J. Hyg., Camb. (1966), **64**, 33
Printed in Great Britain

The influence of unheated guinea-pig serum on the neutralization of Semliki Forest virus by various antisera

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(*Received* 15 *July* 1965)

INTRODUCTION

The presence of fresh unheated animal serum in the neutralization mixture has been reported to reduce the persistent fraction of western equine encephalitis (WEE) virus (Dulbecco, Vogt & Stickland, 1956). Similarly, Sabin (1950) reported that unheated normal serum potentiates the neutralization of dengue virus. Hashimoto & Price (1963) demonstrated that fresh unheated guinea-pig serum did not modify the kinetics of the neutralization of Japanese B encephalitis virus.

In the course of studies of the modifications of the antibody binding capacity of normal and chemically modified Semliki Forest virus, the optimal experimental conditions of Semliki Forest virus neutralization had to be searched for.

The different neutralization curves obtained with different antisera, and the modifications of these curves resulting from addition of unheated guinea-pig serum or of EDTA to the neutralization mixture, are reported here.

MATERIALS AND METHODS

Virus

Purified and crude suspensions of Semliki Forest virus were used. The preparation of purified virus will be described elsewhere (Osterrieth & Calberg-Bacq (to be published). The crude virus suspension was a 10 % (w/v) suspension of infected unweaned mouse brains in saline. After homogenization, the suspension was clarified by centrifugation at 3000*g* for 30 min. The infectious titre of the suspension was 10^{10} plaque-forming units (pfu)/ml. The suspension was stored at −70° C.

Solutions

PBS: Phosphate buffered saline (0·15 M-NaCl, 0·01 M phosphate buffer pH 7·2).

GBSS: Tris Gey's solution of Porterfield (1960) pH 7·6 to which was added 0·1 % (w/v) bovine plasma albumin (Armour fraction V).

EDTA stock solution: 0·1 M ethylene diamine tetraacetic acid in distilled water adjusted to pH 7·0 with M-NaOH.

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Infectivity assays

Chick embryo-cell monolayers were used. The plaque technique that was used will be described elsewhere (Osterrieth & Calberg-Bacq, to be published).

Antisera

Hyperimmune rabbit serum

A rabbit was injected with 5 ml. of the crude suspension of Semliki Forest virus on days 1, 4, 12, 16, 20 and 24. After the second injection, the rabbit fell ill and developed paralysis of the hind legs. The animal was bled on the 45th day. The serum was centrifuged at 105,000*g* for 30 min. and the supernatant was stored at − 20° C. Before use, the serum was diluted 1/10 in PBS and heated at 56° C. for 30 min.

Absorbed hyperimmune rabbit serum

Ten ml. of rabbit antiserum was mixed with the pellet obtained by low-speed centrifugation of 10 ml. of 20 % w/v suspension of normal unweaned mouse brain in saline. The mixture was incubated at 37° C. for 1 hr. and then overnight at 4° C. The next day, the mixture was clarified by a 35 min. centrifugation at 1500*g* and then centrifuged at 105,000*g* for 30 min. The supernatant, called absorbed serum, was stored at − 20° C. Before use, it was diluted and inactivated as the rabbit antiserum.

Rabbit anti-mouse brain serum

A 10 % (w/v) normal unweaned mouse brain suspension in saline was clarified by centrifugation at 1500*g* for 30 min. The supernatant was used for immunization and 5 ml. were injected intramuscularly, on day 1, 5, 9, 13, 17 and 20 to two rabbits. The animals were bled on the 42nd day. The serum was stored at − 20°C. Before use, the serum was diluted and heated as previously described.

Ascitic fluid from hyperimmunized mice

Crude suspension of Semliki Forest virus was mixed with an equal volume of Freund complete adjuvent (Difco), and 0·2 ml. of this mixture was injected into the peritoneal cavity of mice, at 7-day intervals. At the time of the first injection the mice were 4 weeks old. The virus suspension used for the first two injections had been inactivated by a 7-day storage at 4° C. after addition of formalin (0·5 %, w/v). One week after the sixth injection the ascitic fluid was harvested. The average yield per mouse was 7·5 ml. After a few hours at room temperature a slight coagulum appeared, which was removed by low-speed centrifugation. The supernatant was opalescent and was centrifuged at 105,000*g* for 1 hr. The resulting supernatant displayed a fatty top layer that was discarded, and a clear lower layer that was stored at − 20° C. On thawing, a slight coagulum appeared once more, which was removed. Before use, the fluid was heated at 56° C. for 30 min.

