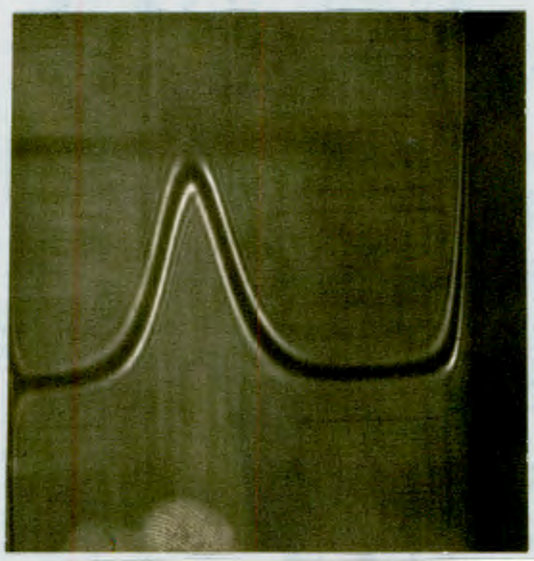

Fig. 16 : Sedimentation pattern of NBS-treated TMVP at a concentration of 16 mg/ml in 0,05 M phosphate buffer, pH 7,5. The photograph was taken 64 min after the rotor reached a speed of 56 000 r.p.m. The sedimentation coefficient was 3S.

coefficient was calculated to be approximately 3S when protein at concentrations of 3,47 mg/ml or 16 mg/ml were examined. No 20S or other aggregates were detected in the samples which were examined within two days of their preparation. In one preparation which had been allowed to stand at 4°C for over two weeks, a faster sedimenting component was however apparent. The 3S component probably consists mainly of monomers in equilibrium with some oligomers.

(b) Preparation of TMVP-phage Conjugates

Haimovich et al., (1970a) have found that glutaraldehyde is a useful bifunctional reagent for preparing protein-phage conjugates and, in the previous section, good results have been obtained by this method in the case of lysozyme-phage conjugates. Attempts to prepare TMVP-T4 phage conjugates by the same method failed, and led to the investigation of two other bifunctional reagents: tolylene-2,4-diisocyanate (TDIC) and bis-diazobenzidine (BDB).

(i) Use of Glutaraldehyde as Bifunctional Reagent

Attempts to couple the protein subunits of TMV to T4 bacteriophage using glutaraldehyde were made under a wide variety of conditions, but no TMVP-phage conjugates sensitive to anti-TMVP antibodies could be produced. Phage that survived the coupling process were allowed to react

with an anti-TMVP serum and were assayed by the direct plating, the "decision" technique and the complex inactivation methods. In a series of experiments the following conditions were used :-

Protein Concentration: Freeze-dried TMVP was dissolved in water and P + G buffer was added to a final concentration of 0,05 M. Undissolved protein was removed by low speed centrifugation (5 000 r.p.m. for 10 minutes) in a Sorvall SS34 rotor. Only non-NBS-treated protein was used. In this way protein solutions of up to 20 mg/ml could be obtained. Such protein was used in coupling experiments at concentrations between 5 and 20 mg/ml. At 5 mg/ml and at 4°C the protein sedimented in the analytical ultracentrifuge as a single component of sedimentation coefficient 4 - 8S.

pH: Coupling to bacteriophage T4 was tried in P + G buffer at pH 6,8; 7,0 and 7,5. It was hoped that the higher pH would minimize aggregation of the protein subunits (Durham and Klug, 1971) and allow a more even "coating" of the phage with protein.

Temperature: Attempts to couple TMVP to phage were made at 4°C and 23°C.

Glutaraldehyde: Both vacuum distilled and commercial glutaraldehyde were used at concentrations of 0,01% to 2,5%

(final concentration in the reaction mixture). The percent viable phage which survived coupling depended upon the glutaraldehyde concentration: 2,5% glutaraldehyde inactivated all the phage, while with 0,05% glutaraldehyde only 0,05% of the pfu remained.

Protein: Protein was also prepared by the cold acetic acid method (Fraenkel-Conrat,1957) from the mutant virus CP-415 which has an extra lysine residue at position 140 compared with the wild-type (Hennig and Wittman, 1972). As glutaraldehyde reacts with free amino groups of proteins (Avrameas and Ternynck, 1969), it was hoped that the use of this mutant protein would lead to more efficient coupling to the phage compared to the wild-type protein. However, in a large number of experiments no TMVP-phage conjugates sensitive to anti-TMVP serum were produced. The "decision" technique and the complex inactivation methods of assaying survivors did not enhance the sensitivity of the phage to anti-TMVP serum.

(ii) Use of Tolylene-2,4-diisocyanate (TDIC) as Bifunctional Reagent

Because of its reduced tendency to aggregate, NBS-treated protein was used in the following experiments:

To 0,1 ml samples (17 mg/ml protein) of NBS-treated TMVP (NBS-TMVP) was added 0,1 ml bacteriophage T4 (10^{13} pfu/ml). To each sample was added 0,3 ml TDIC diluted 0,03 - 3,0%

in dioxane (Merck, Darmstadt), and after thorough mixing the reaction was allowed to proceed at 24°C for 60 minutes. It was stopped by dilution with P + G buffer pH 7,5 and after dialysis against the same buffer with the gelatin omitted, the phage were separated by differential centrifugation. Table 6 shows the percentage of viable pfu in the final supernatant solutions.

Table 6:

Coupling of NBS-TMVP to phage T4 using TDIC as bifunctional reagent.

Sample No.	% TDIC added (0,3 ml)	% pfu in final supernatant solution
1	3	0,09
2	0,3	1,01
3	0,03	46,08

When the surviving conjugates were tested for their ability to be inactivated by anti-TMVP serum, no inactivation was obtained (Table 7).

Table 7:

Reaction of NBS-TMVP-phage conjugates with anti-TMVP serum. The phage were assayed by the direct plating method after 180 minutes at 37°C with

(a) P + G buffer pH 7,5

(b) JOR XIV anti-TMVP serum diluted 1/100.

Sample No.	Dilution at which plated	Pfu Surviving	
		(a)	(b)
1	10^{-4}	740	753
2	10^{-6}	121	110
3	10^{-8}	46	40

It is apparent that the higher the concentration of TDIC added, the less phage survived the reaction.

Although TDIC has been used successfully for preparing various protein-phage conjugates (Haimovich et al., 1970a), no TMVP-phage conjugates sensitive to anti-protein serum could be obtained under the conditions tested here.

(iii) Use of bis-diazobenzidine (BDB) as Bifunctional Reagent

Both glutaraldehyde and TDIC react predominantly with the free amino groups of proteins and the failure of both reagents to couple TMVP to phage may be due to the fact that the N-terminal amino acid of TMVP is acetylated (Narita, 1958) and that there are only two lysine residues in its peptide chain. It was, therefore, decided to try to couple TMVP to phage with a bifunctional reagent which reacts with a wider variety of chemical residues. Bis-diazobenzidine has been used for preparing phage conjugates and for coupling proteins, including TMV to red blood cells for haemagglutination assays (Anderer and Ströbel, 1972; Anderer et al., 1971). BDB acts via tyrosyl, histidyl, amino and carboxyl groups of proteins (Nisonoff, 1967) and although TMVP contains no histidine, the number of residues that are able to participate in the coupling reaction is larger than with glutaraldehyde or TDIC. BDB was tested with both untreated TMVP and NBS-TMVP.

Untreated TMVP: Bis-diazobenzidine was prepared as described in Chapter II by the method described by Herbert (1973) and was diluted 1/15 in phosphate buffer pH 7,5. To a mixture of 0,1 ml of phage (10^{13} pfu/ml) and 0,1 ml of TMVP in P + G buffer pH 7,5 (concentrations as in Table 10), was added 0,03 ml of dilute BDB. After 15 minutes at 37°C, 5 ml of P + G buffer pH 7,5 were added to stop the reaction by dilution. Conjugates were recovered after dialysis by differential centrifugation. One hundred-

fold serial dilutions of the final supernatant fluids containing the conjugates were made and duplicate 0,1 ml samples of each dilution were incubated with 0,1 ml anti-TMVP serum diluted 1/10 and 1/100. After 120 minutes at 37°C, the survivors were assayed by the direct plating method. The results are shown in Table 8. No inactivation was obtained when the TMVP antiserum was allowed to react with non-coupled T4 phage.

Table 8:

Sensitivity of TMVP-phage conjugates (prepared with BDB) to anti-TMVP serum. Phage were assayed by the direct plating method after 120 minutes at 37°C.

TMVP concentration used for preparing conjugates. mg/ml	% Pfu surviving conjugation.	% Pfu surviving after reaction with		
		(a)	(b)	(c)
1,25	0,07	100	ND	100
10,0	0,5	100	71	82

ND = not determined

a = P + G buffer pH 7,5

b = Anti-TMVP JOR IX diluted 1/10

c = Anti-TMVP JOR IX diluted 1/100.

The results in Table 8 indicate that in the case of the preparation made with an original concentration of 10 mg/ml TMVP about 18% of the pfu were inactivated by a 1/100 dilution of antiserum.

NBS-TMVP: After it had been established that TMVP-phage conjugates sensitive to anti-TMVP antibodies could be prepared using BDB as a bifunctional reagent, further coupling experiments were performed with a variety of concentrations of BDB and protein in order to obtain an optimally sensitive preparation of protein-phage conjugates.

NBS-TMVP was used as this was shown to remain in solution as small oligomers up to a concentration of 17 mg/ml. In all the coupling experiments 0,1 ml phage at a concentration of 10^{13} pfu/ml was mixed with 0,1 ml NBS-protein at different concentrations, and various amounts between 0,03 and 0,1 ml BDB were added. After 15 minutes at 37°C the mixture was diluted, dialysed and the protein-phage conjugates were separated by differential centrifugation. The conjugates were diluted 100-fold and 0,1 ml samples of each dilution were allowed to react at 37°C with 0,1 ml of a 1/100 dilution of anti-TMVP serum (JOR XIV). Survivors were assayed by the direct plating method. Details of the conditions used for preparation of the conjugates, and the resulting sensitivity of the conjugates to anti-TMVP serum are shown in Table 9.

As the various conjugates were prepared at different times, different BDB preparations were used. BDB is a highly unstable and reactive molecule and different preparations may vary in their coupling efficiency.

method indicated that it is possible to increase the sensitivity of the assay by incubating for longer than the 2 hours used in the initial tests.

(ii) Effect of Polyethylene Glycol on the Inactivation of TMVP-phage Conjugates.

Franěk and Doskočil (1975) found that when DNP-modified phage was allowed to react with anti-DNP serum in the presence of 8% polyethylene glycol of molecular weight 6 000 (PEG 6 000), the number of survivors measured by the complex inactivation method was decreased. The sensitivity of the DNP-phage to the antiserum was enhanced and DNP antibodies could be detected at 10 - 30 fold lower concentrations than when the inactivation was carried out in buffer.

In order to determine whether the PEG 6 000 would improve the sensitivity of the protein-phage conjugates to antiserum, 0,2 ml of an anti-TMVP serum (JOR XIV) at several dilutions was incubated with 0,1 ml of NBS-TMVP-phage conjugates (± 400 pfu/0,1 ml) and 0,2 ml of 20% PEG 6 000 (final concentration 8%). After 5 h at 37°C, the mixture was plated by the complex inactivation method. Fig. 18 shows a comparison between the pfu that survived inactivation in the presence and absence of PEG.

The percentage of phage conjugates surviving at each serum dilution was markedly decreased by the presence of the PEG.

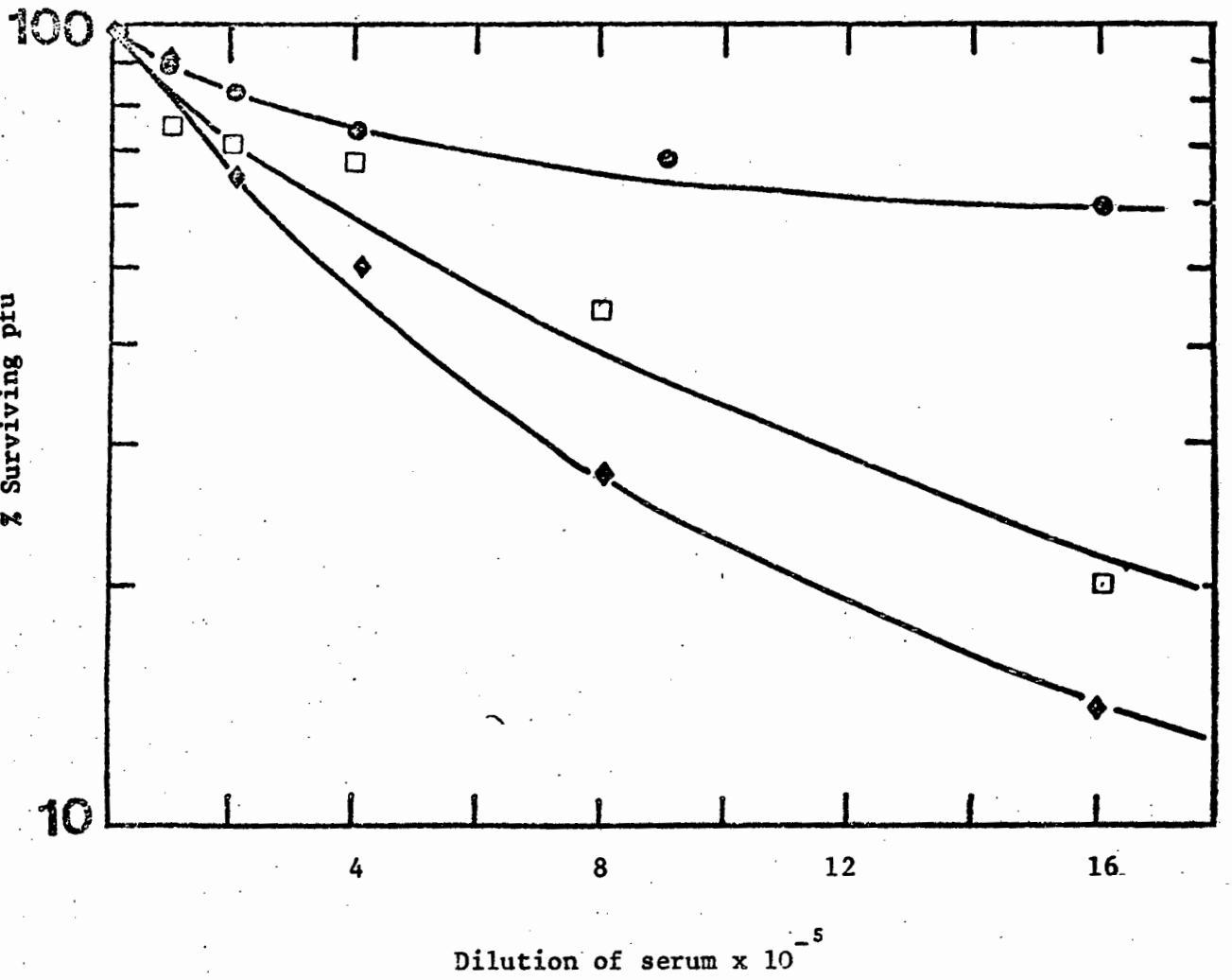

Fig. 18:

Inactivation of phage conjugates by anti-TMVP serum JOR XIV:

● In 0,05 M P+G buffer pH 7,5.

