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A Study of the Basic Aspects of Neutralization of Two Animal Viruses, Western Equine Encephalitis Virus and Poliomyelitis Virus

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A study of the process of neutralization of two animal viruses, Western equine encephalomyelitis (WEE), and poliomyelitis type 1 (P1), was carried out by using the plaque technique as assay method. In all experiments the antibody acted on the virus in a test tube and the proportion of the virus escaping neutralization was determined after having removed the remaining free antibody either by dilution or by washing the assay plates.

The neutralization was found to proceed with time as a first order reaction; its rate was proportional to the concentration of the antibody and was affected by temperature as a process whose activation energy is of the order of 6000 calories. In numerous tests, the virus-antibody complexes were found to be stable at 37°, pH 7.5 and physiological ionic strength. No reaction (P1 virus) or only a slight reactivation (WEE virus) occurred upon dilution of an incubated virus-antiserum mixture. Reactivation occurred, on the contrary, if high titer virus, either active or inactivated by ultraviolet light, was added to an incubated WEE virus-antiserum mixture. This type of reactivation is attributed to the collision of the virus-antibody complexes with free virus particles.

When mixtures of virus and antibody were incubated for a long time, a fraction of the virus remained active, even at highest concentrations of antibody. This persistent fraction could not be attributed to virus particles reactivated by dissociation of the antibody, to absence of cofactors or to presence of inhibitors of virus action, and was found to be nonhereditary. At low antibody concentrations, the final virus survival depended on the antibody:virus ratio. This dependence suggested the introduction of an antibody equivalent of the virus as a measure of the antibody titer.

A theory of neutralization was developed and on its basis some parameters of neutralization were introduced and determined in actual cases. A hypothesis was proposed to explain certain features of experiments of neutralization carried out by the end-point technique, in which the antibody is present in the cultures where the surviving virus is titrated.

The plaque technique for animal viruses allows an accurate quantitative determination of the infectious titer of a given virus suspension; it should, therefore, be suitable for a quantitative study of the basic aspects of the process of inactivation (or neutralization) by antibody.

An extensive study of the neutralization process has been carried out in the past by Burnet et al. (1937); the authors used for their assays the method of counting pocks on the chorioallantoic membrane of the chicken embryo. This method, although less accurate than the plaque technique, is in many respects fairly satisfactory; it has, however, the serious drawback of not permitting certain experiments, which are essential for the study of the process of neutralization. This fact explains part of the discrepancy between the conclusions emerging from the present experiments and those derived by Burnet et al. The subsequent work on neutralization of animal viruses was mainly carried out with the end-point method. As will be discussed in the present article, the latter method is unsuitable for many reasons.

In the present work, much emphasis will be given to the study of the kinetic aspects of neutralization. This will be done in view of the important results obtained by this method in the study of the neutralization of bacteriophage (Delbrück, 1945; Jerne and Avegno, 1956; Mandell, 1955). Furthermore, particular attention will be devoted to the problem of dissociability of the virus-antibody complexes. Methods will be developed for the determination of certain parameters of neutralization. Finally, a hypothesis will be brought forward to explain the marked influence of the cell system on certain results obtained by the end-point technique.

MATERIALS AND METHODS

Solutions and media. The phosphate-buffered saline (PBS), the tissue culture fluid, the chicken-embryo extract, the agar overlay have been previously described (Dulbecco and Vogt, 1954*a*).

Preparation of monolayer tissue cultures. The preparation of monolayer cultures of chicken embryo cells has been given in detail (Dulbecco and Vogt, 1954*b*). The cultures of rhesus monkey kidney cells were prepared according to the modification described by Youngner (1954).

Assay method. The plaque method of assay, as described previously both for Western equine encephalitis virus and poliomyelitis virus (Dulbecco and Vogt, 1954*a*, *b*), was used throughout the work.

Virus. The source of the Western equine encephalitis (WEE) virus

and the preparation of the virus stocks was the same as already described (Dulbecco and Vogt, 1954*b*). In the case of poliomyelitis virus, three different lines of type 1 (P1) virus, strain Brunhilde, were used: wild type, r30, r68. Lines r30 and r68 were obtained from the wild type after 15 and 34 serial passages, respectively, through monkey kidney cultures. (The Mahoney strain was obtained from Albert Sabin.)

Antisera. The following two antisera were used against WEE virus: (a) Horse hyperimmune serum, prepared by The Jensen-Salsbery Laboratories, Inc., Kansas City, Mo. (supplied by Dr. Lennette). (b) Rabbit serum. This serum was prepared in the following way. A rabbit was inoculated twice intramuscularly with 1.1 ml of an emulsion containing 0.5 ml of ultraviolet-inactivated stock virus, 0.5 ml mineral oil and 0.1 ml detergent. Six similar injections in which, however, active virus was used, followed at 2–3 day intervals. After 2 weeks a series of subcutaneous injections of 1 ml active virus without adjuvant was started; 5 injections were given at 2–3 day intervals. After another 2-week period, a third series, similar to the second one, was carried out. The animal was bled 10 days after the last injection.