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Immune guinea-pig serum

A guinea-pig was injected intracerebrally with 0·5 ml. of Semliki Forest virus crude suspension. Two weeks later, 1 ml. of the same suspension was injected intramuscularly. The animal was bled at the end of the third week. The serum was stored at -20° C. Before use, it was heated at 56° C. for 30 min.

Normal guinea-pig serum

Pools were made with the sera of twenty normal male guinea-pigs. These pools were stored in 2 ml. volumes at -20° C.

Neutralization tests

Two or fourfold serial dilutions of serum (or ascitic fluid) were made in PBS, in PBS containing 1% (v/v) normal unheated guinea-pig serum, in 0·002 M EDTA in PBS, in 0·01 M EDTA in PBS, in 0·002 M (or 0·01 M) EDTA in PBS containing 1% (v/v) unheated normal guinea-pig serum, and in PBS containing 1% (v/v) heated normal guinea-pig serum. To 0·5 ml. of these serum dilutions were added equal volumes of Semliki Forest virus suspension. The virus suspensions, either crude or purified, were always diluted in GBSS so as to give, in the neutralization mixture, a virus concentration of about 10^5 pfu/ml. The virus titre was determined from virus–PBS mixtures. Serum–virus mixtures and virus controls were kept at 0° C. for 3 hr. The mixtures were then diluted 1/10 in GBSS and maintained at 0° C., awaiting final dilution and plating. The final dilutions (3·16-fold steps in GBSS) were immediately inoculated to monolayers of chick embryo cells (1 ml. inoculum per Petri dish 8 cm. diameter) previously washed once with 4·5 ml. of GBSS. Adsorption was carried out at room temperature for 90 min. At the end of the adsorption period the excess of fluid was sucked out and the overlay poured.

RESULTS

Kinetics of the neutralization

These experiments were carried out with rabbit absorbed antiserum and purified virus. Final concentrations of serum in the neutralization mixtures were $10^{-3.3}$, $10^{-3.8}$, and $10^{-4.3}$. Incubation times at 0° C. were 15, 30, 60, 90 and 120 min. The results are given in Fig. 1. The curve C showed a descending part and a relatively horizontal part. The descending part is nearly a straight line, compatible with first-order reaction. The horizontal part tends to become parallel to the abscissa, showing that the surviving fraction tends to a minimal value characteristic for each serum concentration, at constant virus concentration. Neutralization appeared almost complete after 90 min. incubation, and 180 min. was chosen as standard incubation time for the other experiments.

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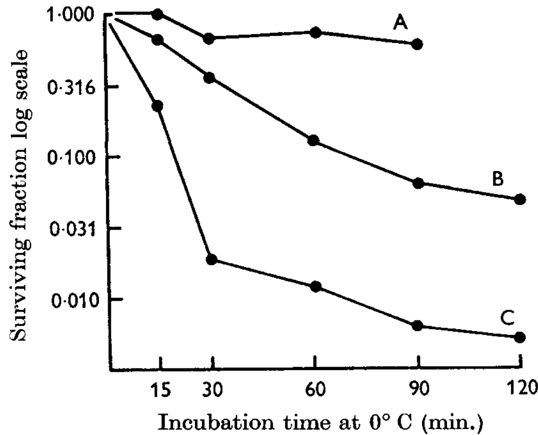

Fig. 1. Kinetics of the neutralization of purified Semliki Forest virus by absorbed hyperimmune rabbit serum. Final concentration of the serum in the neutralizing mixture: curve A $10^{-4\cdot3}$, curve B $10^{-3\cdot8}$ and curve C $10^{-3\cdot3}$.

