

## Neutralization kinetics studies with type SAT 2 foot-and-mouth disease virus strains. 1. Factors that influence the rate and pattern of neutralization

BY M. M. RWEYEMAMU, J. C. BOOTH AND T. W. F. PAY

*The Wellcome Foundation Ltd, Wellcome Foot-and-Mouth Disease Vaccine Laboratory, Pirbright, Woking, Surrey, U.K.*

(Received 28 July 1976)

### SUMMARY

A study of the kinetics of inactivation of foot-and-mouth disease virus type SAT 2 strains revealed that in most cases the rate of neutralization was not rectilinear. Deviations from first-order kinetics observed represented biphasic or parabolic and stepwise reactions. The initial rate was rapid and showed no lag phase or shoulder. The effects of deviations from linearity could be minimized by dilution of antiserum to a suitable extent. Treatment of virus-antibody mixtures with anti-species globulin resulted in enhancement of the rate of neutralization of homologous and heterologous reactions without significantly altering the relation between the two. This treatment also considerably reduced the amount of the persistent fraction. In attempt to disaggregate virus it was observed that sodium dodecyl sulphate inhibited neutralization of virus by specific antiserum.

### INTRODUCTION

A study of the kinetics of neutralization of animal viruses by antibody was first described by Dulbecco, Vogt & Strickland (1956). They demonstrated that neutralization proceeded as a first-order reaction with respect to time but eventually levelled off to reveal a proportion of the virus population which resisted inactivation by antiserum giving rise to the so-called 'persistent fraction'. McBride (1959) elaborated these studies and demonstrated the use of neutralization kinetics in the differentiation of poliovirus strains by comparison of homologous and heterologous rates of neutralization. This method has since found wide application in strain differentiation of such viruses as poliovirus (McBride, 1959; Ozaki, Diwan, McBride & Melnick, 1963), herpes simplex (Wheeler, Briggaman & Henderson, 1969; Ashe & Scherp, 1963), infectious bovine rhinotracheitis (bovine herpes virus 1) (Buening & Gratzek 1967, Potgieter & Maré 1974), rhinoviruses (Cooney, Kenny, Tam & Fox, 1973), and Japanese encephalitis virus (Hashimoto & Prince, 1963).

Capstick, Sellers & Stewart (1959) made the first study of the kinetics of neutralization of foot-and-mouth disease (FMD) virus. They detected anomalies in the rate of virus neutralization. Hence although they considered that strain

differences could be determined based on the calculation of neutralization rate constants they preferred the plaque reduction method.

Bradish, Farley & Ferrier (1963) regarded FMD virus neutralization as being so rapid as to render quantitative kinetic analysis impossible. Recently there has been an increasing interest in the use of neutralization kinetics methods in FMD strain differentiations. But most reports have been concerned with qualitative differences only (Wagner & Cowan, 1971; Martinsen, 1971; Forman, 1975).

The present report describes the kinetics of neutralization of type SAT 2 FMD virus strains and in the accompanying paper the use of the neutralization kinetics in the differentiation of SAT 2 FMD virus strains is described and compared with other serological methods in common use in our laboratory.

#### MATERIALS AND METHODS

##### *Virus strains*

The FMD type SAT 2 virus strains used were from the vaccine bank seeds in contemporary use at the Wellcome Foundation Vaccine Laboratories at Pirbright, U.K., and Nairobi, Kenya. For neutralization kinetics studies most strains were cloned in BHK-21 Cl.13 cells by terminal plaque picking on three successive passages. Stocks were prepared by passaging in IB-RS-2 pig kidney cells (de Castro, 1964) once and small volumes were stored at  $-70^{\circ}\text{C}$ . Virus assay was by the plaque titration method (Mowat, 1962) using 1% agar in tris-buffered Eagle's medium as overlay. The overlay contained 150  $\mu\text{g/ml}$  DEAE-dextran. Plaques were counted after 48 h at  $37^{\circ}\text{C}$ .

##### *Antisera*

Groups of four rabbits were injected intravenously, on two occasions 21 days apart, with BHK-21 cell culture virus harvests that had been concentrated 100-fold by precipitation with ammonium sulphate and pelleting by centrifugation in an MSE 65 Superspeed at 30,000 rev./min. for 1 h. Sera were collected at 7, 14, 21 and 42 days after primary inoculation. Unless otherwise specified, serum from the final bleeding was used as the standard rabbit antiserum.

Antisera were also raised in guinea-pigs against purified 146 S virus antigen (Brown & Cartwright, 1963) rendered non-infectious by treatment with 0.05% acetyleneimine (AEI). Guinea-pigs, in groups of 10, were each inoculated i.m. with 0.2 ml of a mixture of 50:50 antigen:adjuvant. The adjuvant consisted of 9 parts to 1 Marcol:Arlacel dispersed with 1% Tween 80. They were hyper-immunized by a similar injection at 21 days after inoculation. They were bled at 7 days and exsanguinated at 42 days after primary inoculation. Serum collected at the final bleeding was used as the standard hyperimmune guinea-pig serum.