◆ In 0,05 M P+G buffer plus 8% PEG.

□ In 0,005 M P+G buffer plus 8% PEG.

Surviving pfu were assayed by the complex inactivation method.

At a serum dilution of 10^{-4} the percent surviving pfu was reduced from 65% to 22%. Because of this increase in inactivation, PEG 6 000 was added to the reaction mixture in all subsequent experiments.

(iii) Stability of TMVP-phage Conjugates

Not all protein-phage conjugates have been found to be stable over long periods of time. In most cases, however, even when the titre was reduced, the sensitivity of the surviving conjugates to antiserum was not affected (Haimovich et al., 1970a). Attempts were, therefore, made to determine the extent to which the TMVP conjugates lost their activity and sensitivity with time. Since it is known that PEG can inactivate phage (Follansbee et al., 1974), the effect of 8% PEG for 5 h at 37°C on the viability of the phage was determined. The modified phage were found to be reduced in titre from 4,8 x 10^3/ml to 2,3 x 10^3/ml. For this reason, the percentage serological inactivation was determined in each inactivation experiment from the ratio of pfu surviving in the serum/phage/PEG mixture and a control in which the inactivating serum was replaced by normal rabbit serum in P + G buffer. The control was incubated and plated in the same way as the experimental samples. In kinetic experiments the Po value was obtained from a 0,1 ml sample of conjugates of the same titre as that added to the serum, but which was incubated for the duration of the experiment at 37°C with

normal rabbit serum. This control was diluted and plated identically to the serologically inactivated phage.

The conjugates were stored in 0,05 M P + G buffer pH 7,5 at 4°C and at a titre of 10^7 to 10^8 pfu/ml. Dilutions were made to about 5 x 10^3 pfu/ml for use in inactivation experiments, but this titre was found to drop to less than 4 x 10^3 after 10 days at 4°C. These conjugates were thus less stable than some of the conjugates prepared by Haimovich et al. (1970a). However, in spite of the decrease in active phage titre with time, the sensitivity of the conjugates to TMVP antiserum was not reduced after 4 months of storage. A 1/6400 dilution of JOR XIV consistently reduced the number of pfu by 75 to 85% in numerous experiments over a period of 4 months. During the same period the titre of the stock solution dropped from 4,4 x 10^7 to 8,8 x 10^6 pfu/ml, a reduction of 80%.

(iv) Reaction of Modified Phage with Different Antisera

A variety of TMVP antisera from different rabbits, an antiserum directed against the protein prepared from the U2 strain of TMV and an antiserum against whole TMV were tested for their ability to inactivate the protein-phage conjugates. The anti-TMVP sera were JOR XIV, MAL VII and RIC IX. The antiserum MAL VII was raised against TMVP which had been treated with formalin in an attempt to prevent aggregation and to obtain only anti-subunit antibodies.

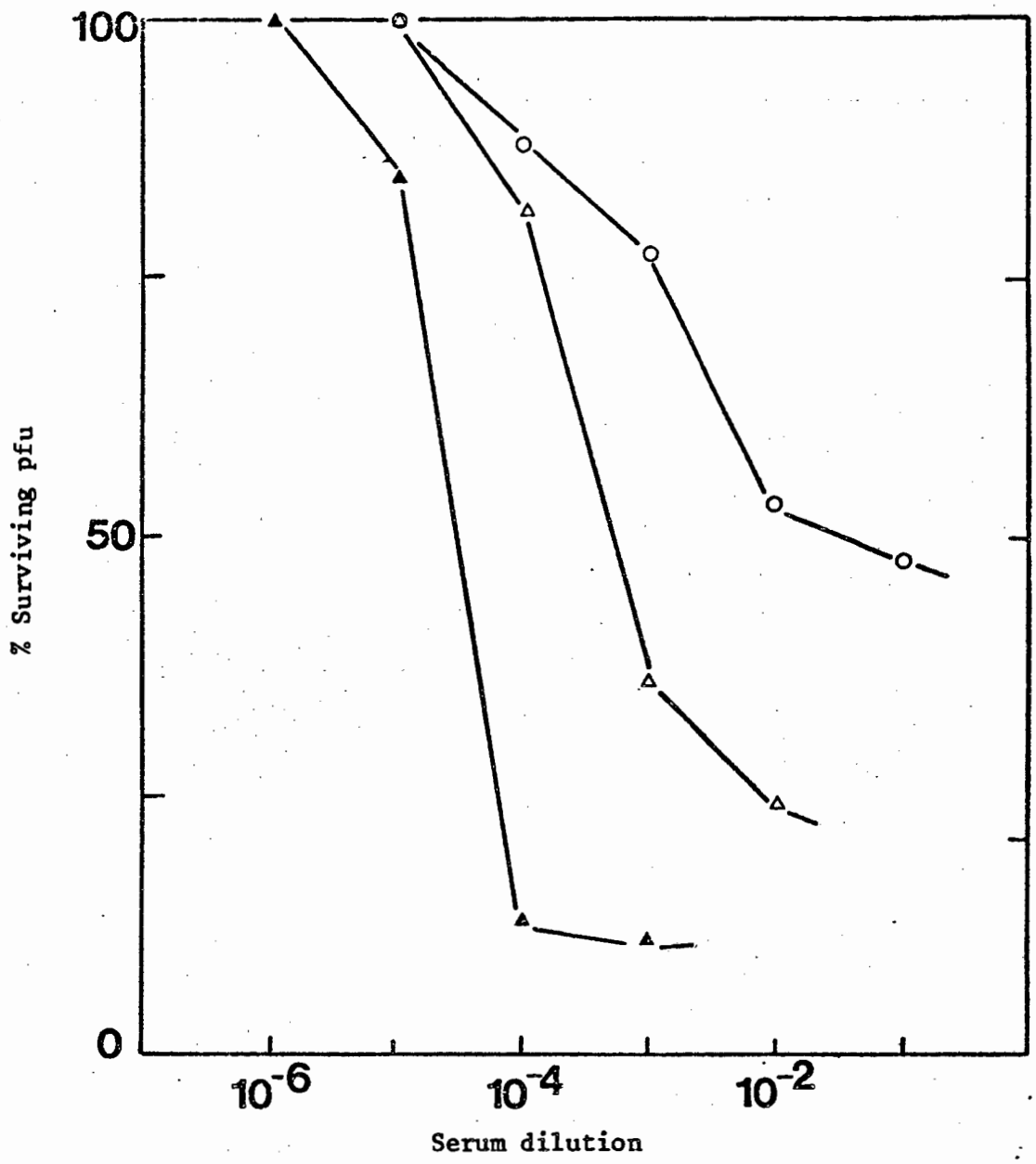

Fig. 19:

Inactivation of TMVP-phage conjugates by anti-TMVP sera from different rabbits:

△RIC IX

○MAL VII

▲ JOR XIV

MAL VII was a serum raised against formalinized TMVP.

The serum AMI XV was raised against whole TMV, but due to breaking up of the virus in the animal, some anti-TMVP antibodies are always present in such sera.

In these inactivation experiments, antiserum was incubated at different dilutions with the phage conjugates for 5 hours. In all subsequent experiments, the phage conjugate preparation referred to as sample 7 in Table 9 was used.

Anti-TMVP Sera: Sera from 3 different rabbits were compared for their ability to inactivate TMVP-T4 phage conjugates. Serial 10-fold dilutions of each serum were made in P + G buffer pH 7,5 and 0,1 ml of each dilution was mixed with 0,1 ml of protein-phage conjugates (diluted to $\pm$ 5 000 pfu/ml) and 0,1 ml of PEG diluted to give 8% final concentration in the reaction mixture. After 5 h at 37°C the phage were assayed by the complex inactivation method. The sera differed markedly in their inactivating abilities, due probably to variations in antibody titre and affinity. JOR XIV caused a greater decrease in pfu over a smaller serum dilution range than the other two sera (Fig. 19).

Of the three sera tested for their inactivating ability, JOR XIV was the most efficient. A dilution series in smaller steps was made and 0,2 ml of each dilution was mixed with 0,1 ml of TMVP-phage conjugates, 0,1 ml P + G buffer pH 7,5 and 0,1 ml of 40% PEG (final concentration

8%) and incubated for 5 h at 37°C. When plated by the complex inactivation method, 50% of the pfu were inactivated by a 4 x 10^{-5} dilution of this serum (Fig. 20). Although use of low ionic strength buffer has been shown to enhance inactivation (Andrieu et al., 1974), no improvement in sensitivity was apparent when a 0,005 M P + G buffer was used (Fig. 18).

Anti-TMV Serum: A serum directed against the whole tobacco mosaic virus was also tested in the same way for its ability to inactivate the phage conjugates. Fig. 21 shows the inactivation at various serum dilutions. This serum at a dilution of 10^{-4} inactivated 50% of the conjugates, but was less efficient than the best anti-TMVP serum.

Anti U2-P sera: Five antisera raised against the protein of the TMV strain U2 (U2-P), were tested for their ability to inactivate the TMVP-phage conjugates. All the sera were diluted 1/1000 and 0,1 ml of each was allowed to react for 5 h at 37°C with 0,1 ml of TMVP-phage (± 120 pfu). The experiment was carried out in 0,005 M P + G buffer pH 7,5 instead of the usual 0,05 M concentration, because the use of low ionic strength buffer has been shown to enhance inactivation (Andrieu et al., 1974). Table 10 shows that the sera COL XXXVI and FRE XXXI were the most efficient inactivators. Some sera have been shown to contain "natural" anti-phage antibodies (Jerne, 1956; Haimovich et al., 1970c) and as COL XXXI was found capable of inactivating non-modified phage, the inactivation of the conjugates

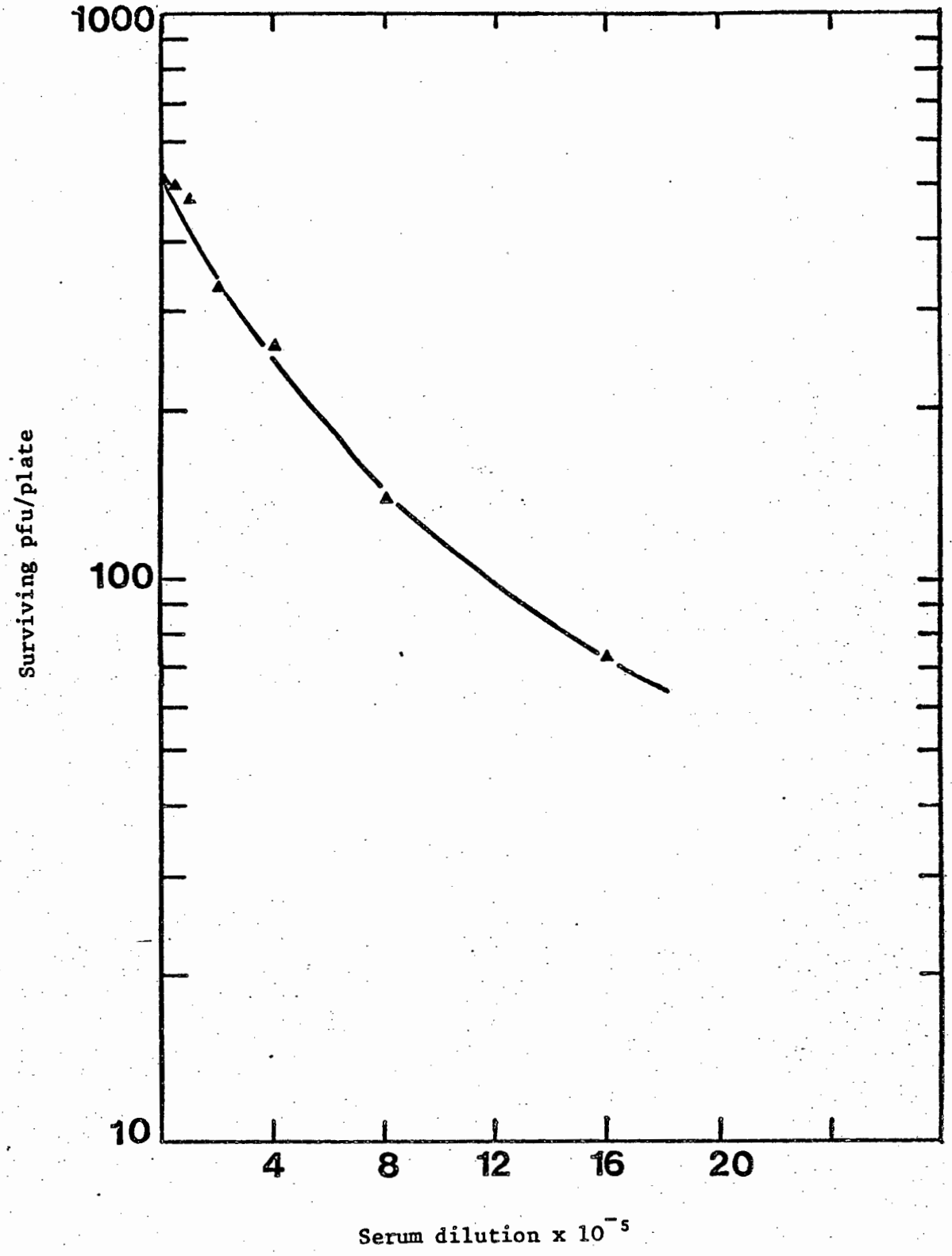

Fig. 20:

Inactivation of TMVP-phage conjugates with anti-TMVP JOR XIV. Inactivation was for 5 h at 37°C in the presence of 8% PEG 6 000. Survivors were assayed by the complex inactivation method.