Neutralization of purified Semliki Forest virus by rabbit absorbed antiserum

Figure 2 shows the neutralization curve obtained when a constant dose of Semliki Forest virus was incubated for a constant length of time with decreasing doses of rabbit absorbed antiserum, and the modifications which were obtained when 0·5 % (v/v) heat-inactivated normal guinea-pig serum, 0·5 % (v/v) unheated guinea-pig serum, EDTA (0·001 M and 0·005 M), or EDTA (0·001 M or 0·005 M) together with 0·5 % (v/v) unheated normal guinea-pig serum were present in the neutralization mixture. The log of the surviving fraction of the virus was taken as ordinate, the dilution of the antiserum in the neutralization mixture as abscissa. The dose of antiserum that neutralizes 90 % of the plaque forming units (pfu), was called neutralizing dose (Fazekas de St Groth, 1961).

When the antiserum concentration rises, the surviving fraction decreases steeply at first, very slowly afterwards. The value of the surviving fraction corresponding to the $10^{-2\cdot3}$ dilution of the antiserum is 0·0032. The neutralizing dose corresponds approximately to the $10^{-4\cdot1}$ dilution of the antiserum.

The presence of 0·001 M and 0·005 M EDTA in the antiserum-virus mixture does not modify the neutralization curve, nor does the presence of 0·5 % (v/v) heated normal guinea-pig serum.

When 0·5 % (v/v) unheated normal guinea-pig serum is added to the virus–serum mixtures, the neutralization curve is completely transformed. The surviving fraction still decreases at first when the antiserum concentration is raised, yet it quickly reaches a minimum value and finally increases although the antiserum concentration is still increased. The minimum value of the virus survival corresponds to a $10^{-4\cdot1}$ dilution of the antiserum. The surviving fraction obtained with the $10^{-2\cdot3}$ dilution of the antiserum is now 30 times greater than it was before addition of unheated normal guinea-pig serum. Moreover, the neutralizing dose of the antiserum that was $10^{-4\cdot1}$ becomes, in presence of normal unheated guinea-pig

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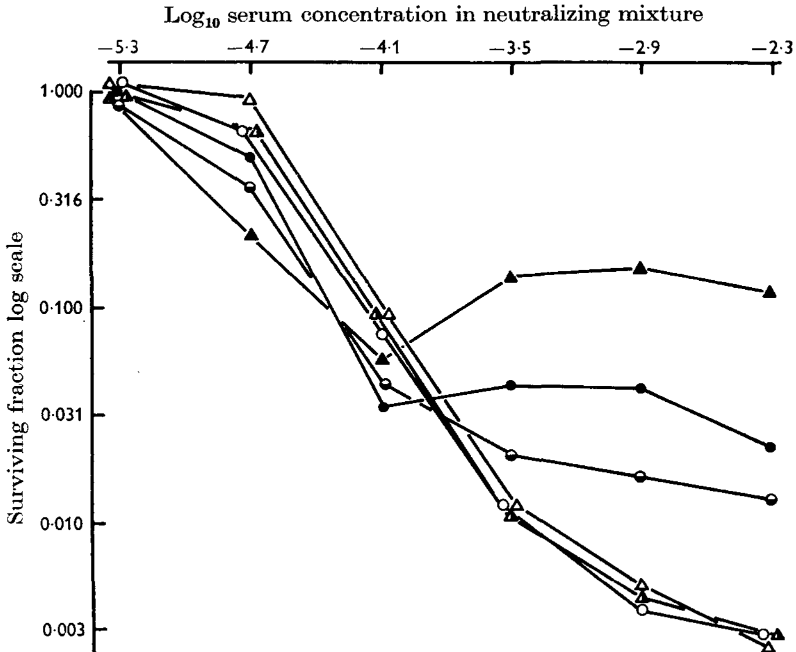

Fig. 2. Neutralization of purified Semliki Forest virus by absorbed hyperimmune rabbit serum. Serum diluent: △, PBS; ▲, PBS containing 1 % (v/v) heated normal guinea-pig serum; ▲, PBS containing 1 % (v/v) unheated normal guinea-pig serum; ○, 0·002 M EDTA in PBS, ⊖, 0·01 M EDTA in PBS containing 1 % (v/v) unheated normal guinea-pig serum; ●, 0·002 M EDTA in PBS containing 1 % (v/v) unheated normal guinea-pig serum.