##### *Neutralization kinetics*

Virus and serum preparations were pre-warmed separately in a water-bath at  $37^{\circ}\text{C}$  for 5 min. The basic neutralization kinetics procedure was to mix virus at a titre of  $10^5$ – $10^{6.5}$  p.f.u./ml with serum at  $37^{\circ}\text{C}$  and sample for virus survival at

varying times. Sampling was done by transferring 0.1 ml of the serum-virus mixture to 9.9 ml serum-free Eagle's medium at 4° C, i.e. 10<sup>-2</sup> dilution, and further diluting with the same medium to 10<sup>-3</sup> and 10<sup>-4</sup>. The samples were assayed for surviving virus by plaque assay on BHK-21 cells with agar overlay containing 150 µg/ml DEAE-dextran. Virus survival was plotted against time in minutes on a semi-logarithmic scale and the neutralization rate constant (*K*) was calculated from the inactivation curve using the formula of McBride (1959):

$$K = 2.3 \times \log \frac{V_0}{V_t} \times \frac{D}{t},$$

where *V*<sub>0</sub> = the number of p.f.u. at time zero, *V*<sub>*t*</sub> = the number of p.f.u. surviving after incubation with antiserum for *t* minutes, *D* = the reciprocal of the final dilution of the antiserum.

Normalized *K* values (NK) were expressed as percentage proportion heterologous to homologous *K* values obtained in the same test using the same antiserum preparation. The control consisted of a mixture of the virus with either heterotypic (type A, strain K18/66) antiserum or normal rabbit serum at the same dilution as the test serum. Sampling was carried out in the same manner as in the text. Hence *V*<sub>0</sub> was the amount of virus in the control mixture sampled at 10–15 s from the onset of the reaction. Virus survival in the control mixture at the end of the test run was required to be within 0.5 log units of the initial titre otherwise the entire test was invalidated.

## RESULTS

### *Pattern of neutralization*

Fig. 1 (*a–c*) illustrate the patterns of neutralization observed. Three types were observed in both homologous and heterologous reactions: linear (Fig. 1*a*), biphasic (Fig. 1*b*) and stepwise (Fig. 1*c*). The biphasic relationship was the most commonly encountered when tests were carried out in the region of antibody excess. After an initial rapid rate of inactivation to 70–90%, neutralization proceeded at a much reduced rate, thus resulting in an overall deviation from first-order kinetics. Such reactions were observed with antisera prepared in rabbits and guinea pigs and with sera collected at 7 days after inoculation or after hyperimmunization.

The stepwise reaction (Fig. 1*c*) was similar to that observed by Capstick *et al.* (1959). The initial rapid rate of inactivation was followed by a period of apparent stability lasting up to 10 min followed by another burst of rapid neutralization. In all reactions observed there was no shoulder or lag phase in the early stages of the neutralization reaction such as has been described by Lafferty (1963). The initial rate was therefore considered to tend to first order kinetics.

### *Effect of serum dilution on the rate of neutralization*

Fig. 2 summarizes the effect of serum dilution on the neutralization kinetics slope of the homologous reaction. With increasing dilution the slope became

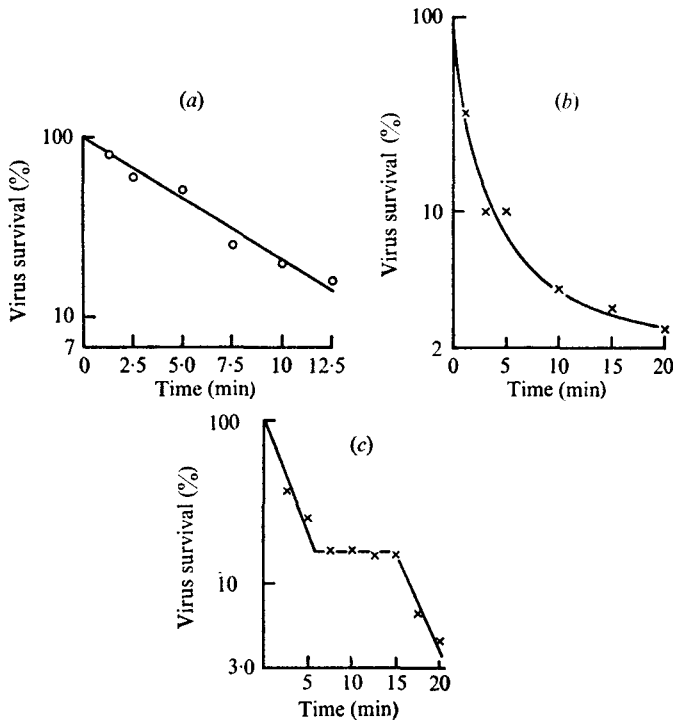


Fig. 1. Patterns of neutralization of FMD type SAT 2 viruses. (a) Neutralization kinetics of SAT 2 Swa 4/69 virus by SAT 2 Swa 1/68 rabbit antiserum demonstrating a first order reaction. (b) Neutralization of FMD SAT 2 Uga 6/70 virus by homologous 7 days post-inoculation rabbit serum demonstrating curvilinear kinetics. (c) Neutralization of FMD SAT 2 Ken 3/57 virus by Tan 5/68 rabbit antiserum demonstrating stepwise kinetics.

shallower so that at the highest dilution depicted (1/128) the kinetics slope was virtually linear. The initial rate of neutralization therefore was considered linear. The  $K$  values calculated on the initial slope at 1/32 and 1/128 dilutions were 55.2 and 56.6, indicating that the use of more dilute serum caused a proportionate decrease in the kinetics slope but did not alter the basic neutralization reaction or cause dissociation of the virus-antibody complex.