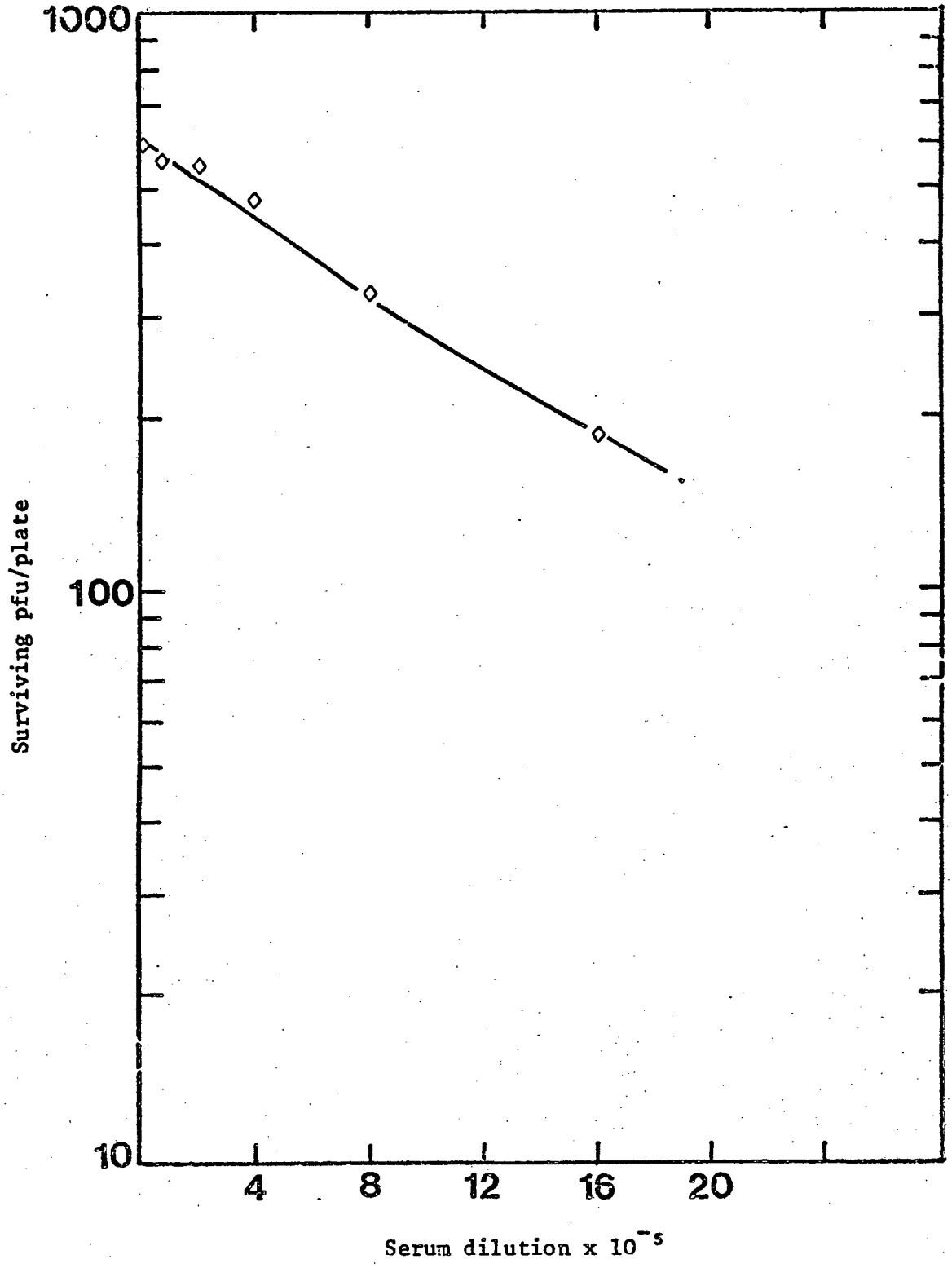

Fig. 21:

Inactivation of TMVP-phage conjugates by anti-TMV serum AMI XV. Inactivation was for 5 h at 37°C in the presence of 8% PEG 6000. Surviving conjugates were assayed by the complex inactivation method.

by this serum may not have been due to the anti-protein antibodies and studies with this serum were not pursued.

Table 10:

Reaction of anti-U2-P sera with TMVP-phage conjugates. Surviving phage were plated by the complex inactivation method.

Serum	% Inactivation
SWA XV	11
COL XXXVI	37
IGO XXVI	0
MAR XXVIII	4
FRE XXXI	62

Fig. 22 shows the results obtained with FRE XXXI in 0,005 M buffer compared with inactivation under the usual conditions. The pfu's surviving at a serum dilution of 10^{-3} were reduced by an additional 30% in the low ionic strength buffer. This enhancement of inactivation was not observed with the anti-TMVP serum JOR XIV (Fig. 18).

In this section, inactivation of TMVP-phage conjugates has been described. The conjugates could be neutralized by TMVP antiserum as well as by serum directed against the intact virus. The cross-reaction of U2-P antiserum with TMVP (vulgare) was illustrated by the inactivation of the

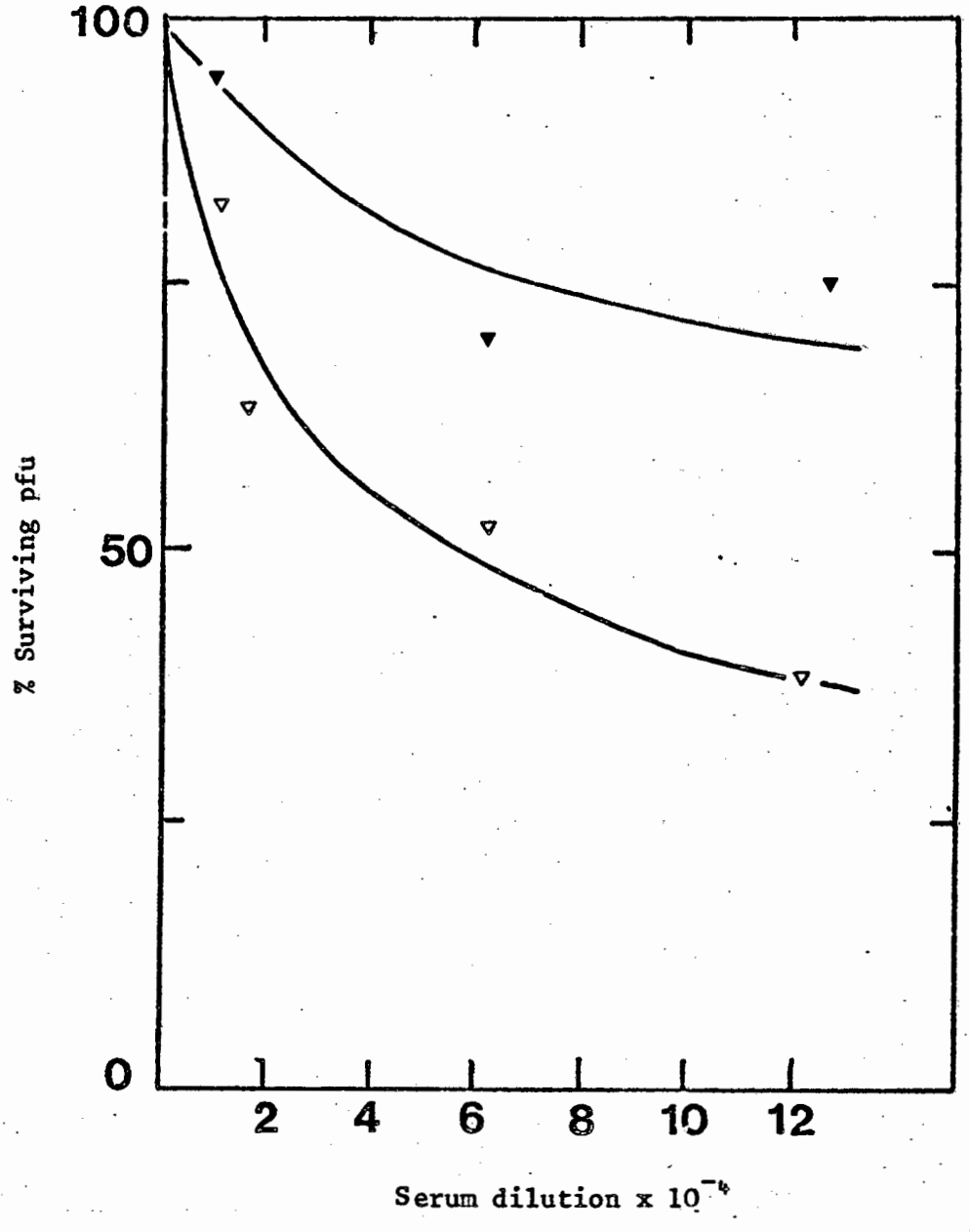

Fig. 22:

Inactivation of TMVP-phage conjugates by anti U2-P serum FRE XXXI. Inactivation was for 5 h at 37°C in the presence of 8% PEG 6000. Surviving conjugates were assayed by the complex inactivation method.

▼ Inactivation in 0,05 M P + G buffer pH 7,5

▽ Inactivation in 0,005 M P + G buffer pH 7,5.

conjugates with U2-P antiserum. Striking differences between sera from different animals have been demonstrated and show the need to test many different sera to select the most suitable ones.

(d) Inhibition of Inactivation

The inhibition of inactivation of protein-bacteriophage conjugates is a useful immunological assay for low concentrations of proteins. The technique has also been applied to the elucidation of the antigenic structure of proteins, for instance by Maron et al. (1970) who compared the capacity of lysozymes from several sources to inhibit the inactivation of hen egg-white lysozyme-T4 conjugates, and by Fuchs et al. (1973), who inhibited the inactivation of nuclease-T4 conjugates with fragments of the enzyme to determine which portions of the molecule have antigenic activity.

The experiments described in the following section were performed to determine whether the modified phage technique could be used to investigate some aspects of TMVP serology. It was important to know whether the NBS modification had any effect on the antigenic characteristics of the molecule, as the protein attached to the phage had been so modified. For this reason the inhibition by NBS-TMVP and untreated protein was compared.

Protein prepared from different strains was also compared for differences in inhibitory capacity and an attempt was made to correlate this information with known amino acid sequence differences. The discriminatory power of the technique was tested by comparing the wild-type strain with certain mutants of TMV differing in only one amino acid. Finally, selected fragments of the TMVP molecule, prepared by tryptic digestion, were tested for their capacity to inhibit the inactivation of the conjugates. Both types of test are used to localise antigenic determinants in globular proteins.

(i) Inhibition of Inactivation with TMVP-vulgare

The inactivation of the protein-phage conjugates can be attributed to specific TMVP antibodies provided the neutralization reaction can be inhibited by homologous protein.

To show the specificity of inactivation, an inhibition experiment was performed using protein which had been spun at 45 000 r.p.m. for 120 minutes to remove all 20S and larger aggregates. The percentage inhibition caused by different dilutions of the protein (Fig. 23) was calculated by the method described by Haimovich et al. (1970b). Fig. 20 was used as a calibration curve. At each dilution of inhibitor, the degree of inactivation was decreased to an amount equivalent to that caused by a higher dilution of serum. The method of calculation is illustrated

by the following example: Anti-TMVP serum at 1/6400 caused a drop in titre from 500 pfu/plate to 74 pfu/plate after 5 hours. In the presence of the inhibiting protein, added at a concentration of $2,5 \times 10^{-4}$ mg/ml, the concentration of surviving phage was decreased to 114 pfu/plate. Turning to Fig. 20, it can be seen that this is equivalent to the inactivation obtained with 62,5% of the initial antibody concentration. Thus 37,5% of the antibody was considered to be inhibited. Each point in Fig. 23 was obtained by this method. Fifty percent inhibition of inactivation was caused by the addition of 0,00063 mg/ml of protein. As 0,1 ml of protein solution was used for inhibition, the amount of protein which caused this inhibition was 63 ng. If 25% inhibition is considered significant, the threshold sensitivity of the assay is about 10 ng of protein.