Table 1. *Effect on the virus titre of the presence in the virus diluent of EDTA, complement, pre-immunization rabbit serum and anti-mouse brain rabbit serum*

	Log_{10} of the virus titres expressed in pfu/ml.		
Diluent	No serum	Normal rabbit serum 10^{-2}	Anti-mouse brain rabbit serum 10^{-2}
PBS	4·90	5·66	5·96
PBS + EDTA (10^{-3} M)	4·83	5·70	5·93
PBS + C′ (1/200)	4·92	5·73	5·90

Each column gives the mean results of two experiments.

serum, $10^{-4\cdot4}$, one half of its previous value. When the antiserum concentration is low, unheated normal guinea-pig serum decreases the surviving fraction and potentiates the neutralization slightly. Conversely, when the antiserum concentration is high, unheated normal guinea-pig serum increases the surviving fraction and inhibits neutralization.

When EDTA is added together with unheated normal guinea-pig serum the neutralization curve tends to return to normality although it still shows some

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distortion. The neutralization mixture was 0·0014 M in Ca^{2+} and 0·001 M in Mg^{2+} The 0·001 M concentration of EDTA was thus a little low, but the 0·005 M concentration was sufficient. Addition of EDTA did not completely suppress the action of unheated normal guinea-pig serum as did heating at 56° C. for 30 min.

The serum obtained from our rabbit before immunization had no effect on the virus titre either in the presence of unheated normal guinea-pig serum or in the presence of EDTA (Table 1). There were no pre-immunization non-specific neutralizing substances.

Unheated normal guinea-pig serum alone and EDTA alone had no effect on the virus titre (Table 1).

A second pool of normal guinea-pig serum was used. The same effect was observed.

Neutralization of crude and purified virus by absorbed and non-absorbed rabbit antiserum with and without unheated normal guinea-pig serum

Figure 3 shows the curves obtained: (A) with absorbed antiserum and purified virus, (B) with non-absorbed antiserum and purified virus and (C) with non-absorbed antiserum and crude virus suspension.

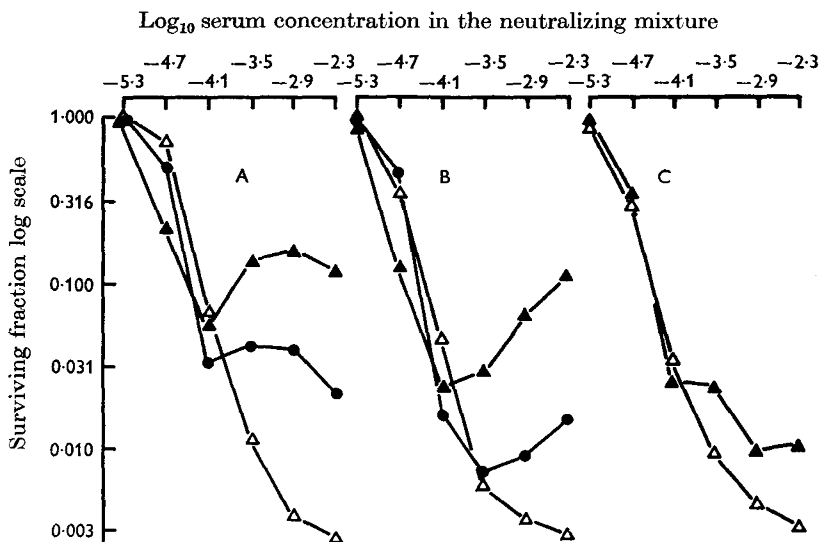

Fig. 3. Neutralization of crude and purified Semliki Forest virus by hyperimmune rabbit serum. Curve A, purified virus, absorbed serum; curve B, purified virus non-absorbed serum; curve C, crude virus, non-absorbed serum. Serum diluent: △, PBS; ▲, PBS containing 1% (v/v) unheated normal guinea-pig serum; ●, 0·002 M EDTA in PBS containing 1% (v/v) unheated normal guinea-pig serum.

Neither the use of absorbed serum nor the use of purified virus modified the relation between the surviving fraction and the serum concentration. The effect of unheated normal guinea-pig serum was similar in all three systems although it was increased by the use of purified virus and still further increased when absorbed

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antiserum was used together with purified virus. EDTA depressed the action of unheated normal guinea-pig serum on the neutralization by rabbit absorbed antiserum as well as by rabbit non-absorbed antiserum.