It was also observed that the dilution of antiserum which was required to produce a neutralization curve which was approximately linear was different for the corresponding homologous and heterologous viruses. For example, in Fig. 3, whereas the heterologous reaction (SAT 2 Ken 3/57 virus *v.* SAT 2 Uga 6/70 serum) was completely linear at 1/8 serum dilution, the homologous reaction was distinctly biphasic with the initial reaction being too rapid for meaningful analysis. It was therefore concluded that for strain comparisons sera would have to be diluted differently for homologous and heterologous reactions unless the viruses compared were serologically closely related.

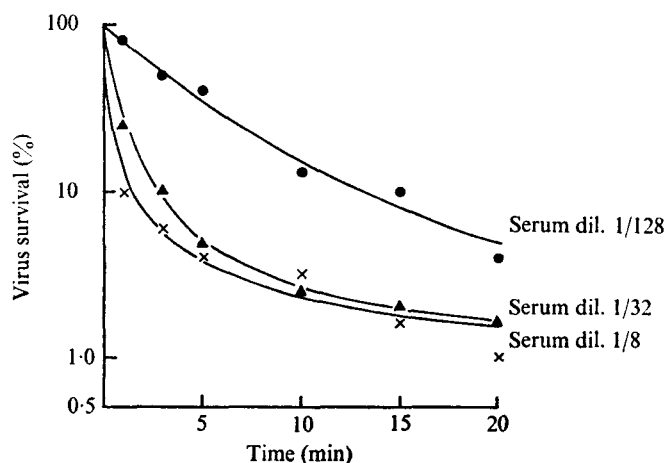


Fig. 2. The effect of serum dilution on the neutralization kinetics slope of FMD SAT 2 Uga 6/70 virus by homologous rabbit antiserum.

#### *Effect of complement*

To determine whether heat-labile factors in serum influenced the neutralization reaction, freeze-dried reconstituted guinea-pig serum with complement titre of about 150 MHD 50 per 0.05 ml was reconstituted and divided into two equal parts. One was heated at 56° C for 30 min to destroy complement effect and the other was used as complement. Each part was mixed with an equal volume of diluted SAT 2 Uga 6/70 rabbit antiserum which was then interacted with homologous virus in the neutralization kinetics.

There was no significant difference in either the kinetics slope or the pattern of neutralization. *K* values obtained for the serum were 40.5 and 36.8 for reaction in the presence of complement and without complement respectively.

#### *Effect of Ca<sup>2+</sup> and Mg<sup>2+</sup>*

Roslycky (1969, 1970) demonstrated a deviation from first-order neutralization kinetics of phages of *Agrobacterium radiobacter* due to the presence of divalent cations in the neutralization mixture. To determine whether divalent cations were responsible for some of the non-linear relationships observed with type SAT 2 FMD viruses, the SAT 2 Uga 6/70 virus and its homologous rabbit antiserum were both diluted in calcium and magnesium free phosphate buffered saline, pH 7.2, containing 0.1% bovine plasma albumin, before being interacted in the neutralization kinetics test. The diluent for the virus-antibody mixtures was also Ca<sup>2+</sup> and Mg<sup>2+</sup> free.

From the results of the test it was evident that neither the shape of neutralization curve nor the *K* value was altered significantly by the omission of the divalent cations in two replicate experiments.

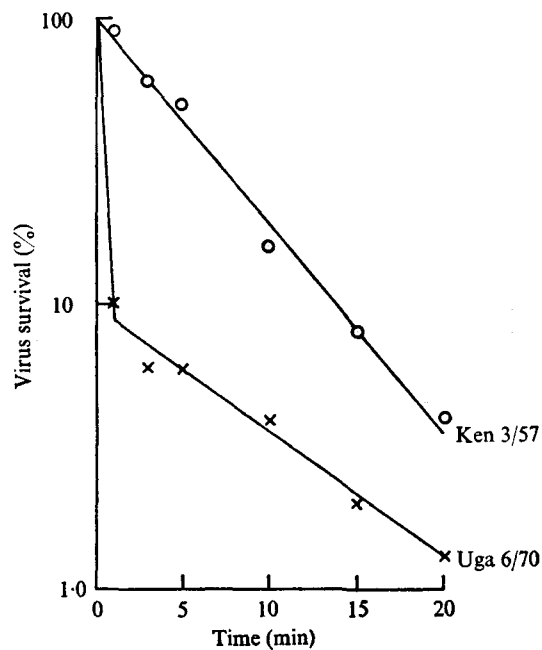


Fig. 3. Neutralization kinetics of FMD SAT 2 Uga 6/70 and Ken 3/57 viruses by Uga 6/70 rabbit antiserum diluted 1/8, demonstrating first-order kinetics for heterologous reaction and a biphasic rate for homologous reaction.