Inhibition of Inactivation with NBS-treated TMVP: As the protein which was attached to the phage had been treated with NBS, it was important to determine whether the antigenic properties of the molecule had been altered by the NBS treatment, particularly as the tyrosine residue 139 which is modified by NBS, lies in a region of the TMVP molecule in which amino acid replacements have been shown to affect the antigenicity of the assembled virus capsid (von Sengbusch, 1965; van Regenmortel, 1967). Although no difference between the treated and native protein could be discerned in gel diffusion experiments (von Wechmar, 1976),

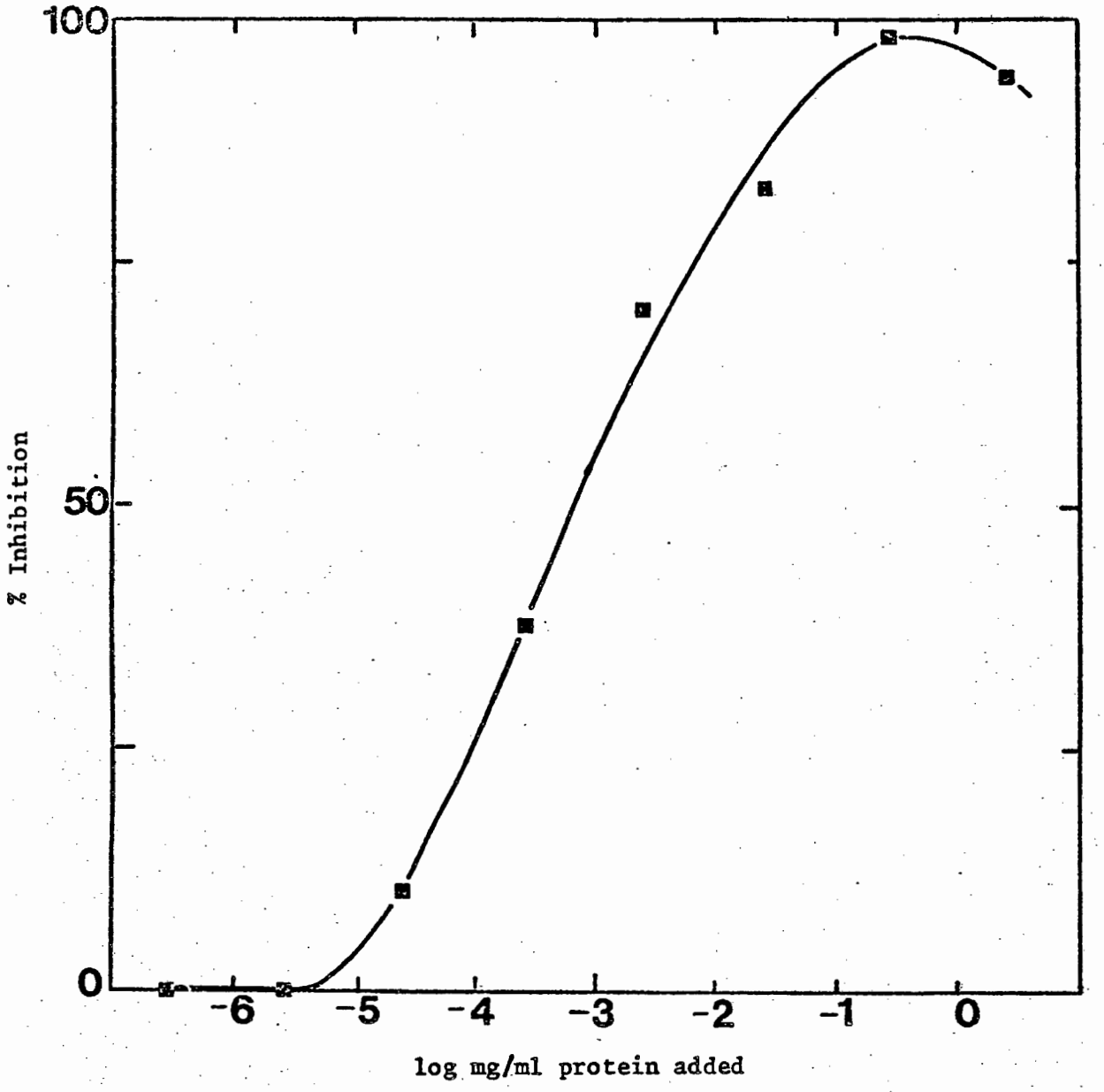

Fig. 23:

Inhibition of inactivation of TMVP-phage conjugates by protein prepared from TMV (vulgare). Percent inhibition was calculated as described in the text.

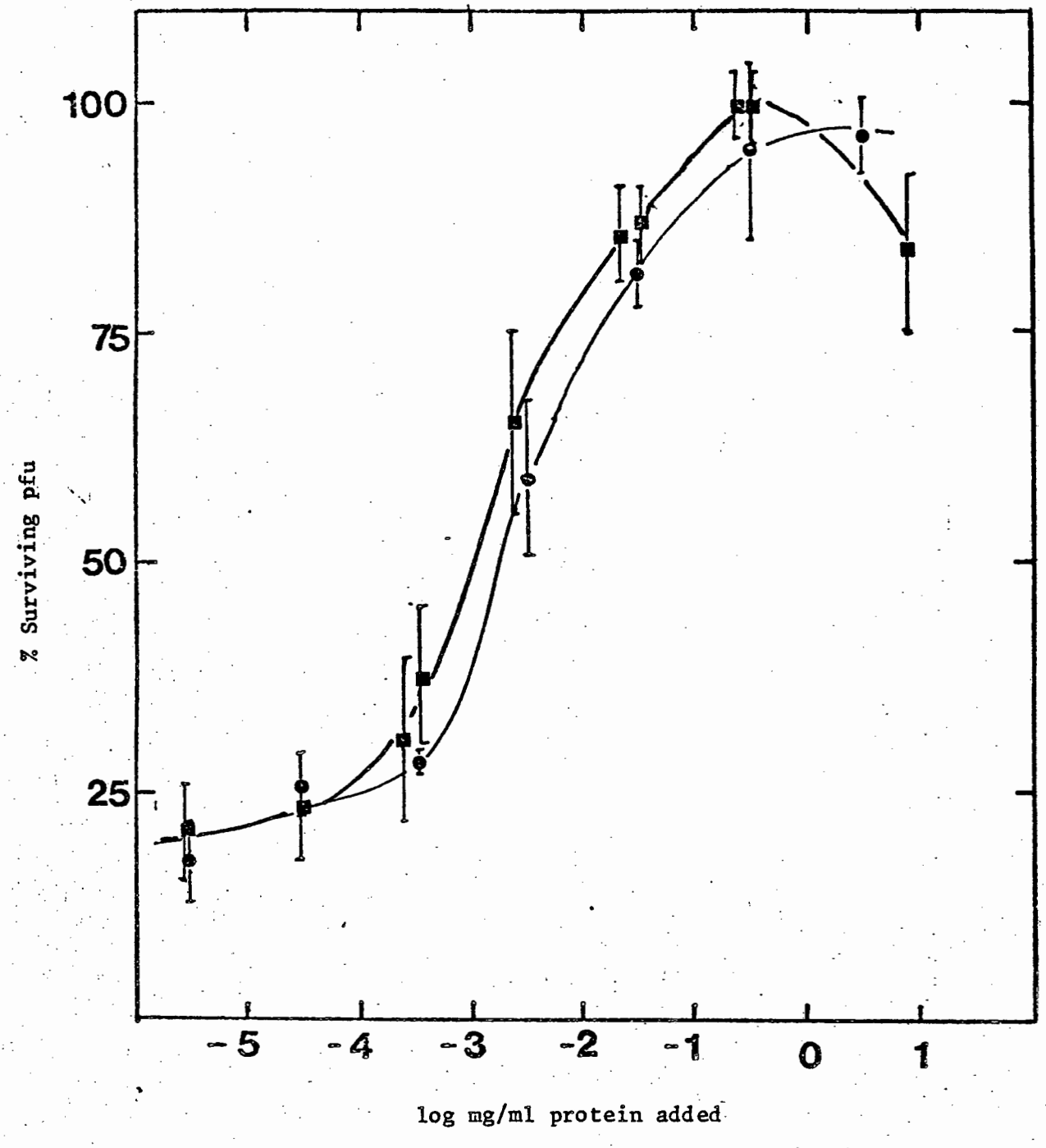

Fig. 24:

Inhibition of inactivation of NBS-TMVP-T4 conjugates by:

■ TMVP (vulgare)

● NBS treated TMVP

Vertical bars indicate standard deviations of the mean.

a difference may have been detectable by the chemically modified phage technique. To test this possibility, NBS-TMVP and normal TMVP which had both been centrifuged at 50 000 r.p.m. for 60 minutes to remove aggregates, were compared for their efficiency to inhibit the inactivation of the phage conjugates. The results of 4 such experiments are shown in Fig. 24. At a final protein concentration of about 0,8 mg/ml in the reaction mixture, a slight decrease in the efficiency of inhibition by the native protein can be observed. This means that fewer antigenic sites are available to the antibody, probably as a result of aggregation occurring with the untreated protein. This decrease is not seen with NBS-TMVP and shows the effectiveness of the NBS treatment in preventing aggregation of the subunits at this concentration. The standard deviations of the mean for at least 6 determinations for native protein, and of 4 for NBS-TMVP were calculated. These values are plotted in Fig. 24. When the results of these experiments were compared, it was found by means of a t-test that there was no statistically significant difference between the inhibition caused by the treated and untreated protein at protein concentrations ranging from 0,3 to 300 µg/ml. This indicates that although the NBS treatment modifies a residue in an immunologically active region of the TMVP molecule, no concomitant differences in the inhibition of inactivation are detectable.

Inhibition of Inactivation with Lysozyme: To show that the

inhibition was specifically caused by TMVP, an experiment was performed to determine whether an unrelated protein could inhibit the inactivation by TMVP serum. Table 11 shows the results of an experiment using lysozyme as the inhibitor. If no inhibition occurred the plaque count per plate was reduced from 250 to an average of 44 (82% inactivation).

Table 11:

Inhibition of inactivation of NBS-TMVP-T4 using lysozyme as inhibitor.

Concentration of lysozyme added (mg/ml).	Pfu/Plate. Average of 2 platings.
2,5	42,5
$2,5 \times 10^{-1}$	40,5
$2,5 \times 10^{-2}$	40
$2,5 \times 10^{-3}$	47
$2,5 \times 10^{-4}$	41,5

Lysozyme, even if added at a concentration of 2,5 mg/ml caused no inhibition of the inactivation. Thus the inhibition caused by TMVP and the related antigens was not caused by some non-specific effect.

(ii) Inhibition of Inactivation by Proteins Prepared From Different Strains and Mutants of TMV.

The aim of the following experiments was to determine whether the inhibition of TMVP-phage inactivation could be used as a means of measuring the degree of serological relatedness of proteins prepared from various strains of TMV. Closely related strains would be expected to inhibit similarly, while those with widely differing serological characteristics should show different degrees of inhibition. It was found that the degree of serological relatedness was correlated with the differences in amino acid sequences of the various strains tested.

On the other hand, protein from mutants differing in only one amino acid exchange could not be distinguished from wild type protein when an anti-TMVP (vulgare) serum was used.

CP-415-P and U2-P: The extent to which inactivation was inhibited by protein prepared from the CP-415 mutant (CP-415-P) and the U2 strain of TMV (U2-P) was compared. Protein from strain U2 differs from vulgare protein by 41 amino acid exchanges. CP-415 is a spontaneous mutant with a single substitution at position 140 (von Sengbusch, 1965; Hennig and Wittmann, 1972). As about 600 pfu were added in this experiment, the calibration curve based on an initial 500 pfu (Fig. 20) was not used to calculate percent inhibition. Instead the percent surviving pfu

was plotted vs. the added protein concentration. The higher the percent survivors, the more effective the inhibition. If no inhibition had occurred, an average of about 110 plaques were expected on the assay plates (i.e. 18% survivors).

As seen in Fig. 25, protein from CP-415 and TMV vulgare were better inhibitors than U2-P. About 75 times more U2-P than vulgare-P or CP-415-P was required to inhibit inactivation so that an arbitrary 50% of the conjugates survived. Both vulgare-P and CP-415-P showed a decrease in inhibitory capacity at concentrations at which aggregation of the subunits could be expected.

Y-TAMV-P and CGMMV-P: The results of a similar experiment with protein prepared from the strains Y-TAMV and CGMMV are shown in Fig. 26. It is clear that Y-TAMV-P was a far more efficient inhibitor than CGMMV-P.

The results of inhibition experiments performed with protein prepared from TMV-vulgare and the related strains Y-TAMV, U2 and CGMMV (cucumber strain) are illustrated for comparison in Fig. 26. The points describing the inhibition curve for vulgare-P were each obtained from an average of at least 6 determinations. The Y-TAMV-P curve was calculated from the results of four experiments and the U2-P and CGMMV-P curves were derived from duplicate determinations.

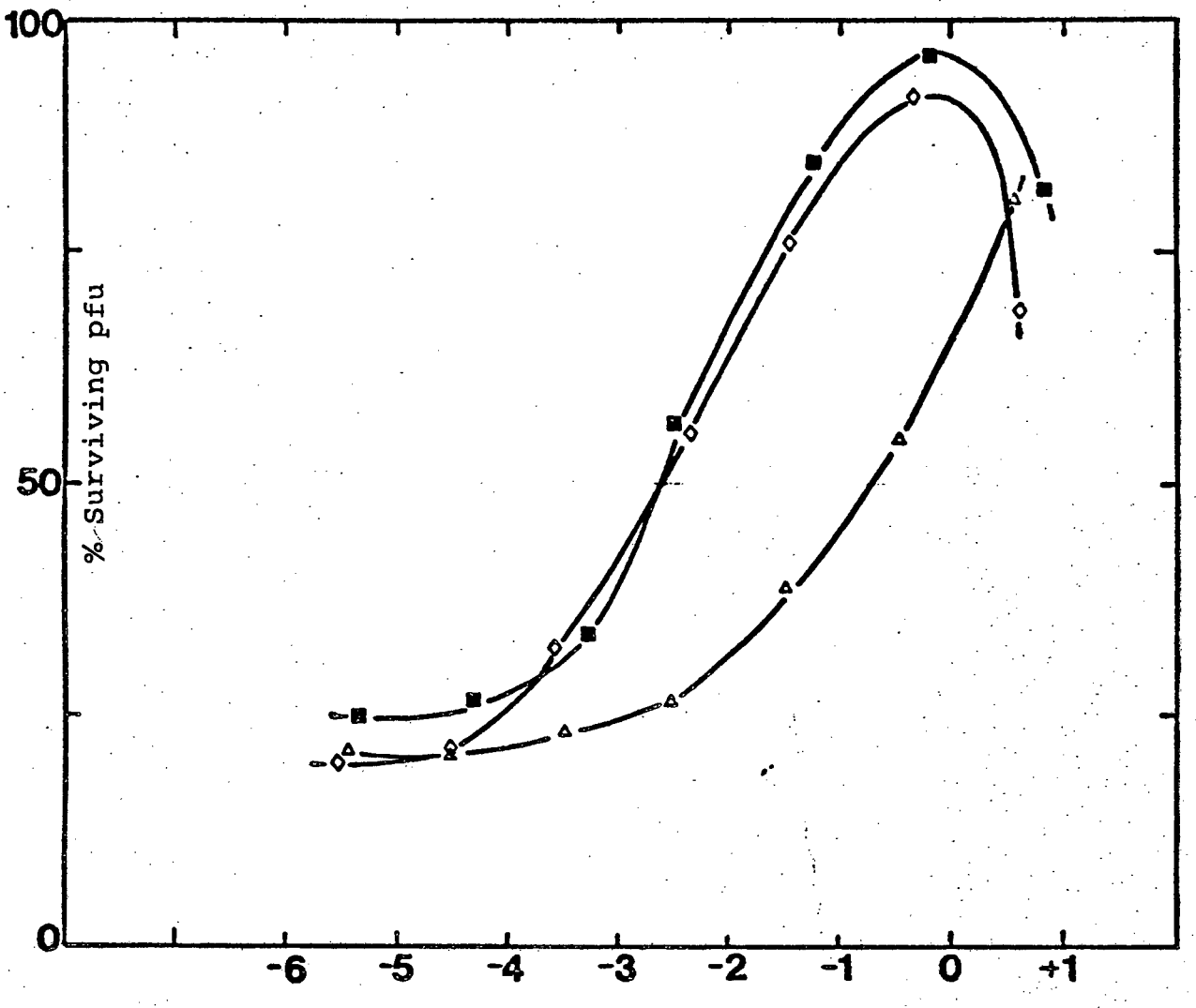

Fig. 25:

Inhibition of inactivation of TMVP-phage conjugates by protein prepared from different strains of TMV:

■ Vulgare

◇ CP415

△ U_2

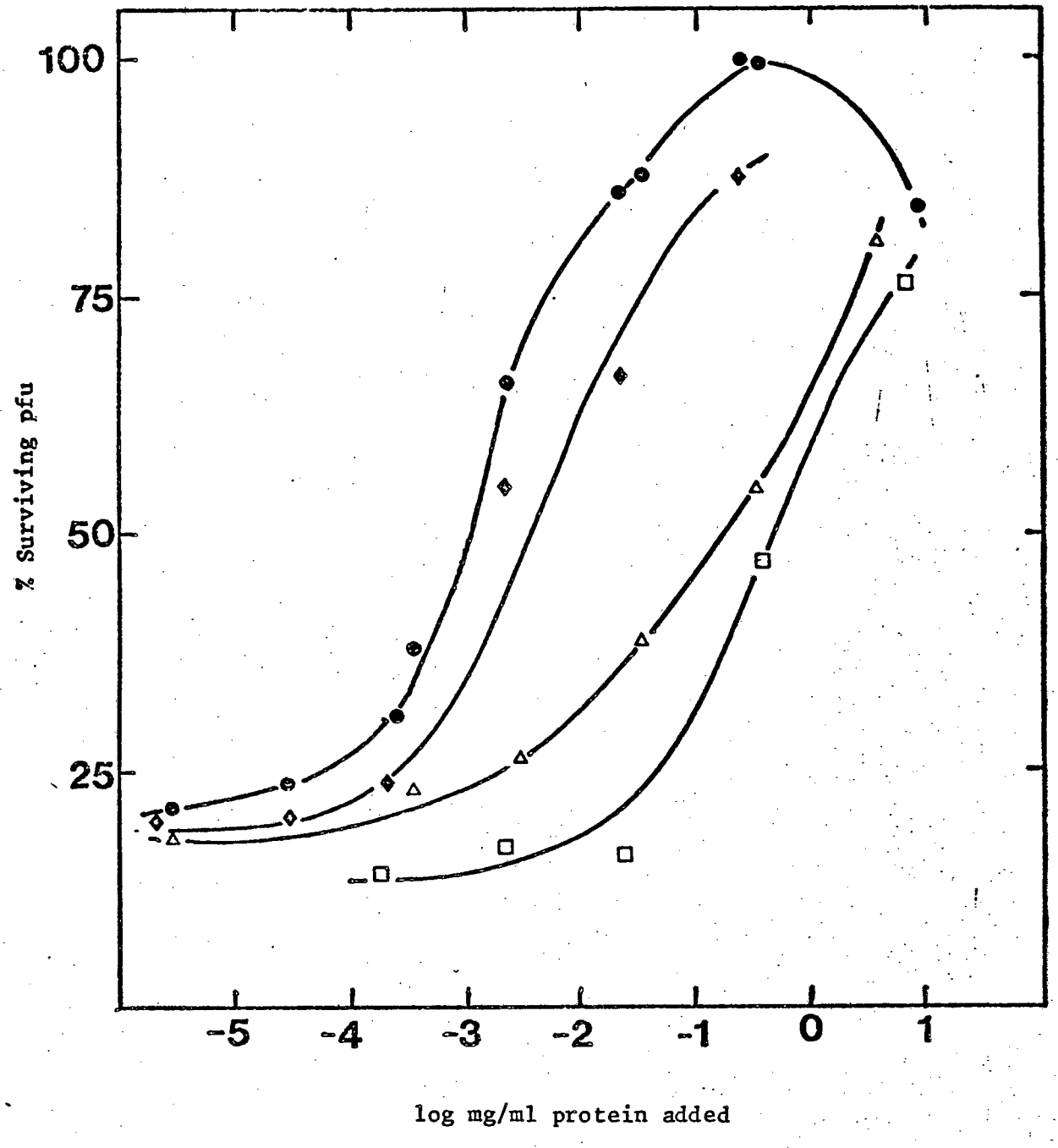

Fig. 26:

Inhibition of inactivation of TMVP-phage conjugates by protein prepared from

● TMV-vulgare

◆ Y-TAMV

△ U_2

□ CGMMV

If the extent of inhibition of inactivation is taken as a measure of the serological relatedness of the inhibitory protein to the protein against which the serum was raised (in this case vulgare-P), it is apparent that Y-TAMV-P is serologically the most similar, followed by U2-P and CGMMV-P. It required the addition of protein at a concentration of about 1 µg/ml to inhibit inactivation so that an arbitrary 50% of the conjugates survived. To inhibit inactivation to the same extent, Y-TAMV-P at 4 µg/ml, U2-P at 63 µg/ml and CGMMV-P at 400 µg/ml had to be added.

However, Figs. 25 and 27 show that it was not possible to distinguish protein from the mutants CP-415 (1 amino acid exchange) or 371 (2 exchanges from the wild-type protein).

The results presented in this section show that inhibition of inactivation was an effective way of discriminating between protein prepared from different strains of TMV. The order, as well as the relative degree of relationship, could be established by comparing the different inhibition curves. It was not possible to distinguish proteins from mutants with 1 or 2 amino acid exchanges. The proteins from the strains Y-TAMV (28 exchanges compared with the vulgare-P), U2 (41 exchanges) and CGMMV (more than 41 exchanges) showed decreasing inhibition capacities with increasing number of sequence differences.

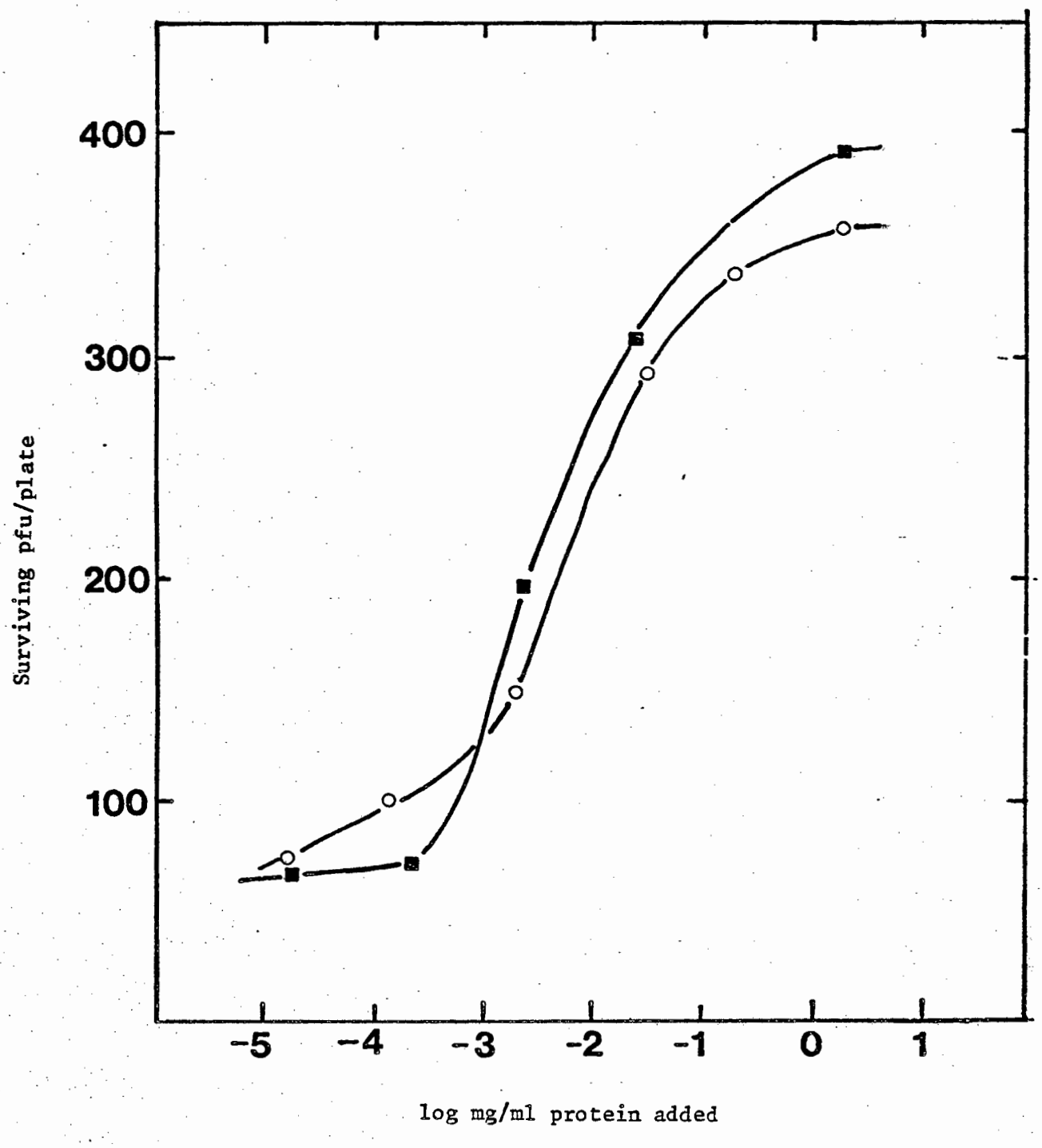

Fig. 27:

Inhibition of inactivation of TMVP-phage conjugates by protein prepared from ■ TMV vulgare

○ Mutant 371

Inactivation was by anti-TMVP serum JOR XIV.

(iii) Inhibition of Inactivation with Intact TMV

Fig. 28 shows the inhibition of a protein antiserum JOR XIV caused by different concentrations of TMV. Weight for weight, the intact virion was a less efficient inhibitor than the protein subunits. This is probably due to the fact that certain determinants on the protein are buried and are not available to react with the corresponding antibodies. To allow 50% of the pfu to survive, about 10 times more virus than protein had to be added to the reaction mixture.

(iv) Inhibition of Inactivation by Tryptic Peptides 4, 8, 11 and 12 of TMVP

It is known that amino acid exchanges in certain regions of the TMVP molecule are serologically detectable. Evidence has accumulated which indicates that regions of the molecule corresponding to the tryptic peptides 4, 8, 11 and 12 contain antigenic determinants (Anderer, 1963a ; von Sengbusch, 1965; van Regenmortel, 1966; Benjamini et al., 1964, 1965). It should be possible to inhibit the binding of a protein antibody to the protein with peptides containing antigenic determinants. In this section an attempt to use the modified phage technique for the detection of such immunological activity is described.

Small quantities of the four tryptic peptides 4, 8, 11 and 12 were purified from a tryptic digest of TMVP by

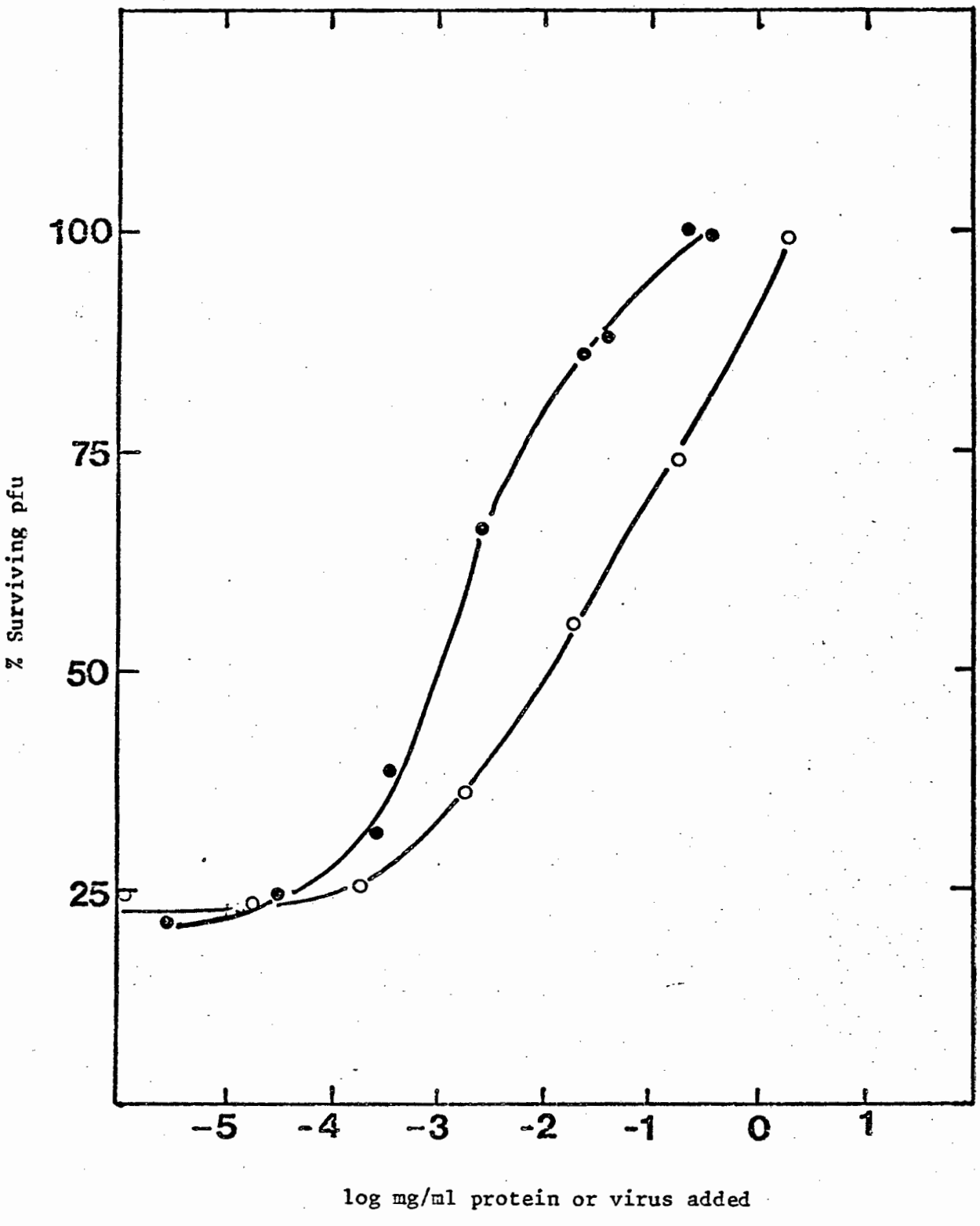

Fig. 28:

Inhibition of inactivation of TMVP-phage by:

● TMVP

○ TMV

The inactivating serum was JOR XIV (Anti-TMVP)

paper electrophoresis. The peptide preparations of unknown concentration, were a gift from Mr. R.C. de L. Milton. An inhibition experiment was performed using different dilutions of peptide 8. Inhibition was allowed to proceed for 40 minutes at 37°C before the phage conjugates were added. The inactivation of the conjugates by antiserum JOR XIV was inhibited by the peptide (Fig. 29). This confirms that peptide 8 contains a major TMVP determinant, as Benjamini et al. (1964) found by the technique of inhibition of complement fixation.