No neutralization was obtained with the serum of rabbits which were immunized with a suspension of brains of non-infected unweaned mice, either in presence of EDTA or in presence of unheated normal guinea-pig serum (Table 1). This shows that the immunological modifications induced in the rabbit by repeated injection of normal mouse brain antigens are not, alone, capable of virus neutralization.

Effect of the concentration of unheated normal guinea-pig serum on the neutralization of a constant dose of virus by a constant dose of serum

As only the inhibition of neutralization was measurable with a reasonable accuracy, when rabbit antiserum was used, the influence of the dose of complement was investigated in this zone. In these experiments we used absorbed rabbit antiserum at the final concentration, $10^{-2.9}$. This concentration corresponds to the

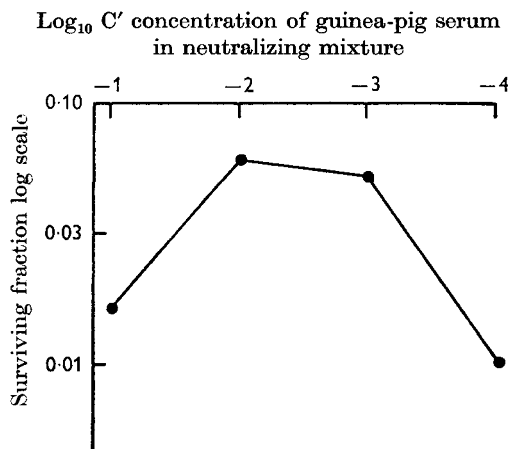

Fig. 4. Effect of the concentration of unheated normal guinea-pig serum on the neutralization of Semliki Forest virus by absorbed hyperimmune rabbit serum. Serum concentration: $10^{-2.9}$.

maximum inhibition of neutralization in presence of 0·5 % (v/v) of unheated normal guinea-pig serum. Figure 4 gives the mean results of three experiments. When the final concentration of unheated normal guinea-pig serum in the neutralizing mixture is raised from 0·01 % to 10 % (v/v) the surviving fraction increases, reaches a maximum value and then decreases. There is an optimal concentration of complement that corresponds to a maximum in the inhibition of neutralization. Above this optimal concentration of complement, the inhibition of the neutralization is less pronounced.

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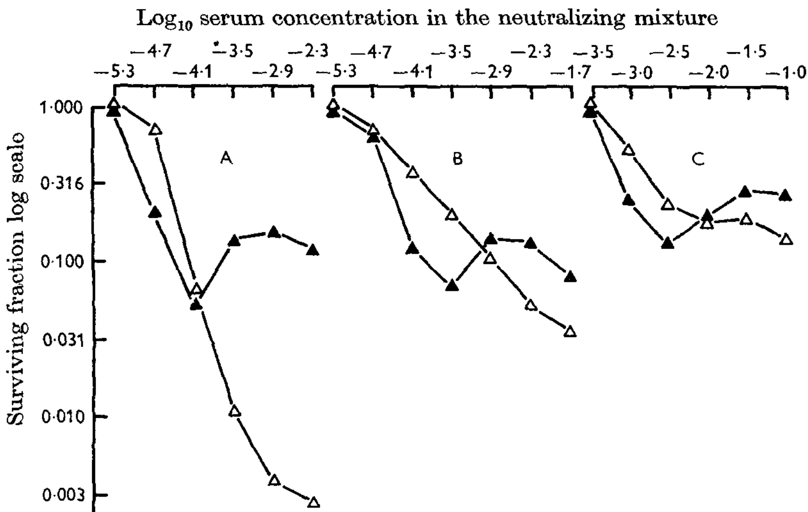

Fig. 5. Neutralization of purified Semliki Forest virus by various antisera. A, Absorbed hyperimmune rabbit serum; B, ascitic fluid from hyperimmunized mice; C, immune guinea-pig serum. Serum diluent: △, PBS; ▲ PBS containing 1% (v/v) unheated normal guinea-pig serum.