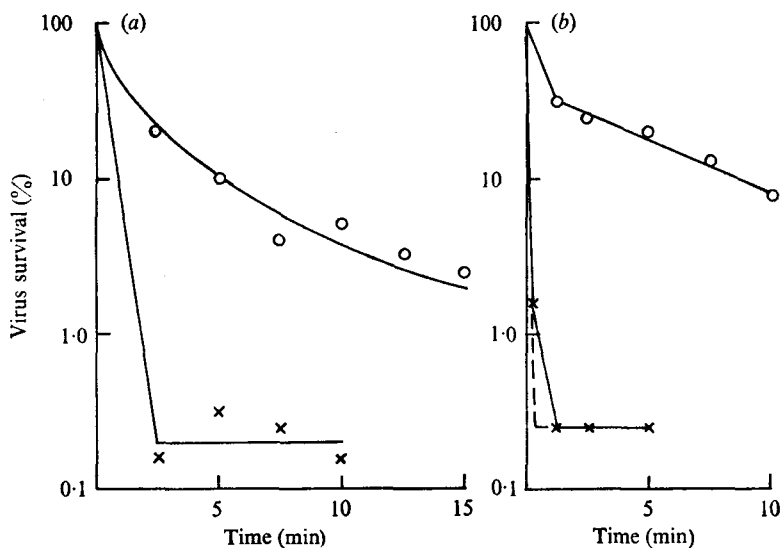


Fig. 4. Effect of anti-rabbit-IgG serum on the kinetics of neutralization of FMD SAT 2 viruses. (a) SAT 2 Tan 5/68 homologous reaction. (b) SAT 2 Uga 6/70 homologous reaction.

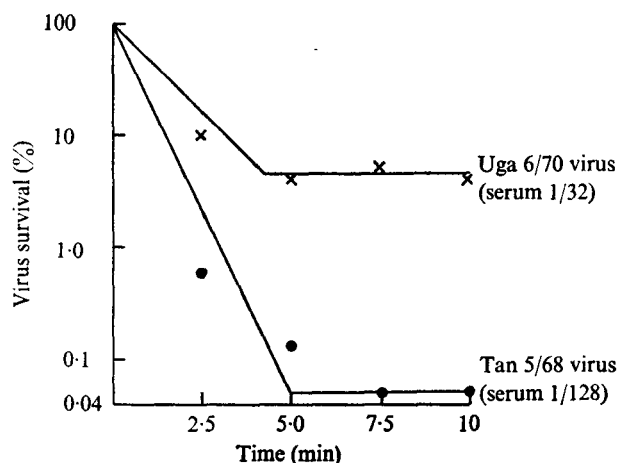


Fig. 5. The effect of anti-rabbit IgG serum on homologous and heterologous neutralization kinetics. Neutralization by FMD SAT 2 Tan 5/68 rabbit antiserum.

### Effect of pH

To determine if the pH at which the virus and antiserum were allowed to interact had any significant effect on the characteristics of the neutralization reaction, two sorts of experiments were carried out. In the first experiment rabbit antiserum to SAT 2 Uga 6/70 virus was diluted 1/32 in 0.67 M Sorensen's buffer at pH 7.0 and in 1 M Tris-HCl buffered saline at pH 7.5, 8.0 and 8.5, before being mixed with homologous virus diluted 1/10 in the same buffers. The kinetics slopes were similar at all four pH values tested as were the  $K$  values (43.5 at pH 7.0, 44.3 at pH 7.5, 41.2 at pH 8.0 and 43.1 at pH 8.5).

In the second type of experiment neutralization was allowed to proceed as usual at pH 7.4 and at varying time intervals replicate samples of the serum-virus mixture were diluted in buffers at pH 7.0, 7.5, 8.0, and 8.5, before assay of infectivity. There was no significant change either in the pattern of neutralization or the neutralization rate constant.

### Effect of anti-globulin serum

The following experiments were undertaken to investigate the role played by infectious virus-antibody complexes on the kinetics of neutralization of homologous and heterologous reactions and on the amount of the persistent fraction.

Rabbit antiserum to SAT 2 Uga 6/70 virus strain was mixed with homologous virus and incubated at 37 °C as in the standard neutralization kinetics test. At varying times after mixing 0.5 ml. was withdrawn and added to 0.5 ml of goat anti-rabbit IgG serum\* at a dilution of 1/20 and then placed on ice for 1 min. At the same time another 0.5 ml sample of the serum-virus mixture was mixed with 0.5 ml of normal goat serum also diluted 1/20 and was similarly left on ice for 1 min.

Finally 0.2 ml of each of the two mixtures (virus with antiserum and either

\* Miles Laboratories Inc., Kankakee, Illinois, U.S.A.