Peptides 4, 11 and 12 were tested at one concentration only. A volume of 0,1 ml of a solution of each of the peptides in buffer was added to 0,2 ml of anti-TMVP serum (JOR XIV). After 40 minutes at 37°C, 0,1 ml of 40% PEG and 0,1 ml of TMVP phage were added. The mixture was plated by the complex inactivation method after 5 h at 37°C, and the results are shown in Table 12. In the absence of inactivating serum, about 1100 plaques were found on the assay plates. When no peptide was added to the serum about 255 plaques/plate were found (i.e. 23% survivors).

All the peptides tested inhibited the inactivation to some extent, but as the concentration of each was not known, it is not possible to compare their efficiency of inactivation.

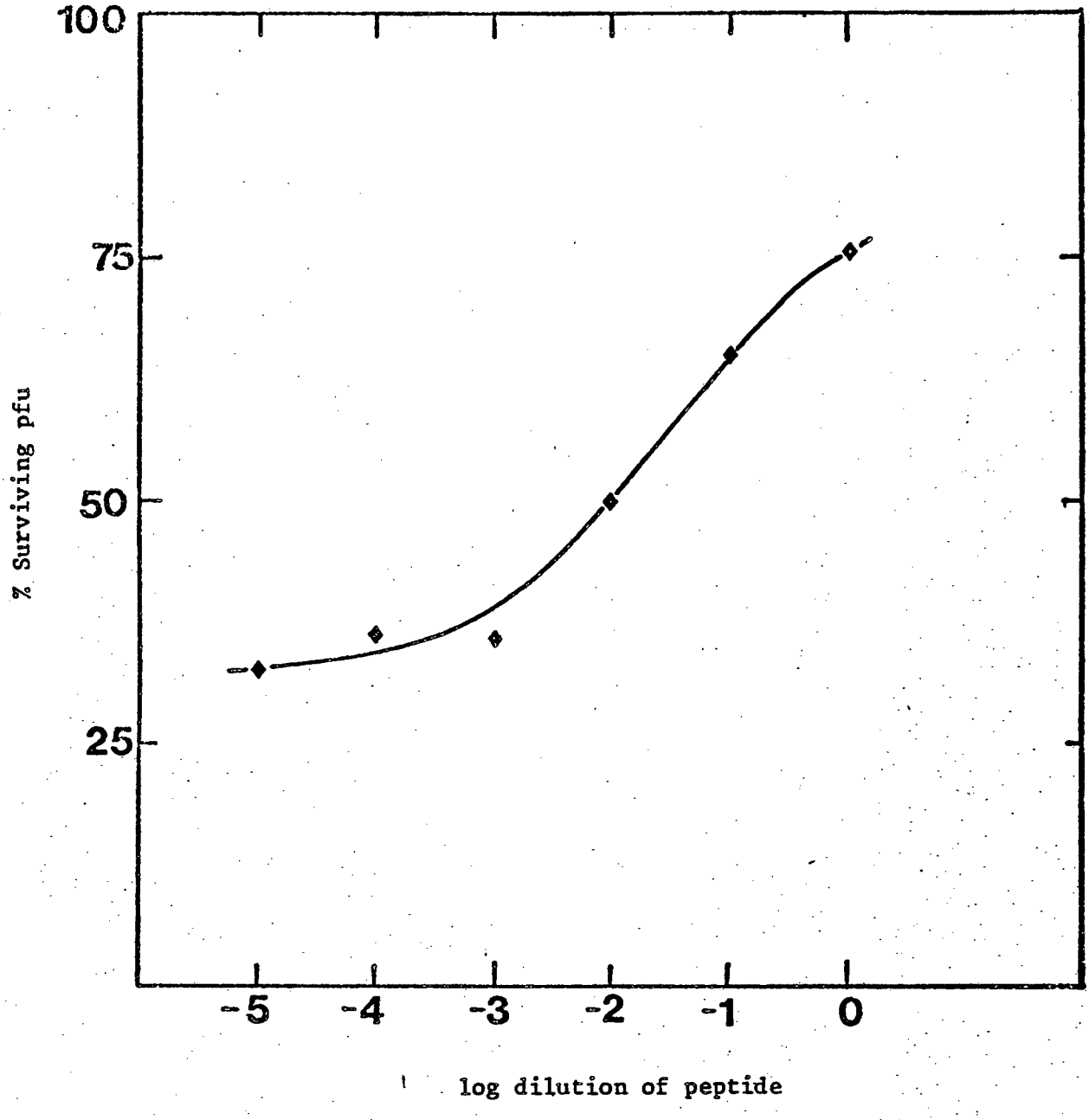

Fig. 29:

Inhibition of inactivation of TMVP-phage by tryptic peptide 8 of TMVP. The inactivating serum was JOR XIV, a TMVP anti-serum

Table 12:

Inhibition of inactivation of TMVP-phage by TMVP tryptic peptides.

Inhibitor	% Surviving pfu
Peptide 4	39
11	30
12	42
Buffer only	23

These experiments do, however, show that the modified phage technique can be used as a relatively simple means of detecting immunological activity in fragments of viral proteins.

(v) Inhibition of Inactivation of TMVP-phage by U2 Antiserum with Different Proteins

Van Regenmortel (1967) found in gel diffusion experiments that it was not possible to differentiate certain chemically induced mutants of TMV from the wild-type virus with a TMV vulgare antiserum, but that anti-U2 sera were capable of distinguishing between the same mutants. Protein prepared from one of these mutants, 371, which has 2 amino acid exchanges at position 138 and 148 respectively, was found to be indistinguishable from wild-type protein by

inhibition of modified phage neutralisation (Fig. 27). The following experiment analyses the ability of an anti-U2-P serum to distinguish between TMVP and mutant 371.

Using antiserum FRE XXXI, at a dilution of 10^{-3}, 40 - 50% of the pfu were neutralized. Protein from the wild-type and the mutant strain was compared for its ability to inhibit this inactivation. The results are shown in Fig. 30. At protein concentrations of about 0,1 mg/ml, the mutant protein inhibited inactivation to a lesser extent than wild-type protein. However, it is likely that the difference observed at this concentration is due to increased aggregation of the mutant protein. At a ten-fold lower concentration there was no difference in the degree of inhibition. It appears, therefore, that the phage neutralization assay has less discriminatory power for comparing coat-proteins than precipitin tests in liquid or gel have for comparing intact virus. Serological differences that are revealed by the formation of spurs in immunodiffusion using intact TMV (van Regenmortel, 1967) are not discernible in the modified phage assay using coat-protein. This is probably due in part to the relatively large errors inherent in the measurement of phage neutralization.

The results nonetheless show that inhibition of inactivation in a heterologous system can serve as a sensitive means of detecting protein. Both U2-P and vulgare-P

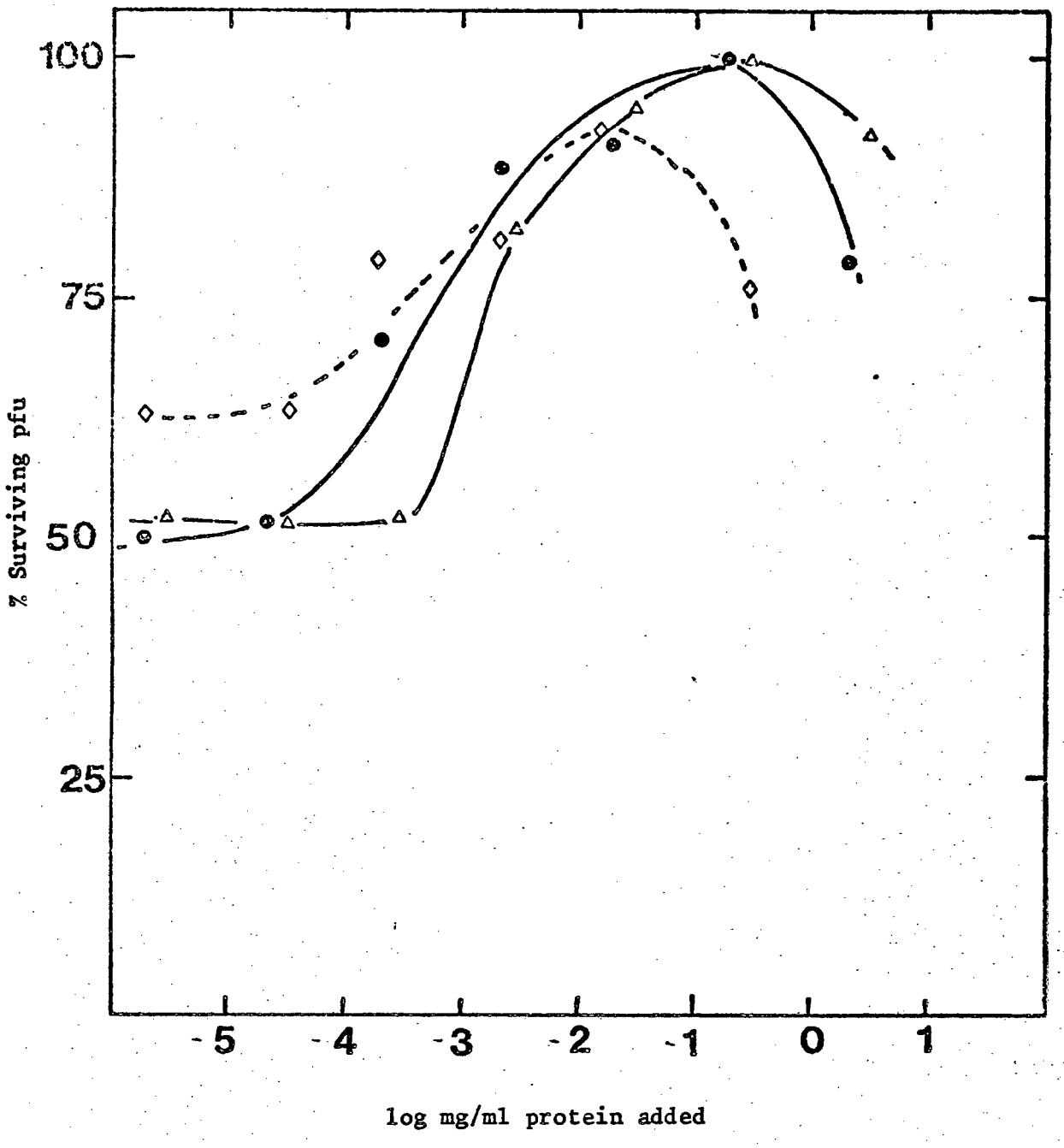

Fig. 30:

Inhibition of inactivation of NBS-TMVP-T4 by protein prepared from ● TMV (vulgare) ◇ mutant 371 △ U2-P.

Inactivation was by Frelimo XXXI, an anti-U_2-Protein serum.

caused significant inhibition at low concentrations: Using U2-P antiserum, U2-P could be detected at a concentration of 1 µg/ml (Fig. 30). In the inhibition of inactivation by TMVP antiserum, U2-P was detected if it was added at 63 µg/ml (Fig. 26). The clear difference in the inhibition caused by vulgare-P and U2-P in the homologous system was not seen in the heterologous system (Fig. 30).

CHAPTER V.

DISCUSSION

1. PHAGE INACTIVATION WITH PHAGE ANTISERA

The purpose of the bacteriophage neutralization experiments was to determine the inactivating strength of various anti-phage sera. These sera were to be used in subsequent "decision" technique plating experiments for which an effective anti-phage serum is essential. At the same time, the kinetics of inactivation by phage antisera were determined and could be compared with the inactivation of chemically modified phage by anti-hapten or anti-protein sera.

The K values of the pooled bleedings of three rabbits were determined at three different stages in the immunization schedule. The K value of the first bleeding (made 9 days after injection) was calculated from the results of a simple experiment which does not establish the kinetics of neutralization. The K value of 7,1 which was obtained is low for an anti-T4 serum (Adams, 1959), but it is known that early serum antibodies may dissociate from the phage particle and that this can lead to a decrease in the rate of inactivation (Jerne and Avegno, 1956). A low K value can also result from a low concentration of antibody in the serum (Rohrmann and Kreuger, 1970) and since the early serum was obtained 9 days after the start of immunization, this could also explain the low K value.