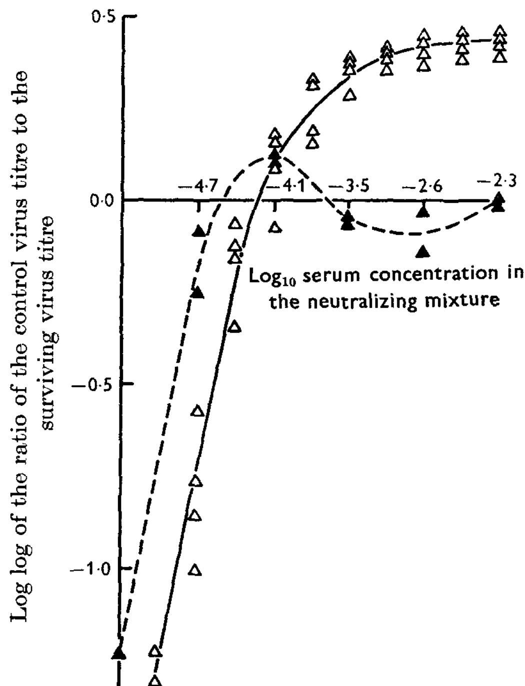

Fig. 6. Log log plot of the neutralization curve of Semliki Forest virus by absorbed hyperimmune rabbit serum. Serum diluent: △, PBS or 0·002 M EDTA in PBS; ▲, PBS containing 1% (v/v) unheated normal guinea-pig serum.

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The effect of unheated normal guinea-pig serum on the neutralization of Semliki Forest virus by antiserum of different origins

We compared the action of unheated normal guinea-pig serum on the neutralization of Semliki Forest virus by: (A) rabbit absorbed antiserum, (B) mouse ascitic fluid, and (C) guinea-pig antiserum (Fig. 5). The two sera and the ascitic fluid had quite different neutralizing potencies, but the shape of the neutralization curves were similar. The effect of unheated normal guinea-pig serum was the same in all instances, decreasing the surviving fraction at low antibody concentrations and increasing it at high concentration of antiserum. The action of unheated normal guinea-pig serum seems independent of the degree of immunization and of the serum donor species, although the extent of the potentiation and of the inhibition is not the same in all three cases.

The log log plot of the neutralization curve

As the neutralization curves obtained with rabbit absorbed antiserum with or without EDTA are identical, we plotted them together (Fig. 6). Log log v_0/v_s was taken as ordinate. v_0 is the virus concentration in the control, v_s the virus concentration in the virus-serum mixture after incubation. The log of the serum concentration was taken as abscissa (Fazekas de St Groth, 1961). The neutralization curve of Semliki Forest virus by absorbed antiserum in the presence of unheated normal guinea-pig serum was plotted in the same way.

DISCUSSION

Studying the neutralization of Semliki Forest virus by rabbit antiserum, we observed that the presence of unheated normal guinea-pig serum slightly potentiated the neutralization when low serum concentrations were used but inhibited the neutralization for greater serum concentrations. The phenomenon was observed with two batches of pooled unheated guinea-pig serum. Heat-inactivated guinea-pig serum had no effect. Addition of EDTA significantly reduced the effect of unheated guinea-pig serum. Thus, the effect of unheated guinea-pig serum on the neutralization appears to be an effect of complement.

When crude virus suspension was used instead of purified virus in the neutralization test, the effect of complement was lessened. Complement could be blocked by the mouse-brain lipids or other substances.

The effect of complement was observed when Semliki Forest virus was neutralized by means of rabbit antiserum, mouse ascitic fluid or guinea-pig antiserum; it is thus not related to the species of the serum donor or to the degree of immunization. Moreover, as on the one hand the effect of complement was more marked when absorbed rabbit serum was used, and on the other hand anti-mouse brain rabbit serum had no neutralizing potency either with or without complement, anti-mouse brain antibodies apparently are not involved in the observed phenomenon. Preimmunization rabbit serum had no neutralizing potency either with or without complement. Complement alone did not modify the virus titre. It thus

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appears that the observed phenomenon requires the presence of specific antiviral antibodies as well as the presence of complement; some neutralizing antibodies probably react differently in the presence of complement.