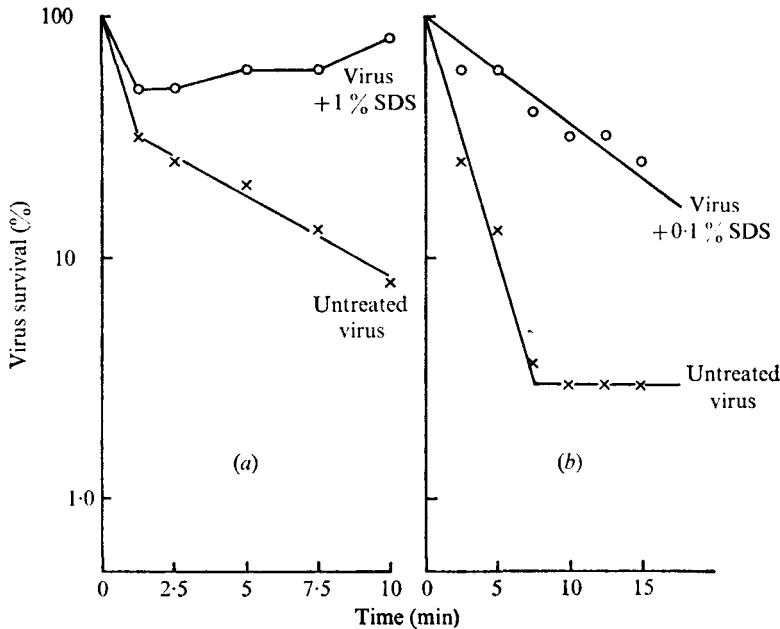


Fig. 6. Effect of SDS on neutralization kinetics of SAT 2 viruses by homologous rabbit antiserum (a) Uga 6/70 serum 1/32; 1% SDS treatment. (b) Tan 5/68 serum 1/32; 0.1% SDS treatment.

antiglobulin or normal goat serum) were transferred to chilled 9.8 ml serum-free Eagle's medium at 4° C and serially diluted tenfold for assay of residual virus in a plaque titration.

The effect of anti-IgG serum on the homologous reaction is illustrated in Fig. 4 for two strains, SAT 2 Tan 5/68 and SAT 2 Uga 6/70. In both cases the persistent fraction was reduced to near extinction. The kinetics slope was also increased about ten-fold.

Fig. 5 illustrates differences between homologous and heterologous kinetics in the presence of anti-IgG serum. Whilst both reactions were enhanced the ratio of heterologous to homologous reaction (normalized  $K$  value) was not altered significantly, being 12% in the illustrated example and 4% in the absence of anti-IgG serum.

#### *Effect of detergents*

Divergency from first-order neutralization due to viral aggregation can be eliminated by the use of monomeric virus obtained after filtration or treatment with detergents. The effect of the non-ionic detergent Nonidet P 40\* and the anionic detergents sodium deoxycholate\* and sodium dodecyl sulphate (SDS)\* on the neutralization kinetics of type SAT 2 FMD viruses was examined. Virus was treated with 1% detergent for 10–15 min. at 37° C before interacting with its homologous serum in the standard neutralization kinetics test.

\* BDH Chemicals Ltd, Poole, England.



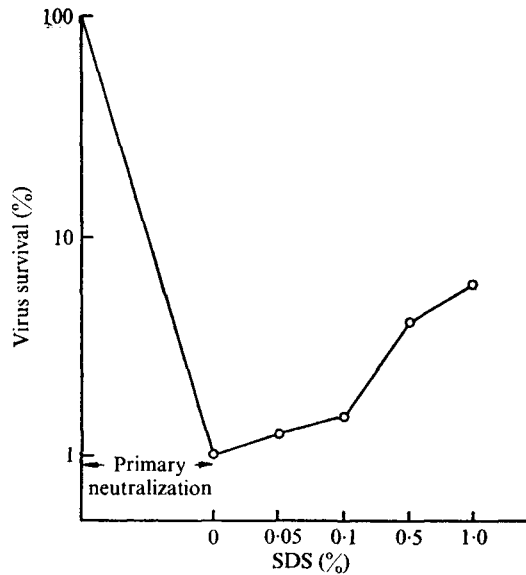


Fig. 7. Dissociation of virus-antibody complexes by sodium dodecyl sulphate (SAT 2 Uga 6/70 virus and homologous rabbit antiserum).

Nonidet P 40 and sodium deoxycholate had no effect on either the shape of the neutralization kinetics slope or the  $K$  value obtained. On the other hand, pre-treatment of the SAT 2 Uga 6/70 virus with 1% sodium dodecyl sulphate (SDS) for 10 min at room temperature was found to inhibit neutralization (Fig. 6). Similar results were obtained with the SAT 2 Tan 5/68 virus pretreated with 0.1% SDS.

To test whether this effect was due to the dissociation of virus-antibody complexes by SDS, SAT 2 Uga 6/70 virus was mixed with homologous antiserum such that antibody was in excess. Neutralization was allowed to proceed at 37° C and then equal samples of the serum-virus mixture were interacted with varying concentrations of SDS at 37° C for 10 min. Assay of residual virus infectivity demonstrated that moderate dissociation of the virus-antibody complex occurred with each successive increase in the concentration of SDS tested (Fig. 7). Such moderate dissociation alone probably did not account for the apparent inhibition of neutralization observed.