The K values of the second and third bleedings are within the range 200 - 1000, typical for T-even phage antisera (Adams, 1959) and increased as immunization proceeded. These sera inactivated the phage according to first order kinetics until at least 99% of the pfu were neutralized.

The K value of the second bleeding was determined at serum dilutions of 10^{-2} and 10^{-3}. The figures obtained for the higher dilutions was 228 compared with 168 for the 10^{-2} dilution. This confirms that the calculated K value increases with serum dilution (Adams, 1959).

The IgG prepared from the second bleeding also neutralised the phage according to first order kinetics (Fig. 8) and the plot of surviving pfu as a function of IgG concentration was linear (Fig. 9). In agreement with the above observations, Haimovich and Sela (1969) found that by contrast to neutralization by IgM, inactivation of phage by IgG occurred according to first order kinetics.

The extreme sensitivity of phage to antibody was confirmed by the fact that 50% of the phage could be inactivated in 2 hours by 46 ng of IgG. The IgG concentration measured reflects total IgG in the serum and only part of this (probably about 10%) is specific for bacteriophage T4. This means that about 5 ng of anti-T4 phage antibody could be detected in the neutralisation test. As the inactivation followed first order kinetics, a lengthening of the time of incubation might have increased the sensitivity of the assay for antibody still further.

2. DINITROPHENYL BACTERIOPHAGE

The main purpose of the work with DNP-modified phage was to gain experience in the methodology of the chemically modified phage technique. The hapten, dinitrophenol, was chosen since it has been shown previously to be highly suitable for this type of study (Carter *et al.*, 1968).

The DNP-phage were prepared using dinitrobenzenesulphonic acid as the haptenating agent. The reaction of DNP-sulphonic acid with protein is less vigorous than the commonly used 1,3 difluoro-2,4-dinitrobenzene. This is an important consideration when modifying phage, as the conditions of reaction must allow some phage particles to remain viable after modification. For the viable hapten-phage conjugates to be sensitive to the anti-hapten serum, it is necessary for the phage coat-protein to be modified in the region of the critical site, so that when an antibody molecule binds to the hapten, the critical site of the phage is occluded and the phage cannot attach to the host bacterium (Sela, 1972). DNB-sulphonic acid reacts with lysyl residues on the phage coat-protein (Carter *et al.*, 1968). The degree of modification depends upon the time allowed for the coupling reaction. Some phage may escape haptenation or may not be sufficiently modified in the vicinity of the critical site. Such particles will be resistant to the anti-hapten serum (Mäkelä, 1966). Carter *et al.* (1968) found that they obtained optimum sensitivity after 10 h of reaction and that 96% of the pfu were inactivated.

In the present study the DNP-phage used in the inactivation experiments had reacted with the DNB-sulphonic acid for 24 hours during which time 96% of the phage had become inactivated. This inactivation is possibly the result of a modification of the phage coat-protein at the critical site. This may prevent subsequent attachment of the phage to the host bacterial cell wall (Carter et al., 1968).

It is also possible that phage particles may become denatured by the conditions used in some hapten coupling procedures. Since it is essential that some of the hapten-phage conjugates survive coupling, there are strict limitations to the range of conditions under which hapten-phage conjugates may be prepared. This often limits the applicability of the modified phage technique and is a distinct disadvantage compared to the passive haemagglutination technique.

The inactivation of the DNP-phage conjugates by anti-DNP serum did not follow first order kinetics. A marked reduction in rate after ± 90% inactivation resulted in curvilinear kinetic plots (Fig. 10). When assayed by the "decision" technique, the neutralisation was more efficient, and steeper plots which were linear over a wider range of inactivation were obtained (Fig. 11).

As mentioned earlier, dissociation of the haptenated-phage/antibody complexes will result in reactivation of the neutralized phage and in an increase in the number of surviving pfu. Dissociation of the complex on the assay plates may also be

the cause of the different plaque sizes which have been observed in direct plating experiments. Dissociation would be expected if the neutralizing antibody has a low affinity for the hapten. However, the "decision" technique limits the time available for dissociation, thereby restoring first order neutralization kinetics.

Another reason for deviations from first order kinetics could be that the modified phage population is heterogenous, i.e. that not all the particles have been optimally modified and that some are insensitive to anti-DNP (Mäkelä, 1966). The fact that the "decision" technique caused improved inactivation at a given concentration of antibody indicates that dissociation of the antibody from the modified phage was probably the reason for the marked deviation from first order kinetics seen in Fig. 10. This conclusion is also supported by the findings of Mossman and Hamer (1973) who experimentally demonstrated that DNP antibody dissociated from DNP-modified phage. It has also been shown that infection of host bacteria by chemically modified phage is a slower process than infection by native phage (Mossman and Hamer, 1973; Andrieu et al., 1974). The DNP-phage will thus infect fewer host bacteria before being inactivated by the strong anti-T4 serum used in the "decision" technique. This may explain why the number of phage which have not reacted with antibody is reduced when the "decision" technique of assay is used.

When different concentrations of anti-DNP were incubated with modified phage and assayed by the "decision" technique, it was

found that 3,9 ng of antibody caused 50% inactivation of the phage (Fig. 12). The original concentration of antibody in the serum was determined by quantitative precipitation with DNP-RSA. Carter et al. (1968) found that they could detect 2 ng of antibody using the direct plating method. However, the inactivation of phage is not a direct measure of the amount of antibody present in the serum and factors such as affinity and class of antibody may affect the sensitivity of the assay (Haimovich and Sela, 1977; Blank et al., 1972).

The specificity of inactivation was established by comparing the inhibition obtained with free DNP and with the structurally related TNP (picryl) hapten. The DNP was an efficient inhibitor of inactivation, whereas TNP inhibited less well (Fig. 13).

In summary, these experiments showed that the modified phage technique could be used to detect low concentrations of antigen and antibody and to distinguish, at the same time, between closely related haptens. The sensitivity of the assay for hapten antibody was of the same order as for phage antibody.

3. LYSOZYME-T4 PHAGE CONJUGATES

Haimovich et al. (1970a), who prepared RNase-T4 and lysozyme-T4 phage conjugates using glutaraldehyde as the bifunctional reagent, found that this coupling agent gave reproducible results and produced conjugates which were efficiently inactivated

by anti-protein antibodies. It was thus envisaged that glutaraldehyde could be used to prepare TMVP-T4 phage conjugates. However, a series of preliminary attempts were unsuccessful and it was decided, therefore, to study the effects of the purity of the glutaraldehyde and of temperature on the conjugation of lysozyme to T4 phage.

Glutaraldehyde, a dialdehyde, has been shown to couple via the free amino groups of proteins (Avrameas and Ternynck, 1969). In addition, the glutaraldehyde molecules polymerize via their carbonyl functions, producing polymers of various lengths (Sachs and Winn, 1970). Distilled glutaraldehyde is expected to consist mainly of monomers, the high molecular weight polymers having a higher boiling point. Vacuum distilled glutaraldehyde was used by Oger et al. (1974) to prepare protein-phage conjugates, but other workers have used commercial grade reagent which is probably predominantly polymeric (Richards and Knowles, 1968; Sachs and Winn, 1970). The effect of temperature on coupling efficiency is also an important factor when TMVP is to be conjugated, as low temperatures (4°C) inhibit polymerization of this protein. It is conceivable that large TMVP polymers could sterically inactivate the phage particles without the need for antibodies.

From a comparison of lysozyme-phage conjugates prepared at 4°C and 23°C with either non-distilled commercial reagent or glutaraldehyde which had been distilled under vacuum it appears that non-distilled glutaraldehyde was an efficient coupling

agent, particularly at 4°C (sample No. 10, Table 5). These conditions resulted in conjugates of which 94% could be neutralized by lysozyme antiserum. The sensitivity of protein-phage conjugates to protein antiserum is not a direct measure of a bifunctional reagent's coupling efficiency per se, but rather indicates that sufficient protein has been attached to the phage in the region of the "critical site" to make the phage sensitive to antibody.

The results obtained with lysozyme were useful in that they showed that there was no advantage to be gained by the use of vacuum-distilled glutaraldehyde. Polymeric glutaraldehyde is thought to owe its efficiency as a coupling agent to the fact that it can react with free amino groups some distance apart and that it can fit flexibly between protein chains without distorting the tertiary structure to any great extent (Sachs and Winn, 1970). Another important fact which was established was that this bifunctional reagent can be used to prepare protein-phage conjugates at 4°C.

When the effect of different serum dilutions on the inactivation of a sample of lysozyme-phage conjugates was determined, it was found that plating by the complex inactivation and direct plating methods gave a similar number of survivors at each serum dilution. This may mean that the phage were optimally coupled, or that the lysozyme antibodies had a high affinity for the attached protein. The sensitivity of these conjugates was such that 50% could be inactivated by a 4×10^{-6} dilution of serum (Fig. 14). The specificity of the inactivation was

established by the ability of the homologous antigen to inhibit neutralization.

4. TMVP-T4 PHAGE CONJUGATES

(a) Preparation of Conjugates

The results of the experiments with lysozyme indicated that non-distilled commercial glutaraldehyde could couple efficiently proteins to phage and that the conjugates were sensitive to protein antibodies. However, numerous attempts to prepare TMVP-T4 conjugates with glutaraldehyde were unsuccessful. There are two possible reasons for this: The glutaraldehyde failed completely to couple the protein to the phage, or protein-phage conjugates were produced which were not sensitive to TMVP antibodies.

Since the TMV subunit has an acetylated N-terminal amino acid (Narita, 1958), no free α -amino group is available for reaction. The wild-type protein has two lysine residues at positions 53 and 68, each having a potentially reactive ϵ -amino group if the protein is disaggregated (Perham and Richards, 1968). In the intact virus the lysine residues are sterically masked and one (Perham and Richards, 1968), or both (Anderer, 1963b) are not available for reaction. Thus if the protein which is to be coupled (via the lysine residued of the phage coat

protein) is aggregated to any extent, the number of amino groups available for reaction with the glutaraldehyde carbonyl function will be reduced. Furthermore, Avrameas and Ternynck (1969) suggest that in most proteins only a few free amino groups are capable of reacting with the glutaraldehyde. They also found that proteins with few free amino groups, like glucose oxidase, could not be cross-linked by glutaraldehyde to form insoluble polymers. Avrameas et al. (1969), who used glutaraldehyde as a coupling agent for the preparation of sensitized erythrocytes, also found that if the protein to be coupled had very few lysines, the sensitization of their erythrocytes was not very efficient. These findings suggest that the negative results obtained with TMVP are due to an insufficient number of lysyl residues in this protein. Another possibility is that the protein was in fact coupled to the phage, but the resulting conjugates were not sensitive to the action of the antiserum. This is expected if the protein is conjugates at a position on the phage which is not sufficiently near to the critical site. However, many attempts with different proportions of reactants were all unsuccessful and since a different bifunctional reagent, TDIC, which also couples via free amino groups (Gyenes and Sehon, 1964; Schick and Singer, 1961) failed as well, it seems likely that the TMVP was not coupled to the phage at all. Attempts to conjugate the CP415 mutant protein which as an extra lysine residue was also unsuccessful.

The preparation of TMVP-phage conjugates was successful

when the highly reactive BDB was used as bifunctional reagent. This chemical has been found to produce conjugates which are less sensitive to the action of serum than those prepared with glutaraldehyde or TDIC (Haimovich et al., 1970a) BDB couples via the tyrosyl, histidyl, amino and carboxyl groups on proteins via diazo-linkages (Gordon et al., 1958; Nisonoff, 1967). The large number of residues available for reaction may in some cases lead to the steric blocking of large areas of the protein molecule because of attachment to the phage. It is possible that the BDB could modify regions of the protein molecule which contain antigenic determinants. In either case there would be less surface area of the coupled protein molecule available to the antibody than if it were attached to the phage only by way of certain available amino groups. Another possible reason for the lower sensitivity of phage conjugated with BDB is that the surface of the phage close to the "critical site" may have few chemical groups with which BDB can react. If this is the case, better results may be obtained with some other phage than T4.

When the TMVP-phage conjugates were assayed by the direct plating method, even a 1/100 dilution of anti-TMVP serum caused only minimal inactivation after 3 hours (Fig. 17). The complex inactivation, and to a lesser extent, the "decision" technique improved the inactivation (Fig. 17), but it is only when PEG was added to the reaction mixture

and incubation was continued for 5 hours, that very dilute serum had any marked effect on the number of surviving conjugates (Fig. 18). After 5 hours, about 85% of the conjugates were inactivated by a $1,6 \times 10^{-4}$ dilution of serum. PEG is thought to act at the stage in the complex inactivation method of assay when the anti-rabbit IgG is added, i.e. when the complex on the phage is formed. Fránek and Doskočil (1975) suggest that there is a connection between the enhancement of serological precipitation by PEG and the effect of PEG on the neutralization of modified phage. However it has been shown that PEG can prevent the adsorption of phage to the host bacteria (Follansbee et al., 1974) and it seems likely that this is a major factor contributing to its effect on the conjugates. The 8% PEG concentration used in the modified phage experiments may not prevent adsorption, but could merely reduce the tendency of the phage to adsorb to its host, possibly causing a retraction of some of the tail fibres (Follansbee, et al. 1974). Phage with all tail fibres retracted are effectively neutralized (Kellenberger et al., 1965), but those with some of the 6 fibres in the extended configuration can adsorb to and infect the host (Wood and Henninger, 1969). As most of the neutralizing activity of T-even phage antiserum is directed against the phage tail fibres (Franklin, 1961; Edgar and Lielausis, 1965) it is possible that phage with only some of the fibres in the extended configuration would be more readily inactivated by the antibody.

(b) Inactivation of TMVP-T4 Phage Conjugates

The phage conjugates were allowed to react with TMVP antiserum at different dilutions. The sera were obtained at various times from three different rabbits and differed markedly in their inactivating abilities. It has been found that the inactivation of modified phage by antiserum depends not only on the amount of antibody present, but also on the class and affinity of the antibodies (Haimovich and Sela, 1969a, 1977; Blank et al., 1972). These parameters are likely to vary according to the immunization schedule used to obtain the antisera and are bound to differ in different animals (van Regenmortel and von Wechmar, 1970; van Regenmortel, 1975). For this reason the inactivation of modified phage can give misleading results if used as a direct measure of the amount of antibody present in an unknown serum.