'Complement-requiring neutralizing antibodies' which are potentiated by complement have been described by Yoshino & Taniguchi (1965*a*) working with herpes simplex virus. These antibodies are present in greater proportion in the serum of animals which are bled early after infection than in the serum of hyperimmune animals. Such antibodies are probably also present in the antisera described here, because the potentiation of neutralization by complement at low serum concentration was more marked when immune guinea-pig serum was used instead of hyperimmune rabbit serum. But, in addition, we observed that neutralization by high concentrations of serum was inhibited by complement. This inhibition of neutralization, which is more marked when late serum is used instead of early serum, is probably related to another kind of antibody. These antibodies, unlike the complement-requiring neutralizing antibodies of Yoshino & Taniguchi (1965*a*), would be present in greater proportion in late serum than in early serum. A similar inhibition of neutralization was perhaps observed by Dozois, Wagner, Chemera & Andrew (1949), who noticed that the potentiation of the neutralization of WEE virus in the presence of complement disappeared when the serum concentration in the neutralization mixture was raised to 25 % (v/v).

When the neutralization is plotted on the log log scale, the resulting curve is very similar to the theoretical and experimental curves described by Fazekas de St Groth (1961). The theoretical curve is based on a theory assuming that primary neutralization is a state of equilibrium between association and dissociation of virus–antibody complexes. As our results fit well with this curve, they do not contradict the theory. Moreover, as the values of the surviving fraction obtained with high serum concentrations are lower when undiluted virus–serum mixtures are inoculated to the cells, instead of dilutions of the neutralization mixtures (unpublished results), we have experimental evidence that dissociation does occur. In our tests we measured the surviving fraction of virus after dilution of the neutralizing mixture, so our results were biased towards the measurement of secondary irreversible neutralization. We measured a compound of secondary irreversible neutralization and of the equilibrium neutralization to be found in the final dilution of the virus serum mixture. Because our infectivity assay was always carried out with dilutions of the neutralizing mixture, as is the case in studies of the kinetics of the neutralization, the minimum surviving fraction obtained with high serum concentrations corresponds to the non-neutralizable fraction of Dulbecco *et al.* (1965). According to Fazekas de St Groth & Reid (1958), Fazekas de St Groth (1962) and Lafferty (1963*a*, *b*, *c*) this non-neutralizable fraction is the result, first, of the presence in the antiserum of non-neutralizing antibodies, secondly, of the decreasing probability of irreversible virus–antibody unions as the virus surface becomes saturated with antibodies and, finally, of the increasing importance of non-avid antibodies when the serum concentration is raised. The modification of the neutralization curve in the presence of complement is in good agreement with these hypotheses. The observed potentiation of neutralization would be an

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increase of the secondary irreversible binding of avid neutralizing antibodies. The inhibition of neutralization which is, on account of the experimental design, an inhibition of irreversible neutralization, would be either an increase of neutralization by non-avid antibodies (followed upon dilution by dissociation) or less probably an increase of irreversible binding of non-neutralizing avid antibodies. The theory of Yoshino & Taniguchi (1965*b*) assuming that antibodies are always irreversibly attached to the virus but that the neutralization is a secondary process which can be enhanced by complement, could explain the potentiation but could not easily explain the inhibition of neutralization by complement. The lower inhibition of neutralization observed with high concentration of complement could be due to the fact that at the final dilution, when the diluted neutralizing mixture is in contact with the cells, a decrease of the irreversible neutralization is masked by an increase of an equilibrium neutralization involving a joint action of complement and antibody. This is compatible with the fact that this phenomenon was observed at high concentration of serum as well as of complement.

SUMMARY

The neutralization of a constant dose of Semliki Forest virus by various doses of different antisera was studied. The presence of complement (1/200, v/v) in the neutralization mixture inhibited the neutralization by high concentration of antiserum and somewhat potentiated neutralization by low serum concentration. Because the experiments were biased towards a measure of irreversible neutralization, the inhibition observed in the presence of complement appeared to be an inhibition of irreversible neutralization. This inhibition was interpreted as a dissociation of a complement binding virus–antibody complex. The antibodies involved appeared to be virus-specific.

We are indebted for their technical aid to Mrs M. Heusden, Mr R. Mommens and Mr J. L. Renson.

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Note added in proof. Similar findings on the inhibition of virus neutralization were recently described by Westaway, A. G. (1965). The neutralization of arboviruses. I. Neutralization in homologous virus-serum mixtures. *Virology*, **26**, 517.

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