One possibility was that SDS enhanced the infectivity of virus-antibody complexes without breaking the antigen-antibody bond. To test such a hypothesis SAT 2 Uga 6/70 virus was treated with 1% SDS for 10 min at 37° C and then interacted with serum such that antibody was in excess. At varying time intervals samples of the serum-virus mixture were interacted with anti-species IgG serum before assay of residual virus. The results of the experiment are summarized in Fig. 8. It will be observed that in the absence of SDS, anti-IgG serum demonstrated virus-antibody combination to have been almost instantaneous. SDS, in the absence of anti-IgG, completely inhibited neutralization. Although anti-IgG demonstrated some neutralization in the presence of SDS the rate of neutralization

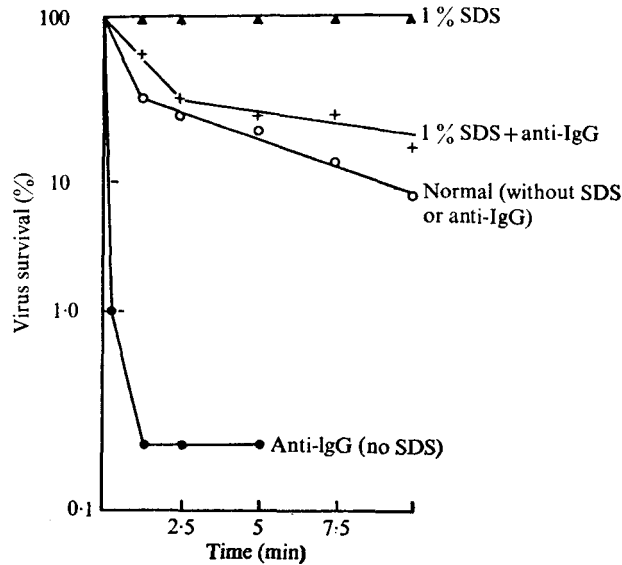


Fig. 8. Effect of SDS and anti-IgG on the neutralization kinetics of SAT 2 Uga 6/70 virus by homologous rabbit antiserum.

was about 10 times less than that achieved in the absence of SDS; it was even slower than the normal kinetics of interacting virus and serum without the enhancement of anti-IgG serum. The action of SDS was therefore considered not to be principally through the enhancement of infectivity of virus-antibody complexes

An alternative hypothesis was that SDS combined with or denatured antibody. SAT 2 Uga 6/70 rabbit serum was treated with 1% SDS for 30 min at 37°C. Excess SDS was removed by precipitation at 0°C with saturated potassium chloride. The neutralizing effect of the supernatant was compared with that of untreated serum in a neutralization kinetics. Identical reactions were obtained with SDS treated and untreated serum against homologous virus.

It was similarly demonstrated that SDS did not inhibit neutralization by either blocking or damaging antibody-combining sites on the virus. SAT 2 Uga 6/70 virus was pretreated with 1% SDS and excess SDS removed by cooling and precipitation with KCl. The neutralization kinetics of such pretreated and untreated virus against homologous serum were identical.

#### DISCUSSION

An investigation of the factors which influence the kinetics of neutralization of FMD virus by antibody was considered necessary before the technique could be confidently applied to a study of the serological differentiation of strains of FMD virus.

In our study two types of deviations from linearity were observed, a biphasic or parabolic reaction and a stepwise reaction. The size of the persistent fraction detected in most tests was high, often amounting to 5–10% of initial virus.

Deviations from linearity in many virus-antibody systems have been attributed to a variety of causes including viral aggregation (Wallis & Melnick, 1967), heterogeneity of antibody charge (Lafferty, 1963) and antibody class (Haimovich & Sela, 1969), heat-labile factors in serum and the pH of the neutralization mixture (Heinman, 1967; Hashimoto & Prince, 1963; Mandel, 1961; Hahon, 1969; Takabayashi & McIntosh, 1973).

Treatment of FMD viruses with sodium deoxycholate or Nonidet P 40 to disperse viral aggregates (Gwaltney & Calhoun, 1970) did not result either in enhancement of the neutralization reaction or elimination of the non-linear kinetics curves. Similarly there was no change in either pattern or slope of the inactivation curve when calcium and magnesium ions were omitted from the neutralization mixture, or when guinea-pig complement was added, or when neutralization was allowed to proceed at pH values ranging from 7.0 to 8.5. Furthermore a biphasic response was demonstrated with 7-day (essentially IgM) antiserum as well as hyperimmune serum (predominantly IgG), and with rabbit as well as guinea-pig antisera. That the initial rate of neutralization, at any rate, was of the first-order kinetics was demonstrated by using serum at high dilutions. Hence, whereas non-linear inactivation curves present problems when serological differences between strains are required to be expressed quantitatively, in practice such difficulties could be readily overcome since most antisera gave rectilinear neutralization reactions upon being diluted to a sufficient extent.