The difference in inactivation caused by 3 anti-TMVP sera is illustrated in Fig. 19. The serum JOR XIV was the most efficient inactivator of the conjugates. This serum inactivated over 80% of the conjugates in 5 h and also caused maximum inactivation over a narrower dilution range than the other two. As this serum could be diluted to 10^{-5} and still cause $\pm$ 20% inactivation, it probably had a higher titre of antibody than the other two, both of which caused no inactivation at this dilution. The fact that it inactivated the phage over a less wide dilution range (10^{-6} - 10^{-4}) could also reflect a high

affinity for the antigen. None of the sera tested had any effect on non-modified phage, an important consideration as it is known that some sera contain "natural" phage antibodies (Jerne, 1956; Haimovich et al., 1970).

The difficulty of correctly interpreting modified phage neutralization data, particularly in work with antigens such as virions and virus coat protein, becomes apparent when the inactivation of conjugates by TMVP anti-serum and a serum directed against intact virus is compared (Figs. 20 and 21). The TMV antiserum inactivated the TMVP-phage conjugates to a lesser extent than did the TMVP antiserum. This may be due to the anti-virus serum having an antibody population directed only against determinants available on the surface of the virus. Anti-TMVP serum, however, would have a population of antibodies, absent in the TMV antiserum, which are directed against determinants hidden in the virus (i.e. cryptotopes). There would thus be less antibody in a virus antiserum available to inactivate the TMVP-phage conjugates. Antibodies specific for new determinants on the virus surface (i.e. neotopes) would likewise not react with TMVP and so would not inactivate the phage.

When the differences in inactivating ability of individual TMVP sera are considered (Fig. 19), it is apparent that individual serum differences in titre and affinity can affect the efficiency of inactivation to a large extent. A further complicating factor is the difficulty encountered

in raising sera which are specific for only virus or subunit determinants and which arises because of virus disruption and protein aggregation in the preparations used for immunization (van Regenmortel and Lelarge, 1973). Taking all factors into account, the difference in neutralization of the TMVP-phage by anti-TMVP and anti-TMV sera merely reflects the well-known serological differences that exist between TMV and its disaggregated coat-protein.

The results have also shown that cross-reactivity between related viral antigens can be studied by the inactivation of modified phage. Serum prepared against protein of strain U2 also inactivated TMVP-phage. In this case the reaction with the conjugates was much weaker, and a 10^{-3} dilution of serum resulted in only 25% inactivation. The enhancement of phage inactivation in low ionic strength buffer is a long established fact (Jerne, 1952; Jerne and Skovsted, 1953; Cann and Clark, 1954) and has been used to improve the sensitivity of the modified phage assay (Andrieu et al., 1974). Salt concentration, like PEG, is thought to affect the phage tail fibre extension and retraction (Conley and Wood, 1975). Using a 0,005 M buffer instead of the usual 0,05 M concentration, it was possible to increase inactivation by heterologous serum to about 60% (Fig. 22). However, when the inactivation with homologous TMVP antiserum was performed in low ionic strength buffer, the sensitivity of the conjugates to antibody was not improved (Fig. 18).

The increase in the efficiency of reaction in low ionic strength buffers has been interpreted as an increase in the probability that a collision between a hapten antibody and a modified phage will result in specific binding (Andrieu et al., 1974). If the antibody already has a high affinity for the determinant on the modified phage, it may be that its chances of binding will not be further improved by the use of low molarity buffer. Since the U2-P antibodies probably do not have a high affinity for the heterologous antigen, the low ionic strength may in this case lead to an improvement in inactivation.

(c) Inhibition of Inactivation

The inhibition of inactivation of protein-phage conjugates with protein can serve as a simple method for the detection and quantitation of protein antigens. The system is very sensitive and allows one to compare the immunogenic reactivity of related macromolecules.

An antiserum which did not neutralize T4 phage but which caused ± 80% inactivation of the TMVP-phage conjugates was used in the inhibition experiments. The sensitivity of the phage assay for the detection of antigens depends upon the affinity or avidity of the antibodies used (Sela and Haimovich, 1970). The less antibody needed to obtain significant inactivation, the more sensitive will be the assay. The serum which was used inactivated about 80%

of the phage conjugates in 5 h at a dilution of 1/6400 (Fig. 20). This inactivation could be inhibited by TMVP at different concentrations (Fig. 23). Significant inhibition (25%) was caused by the addition of 0,1 ml of protein at 0,1µg/ml, which demonstrates the extreme sensitivity of the assay for protein antigens. Experimental data of the type shown in Fig. 23 can be used as a calibration curve to measure the amount of TMVP in unknown solutions. As well as serving as a very sensitive means of detecting and assaying TMVP, the inhibition of inactivation also shows that inactivation of the conjugates is specifically due to TMVP antibody.

It has been shown by quantitative precipitation (von Sengbusch, 1965) and precipitation in gel (van Regenmortel, 1967) that amino acid exchanges due to mutation in positions 135 - 140 of the TMVP polypeptide chain, affect the antigenic properties of the intact virion. The TMVP attached to the phage was treated with NBS to prevent its aggregation at the high concentrations used in coupling. As NBS treatment modifies the tyrosyl residue in position 139 (Ohno et al., 1972), it was crucial to determine the effect of this chemical modification on the antigenicity of the TMVP. No difference between NBS-TMVP and native protein was discernible by double diffusion in gel (von Wechmar, 1976), nor was there any significant difference in the inhibition caused by the modified and non-modified proteins (Fig. 24). These results may indicate that the inhibition of TMVP phage inactivation is not sufficiently

sensitive to detect the chemical modification of a single residue. This seems likely as protein from CP415, a mutant with an amino acid exchange at position 140, and mutant 371 which has 2 exchanges in position 138 and 148 (Hennig and Wittmann, 1972), could not be distinguished from the wild-type protein (Figs. 25 and 27).

A further possibility is that the amino acids in positions 138, 139, 140 and possibly 148, do not lie in the region of an antigenic determinant of TMVP. This would mean that peptide 11 contains a neotope which has no immunological activity in the viral subunit. The validity of such a conclusion will depend on the relative sensitivities of the methods used to detect immunological changes in the protein and virus capsid respectively. A single exchange in the isolated protein may not be detectable, even by a method as sensitive as the modified phage technique. However, because of the 2130 subunits in the virus (and therefore 2130 exchanges per particle), this same exchange may be more easily detectable by spur formation in gel diffusion experiments.

It appears that for inhibition of phage inactivation to discriminate between proteins that differ only in a few amino acids, a certain minimum number of exchanges or modifications is required. For example Maron et al. (1972) found that unless antibodies specific for the active site region of lysozyme were used, affinity labelling of the enzyme did not affect its inhibitory properties.

Similarly, two duck lysozymes, differing only by a single amino acid in the "loop" region of the molecule, were not distinguishable by inhibition of an anti-lysozyme serum. It required 7 exchanges to distinguish turkey from hen lysozyme (Maron et al., 1970). These results, and those obtained with the mutants of TMVP suggest that even if one or two differences occur in an immunologically active region of the TMV subunit they will not be detectable by the modified phage technique.

The inhibition of inactivation of a TMVP antiserum could, however, distinguish protein from various strains of TMV. The inhibitory efficiency of the different proteins reflects the antigenic similarity of the inhibitor to the determinants on the immunogen. Therefore, the order of inhibition seen in Fig. 26 is a direct measure of the similarity of the inhibitors to TMVP (vulgare). The order of relationship for protein from Y-TAMV, U2 and CGMMV is the same as that found by van Regenmortel (1975) in precipitin tests, using many bleedings from different rabbits collected over a long period of time. Protein from Y-TAMV was a better inhibitor than U2-P which in turn was better than CGMMV-P. This order of relationship can also be correlated with the extent of homology in the amino acid sequence of the corresponding coat-proteins. Thus even though not all residues play a part in the immunogenicity of the TMVP molecule (von Sengbusch, 1965; van Regenmortel, 1967). There is a distinct correlation

between similarity in sequence and extent of neutralization inhibition. The modified phage technique in this case clearly established the order of relationship of the proteins of different strains, using a single bleeding from a single rabbit.

This discriminatory power, however, was not apparent in the inhibition of a heterologous system. Both U2-P and vulgare-P inhibited the neutralization by an anti-U2 serum to a very similar extent. This system could also not differentiate protein from mutant 371 from that of the wild-type (Fig. 27). The assay for U2-P was about 60 times more sensitive than when anti-TMVP serum was used. A disadvantage of this heterologous system is that only 50% of the pfu were neutralized by the U2-P antiserum. Thus the difference between the number of plaques on the assay plates for the inhibited and uninhibited inactivation reaction is reduced, and any error or scatter becomes more pronounced.

The inhibition of inactivation by TMVP antiserum was also used to demonstrate immunological activity in the tryptic peptides 4, 8, 11 and 12 of TMVP. Since all these peptides inhibited the inactivation to some extent, they must all contain antigenic determinants (Fig. 29, Table 12). As the concentration of the peptides was not known, it is not possible to comment on the relative activity of each.

When the inhibition of inactivation by virus and protein

was compared, approximately 10 times more TMV than TMVP was needed to inhibit inactivation so that 50% of the pfu survived (Fig. 28). Assuming a valence of 780 for the virus (van Regenmortel and Hardie, 1976) and 4 for the protein (van Regenmortel and Lelarge, 1973), it can be calculated from the relative molecular weights (17 500 and 40 x 10^6) that in a given amount of protein, there are about 10 times as many sites available for antibody binding. In effect, this means that equivalent inhibition is obtained when the same total number of sites of protein or virus are used (Fig. 28). However, only one of the four antigen sites of the protein exists at the surface of the virus since the other three are hidden. Therefore, the equivalent inhibition caused by protein and virus referred to above, requires about 4 times more "surface sites" of the virus. This may be due to the fact that the surface site is different in the virus (i.e. neotope), or that the absence of the hidden sites has to be compensated by a larger number of surface sites to achieve equivalent inhibition.

Since the virus cannot remove antibodies directed to TMVP cryptotopes from the antiserum, it should not be able to inhibit inactivation completely, unless only antibodies directed against virus surface sites are responsible for the inactivation of the modified phage. This would be the case if the protein subunit were attached to the phage particle in such a way that only the area which forms

the exterior surface in the virus is available to the antibody - an unlikely situation. It is far more likely that the virus preparation which was used in the inhibition contained some disaggregated subunits.

To conclude, this work has shown that the modified phage technique can be used for studying the serological properties of viral antigens. The wide range of affinities of individual antisera necessitates careful selection of antisera with the highest neutralizing ability. The method detects viral antigens and antibodies in the nanogram range and its sensitivity is thus comparable to that of passive haemagglutination assays. The modified phage technique has the advantage that it can be used with concentrations of virus protein at which aggregation is minimized. The various manipulations, once the modified phage have been prepared, are fairly simple to perform.

The preparation of the phage-conjugates is complicated by the need to prevent aggregation or precipitation of the viral subunits. In the present work this was achieved by treating the viral protein with NBS. The preparation of modified phage is essentially an empirical process and suitable conditions must be determined by experiment.

The inhibition of inactivation with homologous and related antigens can be used to detect small amounts of protein and virus, as well as to discriminate between protein from different strains, mutant proteins differing in one or two

amino acids can probably not be distinguished. The discriminatory ability may be improved if intact virus is used for inhibition, but this possibility was not tested. The data obtained by the use of modified phage has in most cases correlated well with that obtained by classical methods.

SUMMARY

1. The neutralization of native T4 phage by its antiserum was studied. Inactivation was found to follow first order kinetics. The inactivating strength of anti-T4 sera increased as immunization proceeded. As little as 10 µg of phage antibody could be detected.

2. T4 phage were modified by attaching the DNP hapten and were neutralized by DNP antibodies. Inactivation of the DNP phage did not follow first order kinetics, but showed a decrease in rate as inactivation proceeded. Neutralization was enhanced when the phage were assayed by the "decision" technique. Using this method of assay 4 ng of DNP antibodies could be detected. The inactivation of the modified phage was inhibited by the homologous DNP and the cross-reacting TNP hapten. This established the specificity of inactivation and showed that closely related haptens could be distinguished.

3. Lysozyme was attached to phage in an attempt to determine the effect of various conjugation parameters on the sensitivity of protein-phage conjugates to antiserum. It was found that glutaraldehyde functioned as a bifunctional reagent at both $4^{o}C$ and $23^{o}C$.

4. Conjugation of tobacco mosaic virus protein to phage could not be obtained using glutaraldehyde or TDIC as bifunctional reagent but was successful with BDB. The protein which

was coupled to the phage was treated with NBS to prevent aggregation of the subunits at high concentrations necessary for coupling. The sensitivity of the conjugates to antiserum was enhanced by performing the inactivation in the presence of PEG. The conjugates could be inactivated by antisera raised against TMVP, intact TMV and protein prepared from the U2 strain of TMV. Individual sera were found to differ greatly in their inactivating abilities.

The inhibition of inactivation of the TMVP phage was used to assay low concentrations of protein and to differentiate between protein prepared from different strains of TMV. Mutant protein with one or two amino acid exchanges could not be distinguished from wild-type virus protein. The presence of immunological activity in four tryptic peptides was demonstrated by their ability to inhibit the neutralization of the TMVP modified phage.

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APPENDIX

EXTINCTION COEFFICIENTS

For 1 cm path length cuvettes.

	Wavelength (mm)	$E^{0,1\%}$	Reference
TMV	260	3,1	Hardie and van Regenmortel, (1975)
TMVP	280	1,27	Fraenkel-Conrat (1957)
NBS-TMVP	281	1,13	Inoue *et al.* (1974)
Y-TAMVP	282	1,31	Hendry (1976)
CGMMVP	280	0,92	Determined using immersion refractometer
IgG	280	1,4	Hardie and van Regenmortel (1975)
Bacteriophage T4	260	E = 1,0 for 1 x 10^{11} particles	Haimovich *et al.* (1970a).

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