The addition of anti-species-IgG serum to the neutralization test mixture influenced the shape of the neutralization curve and resulted in a definite enhancement of the rate of neutralization. It also brought about the almost total obliteration of the persistent fraction, indicating that in foot-and-mouth disease virus-antibody interaction, the persistent fraction seems to be mainly due to infectious virus-antibody complexes as demonstrated in other virus-antibody systems (Ashe & Notkins, 1966, 1967; Philipson, 1966; Hahon, 1969; Cihak, 1973). The increase in the rate of neutralization is considered to reflect the reaction of neutralization as being two-step; the initial reaction being the attachment of antibody to virus or sensitization (Ashe & Notkins, 1967) and the final reaction to be neutralization of infectivity. The use of anti-IgG serum demonstrated FMDV-antibody attachment to be virtually instantaneous; it was complete within a few seconds if antibody was in excess. Of particular interest, from a practical point of view, was the finding that relative differences in the inactivation rate constants recorded in comparisons of homologous and heterologous strains were identical with or without the incorporation of anti-IgG serum in the test. It was inferred that serological relationship between two FMD virus strains depends on the extent to which they share common antigenic determinants rather than on the quality of their respective antisera. This also indicates that treatment with anti-globulin could be used to enable accurate serological comparisons of different strains to be made in the neutralization kinetics test with antisera which displayed low titres of neutralizing antibody. The application of this enhanced technique in the serological analysis of strains of FMD virus type SAT 2 is described in a further paper (Rweyemamu, Booth, Parry & Pay, 1977).

The influence of sodium dodecyl sulphate on the kinetics of neutralization of FMD type SAT 2 viruses by antisera is not readily explicable. SDS was found to have no significant, irreversible deleterious effect on either antibody or virus alone and did not enhance the infectivity of the virus-antibody complexes. It was found to inhibit neutralization strongly when antigen and antibody were allowed to combine in the presence of SDS but to exert only a moderate effect when interacted with established virus-antibody complexes. It seems to affect the association of antigen with antibody probably by reversibly altering the molecular charge of antibody. That SDS interacts with serum proteins has been well documented. For example, Putnam & Neurath (1944) demonstrated that serum proteins could be precipitated by SDS at concentrations ranging from 0.1 to 1% and that the action was related to the anionic properties of SDS. They also showed that the detergent-protein complex could be dissociated by barium chloride; the recovered protein was found to be only slightly denatured. Our results are in agreement with this and lead us to the conclusion that SDS under conditions of our tests did not significantly denature antibody in respect of its ability to combine with antigen. In this connection also Erickson & Neurath (1943) found antibodies which had been denatured by guanidine chloride to retain their serological activity. Another reaction of SDS with serum protein which has been of interest in this context is its precipitation of immunoglobulin as demonstrated in agar double diffusion test (Palmer, Martin, Hierholzer & Ziegler, 1971; Cho & Feng, 1974). But to the best of our knowledge the inhibitory effect of SDS on antigen-antibody reactions has not been reported previously.

We are grateful to Mr N. Parry, B.Sc., and Mr M. Francis for technical assistance.

#### REFERENCES

- ASHE, W. K. & SCHERP, H. W. (1963). Antigenic analysis of herpes simplex virus by neutralization kinetics. *Journal of Immunology* **91**, 658-65.
- ASHE, W. K. & NOTKINS, A. L. (1966). Neutralization of an infectious herpes simplex virus-antibody complex by anti-globulin. *Proceedings of the National Academy of Sciences of the U.S.A.* **56**, 447-51.
- ASHE, W. K. & NOTKINS, A. L. (1967). Kinetics of sensitization of herpes simplex virus and its relationship to the reduction in the neutralization rate constant. *Virology* **33**, 613-17.
- BRADISH, C. J., FARLEY, J. O. & FERRIER, H. E. N. (1962). Studies on the nature of the neutralization reaction and the competition for neutralizing antibody between components of the virus system of foot-and-mouth disease. *Virology* **18**, 378-400.
- BROWN, F. & CARTWRIGHT, B. (1963). Purification of radioactive foot-and-mouth disease virus. *Nature, London* **199**, 1168-70.
- BUENING, G. M. & GRATZEK, J. B. (1967). Comparison of selected characteristics of four strains of infectious bovine rhinotracheitis virus. *American Journal of Veterinary Research* **28**, 1257-67.
- CAPSTICK, P. B., SELLERS, R. F. & STEWART, D. L. (1959). The neutralization of the virus of foot-and-mouth disease by immune serum. *Archiv für die gesamte Virusforschung* **9**, 606-20.
- CHO, C. T. & FENG, K. K. (1974). Non-immunological precipitation of serum by sodium dodecyl sulphate in agar diffusion. *Applied Microbiology* **28**, 557-60.
- CHAK, J. (1973). Neutralization of infectious ME (Maus-Elberfeld) virus-antibody complexes by anti- $\gamma$ -globulin antibody. *Medical Microbiology and Immunology* **159**, 73-82.

- COONEY, M. K., KENNY, G. E., TAM, R. & FOX, J. P. (1973). Cross relationships among 37 rhinoviruses demonstrated by virus neutralization with potent monotypic rabbit antisera. *Infection and Immunity* **7**, 335-40.
- DE CASTRO, M. P. (1964). Behaviour of the foot-and-mouth disease virus in cell cultures; susceptibility of the IB-RS-2 cell line. *Archivos do Instituto biológico, São Paulo* **31**, 63-78.
- DULBECCO, R., VOGT, M. & STRICKLAND, A. G. R. (1956). A study of the basic aspects of neutralization of two animal viruses, western equine encephalitis virus and poliomyelitis virus. *Virology* **2**, 162-205.
- ERICKSON, J. O. & NEURATH, H. (1943). The serological activity of denatured antibodies. *Science, New York* **98**, 284-5.
- FORMAN, A. J. (1975). A comparison of some immunological methods for the differentiation of strains of foot and mouth disease virus. *Journal of Hygiene* **74**, 215-25.
- GWALTNEY, J. M. & CALHOUN, A. M. (1970). Viral aggregation resulting in the failure to correctly identify an unknown rhinovirus. *Applied Microbiology* **20**, 390-2.
- HAHON, N. (1969). The kinetics of neutralization of Venezuelan encephalomyelitis virus by antiserum and reversibility of the reaction. *Journal of General Virology* **4**, 77-78.
- HAIMOVICH, J. & SELA, M. (1969). Inactivation of bacteriophage T4, of poly-d-ananyl bacteriophage and of penicilloyl bacteriophage by immunospecifically isolated IgM and IgG antibodies. *Journal of Immunology* **103**, 45-55.
- HASHIMOTO, N. & PRINCE, A. M. (1963). Kinetics studies on the neutralization reaction between Japanese encephalitis virus and antiserum. *Virology* **19**, 261-72.
- HEINEMAN, H. S. (1967). Herpes simplex neutralizing antibody-quantitation of the complement-dependent fraction in different phases of adult human infection. *The Journal of Immunology* **99**, 214-22.
- LAFFERTY, K. J. (1963). The interaction between virus and antibody. I. Kinetics studies. *Virology* **21**, 61-75.
- MANDEL, B. (1961). Reversibility of the reaction between poliovirus and neutralizing antibody of rabbit origin. *Virology* **14**, 316-28.
- MARTINSEN, J. S. (1971). Two plaque-size variants of foot-and-mouth disease virus differing in neutralization by guinea-pig antisera. *Research in Veterinary Science* **12**, 399-400.
- MCBRIDE, W. D. (1959). Antigenic analysis of poliovirus by kinetics studies of serum neutralization. *Virology* **7**, 45-58.
- MOWAT, G. N. (1962). Growth of foot-and-mouth disease virus in a fibroblastic cell line derived from hamster kidneys. *Nature, London* **194**, 253.
- OZAKI, Y., DIWAN, R., MCBRIDE, W. D. & MELNICK, J. L. (1963). Antigenic characterization of oral polio-vaccine progeny by neutralization kinetics. *Journal of Immunology* **90**, 288-96.
- PALMER, E. L., MARTIN, M. L., HIERHOLZER, J. C. & ZEIGLER, D. W. (1971). Nonspecific precipitation of serum proteins by sodium lauryl sulphate in agar diffusion and immunoelectrophoresis. *Applied Microbiology* **21**, 903-6.
- PHILIPSON, L. (1966). Interaction between poliovirus and immunoglobulins. II. Basic aspects of virus-antibody interaction. *Virology* **28**, 35-46.
- POTGIETER, L. N. D. & MARÉ, C. J. (1974). Differentiation of strains of infectious bovine rhinotracheitis virus by neutralization kinetics with late 19S rabbit antibodies. *Infection and Immunity* **10**, 520-27.
- PUTNAM, F. W. & NEURATH, H. (1944). The precipitation of proteins by synthetic detergents. *Journal of the American Chemical Society* **66**, 692-7.
- ROSLYCKY, E. B. (1969). Anomaly in the neutralization kinetics of phages of *Agrobacterium radiobacter*. *Canadian Journal of Microbiology* **15**, 1033-42.
- ROSLYCKY, E. B. (1970). Radiobacterphage neutralization anomaly in relation to cations. *Canadian Journal of Microbiology* **16**, 527-33.
- RWEYEMAMU, M. M., BOOTH, J. C., PARRY, N. & PAY, T. W. F. (1977). Neutralization kinetics studies with type SAT 2 foot-and-mouth disease virus strains. II. Antigenic differentiation of vaccine strains. *Journal of Hygiene* (In the press).
- TAKABAYASHI, K. & MCINTOSH, K. (1973). Effect of heat labile factors on the neutralization of vaccine virus by human sera. *Infection and Immunity* **8**, 582-9.
- WAGNER, G. G. & COWAN, K. M. (1971). Immunochemical studies of foot-and-mouth disease. IX. Differences in neutralizing activities of guinea pig and bovine 19S and 7S antibodies. *Journal of Immunology* **106**, 656-66.

WALLIS, C. & MELNICK, J. L. (1967). Virus aggregation as the cause of the non-neutralizable persistent fraction. *Journal of Virology* **1**, 478–88.

WHEELER, C. E., BRIGGAMAN, R. A. & HENDERSON, R. R. (1969). Discrimination between two strains (types) of herpes simplex virus by various modifications of the neutralization test. *Journal of Immunology* **102**, 1179–92.