For P1 virus, the following two antisera were used: (a) Monkey hyperimmune serum (M.S.), kindly supplied by Dr. Pait (Pool A 453, 1/23/50); (b) Rabbit antiserum (R.S.), obtained after three series of 9 subcutaneous injections, at 2–3 day intervals, of 1 ml undiluted Brunhilde virus tissue culture stock.

In some experiments serum heated at 56° for 30 minutes was used; in others, unheated serum. Both sera gave the same results.

The amount of antibody used in the experiments is expressed in antibody equivalents. One equivalent is the amount of antibody which, when mixed with virus and completely absorbed on it, inactivates the virus to a survival of e^{-1}. The reason for this choice will be indicated later. The equivalent is not an absolute measure, but determines a fixed ratio between the number of interacting antibody molecules and of virus particles.

RESULTS

Requirements for the Quantitative Study of the Interaction between a Virus and its Antibody

In the quantitative study of the interaction between a given virus and its antibody, we have to distinguish between two systems: 1. the *reaction system*, represented, for example, by the neutralization tube in

which virus and antibody are mixed and allowed to interact for various periods of time; 2. the *measuring system*, which measures the degree of interaction that has taken place in the neutralization tube within the time interval studied.

In our case, the measuring system consisted of monolayer cultures on which the surviving virus produced plaques.

If large quantities of antibody are carried from the reaction system to the measuring system, the extent of neutralization that took place in the reaction system cannot be measured as already shown by Burnet *et al.* (1937) in the case of vaccinia virus. The following experiment further illustrates this point.

Mixtures of serum and WEE virus were prepared, containing closely spaced serum concentrations and virus in a concentration adequate to obtain a countable number of plaques (the proper concentration of the virus for the different serum concentrations had been determined in preliminary experiments). In all mixtures, the amount of antibody was in excess over the virus, as will be shown later. The tubes containing the virus-serum mixtures were incubated for a time sufficient for the completion of the neutralization reaction (as shown by other experiments). Aliquots, in two cases at a dilution 1:500, of each tube were then plated onto two plates. After an incubation period of 30 minutes, one plate of each pair was overlaid with agar, whereas the other was first washed by three changes of 10 ml PBS, and then overlaid.

As may be seen from Table 1 (last column), the virus survival, as determined on the washed and unwashed plate, respectively, was approximately equal when the serum was carried to the plate at a concentration of 10^{-3} or less (last two rows); at higher serum concentrations, the survival measured on the washed plates was higher than that determined on the unwashed plates, the difference becoming very large at high concentrations of the antiserum (first row). Since, as shown later, the virus-antibody complexes studied in this system, are practically undissociable under the conditions of the experiment, the reduction of the number of plaques on the unwashed plates must be attributed to the inactivation—by the carried-over antibody—of the progeny virus released by some of the infected cells. The same cells, in the absence of antibody, would have given rise to plaques.

The above experiment shows, therefore, that in this special system, antiserum at a concentration higher than 1×10^{-3} must not be carried onto the plate. This can be achieved either by diluting the virus-serum

TABLE 1

EFFECT OF CARRIED-OVER ANTIBODY ON THE DETERMINATION OF THE SURVIVING VIRUS FRACTION

Horse antiserum and WEE virus were mixed in the indicated proportions in separate neutralization tubes. After 2 hours' incubation, 0.6 ml of each tube was plated, at the indicated dilution, onto monolayer chicken embryo cultures. After an incubation period of 30 minutes at 37° to allow adsorption of the virus to the cells, some cultures were immediately overlaid with nutrient agar (= unwashed plates), whereas the others were first washed three times with 10 ml PBS and then overlaid (= washed plates).

Neutralization tubes		Dilution factor	Number of plaques on		Surviving virus fraction on		Ratio of (6) to (7)
Serum concentration (1)	Virus concentration (pfu)[a]/ml (2)	(3)	Washed plates (4)	Unwashed plates (5)	Washed Plates (6)	Unwashed plates (7)	(8)
5×10^{-2}	2.6×10^{7}	500	90		2.9×10^{-3}		
5×10^{-2}	2.6×10^{7}	1	confluent (>500)	0	$>3.2 \times 10^{5}$	$<7 \times 10^{-8}$	$>4.6 \times 10^{2}$
5×10^{-2}	2.6×10^{4}	1	9		5.8×10^{-4}		
1.7×10^{-2}	2.6×10^{7}	500	124		4.0×10^{-3}		
1.7×10^{-2}	1.3×10^{6}	1	confluent (>500)	176	$>6.4 \times 10^{-4}$	2.3×10^{-4}	>2.8
1.7×10^{-2}	2.6×10^{4}	1	16				
5.0×10^{-3}	6.2×10^{5}	1	68	260	1.8×10^{-3}	7×10^{-4}	2.6
1.0×10^{-3}	2.6×10^{4}	1	11	19	7.0×10^{-4}	1.2×10^{-3}	0.6
5.0×10^{-4}	2.6×10^{4}	1	17	19	1.1×10^{-3}	1.2×10^{-3}	0.9

[a] pfu = plaque-forming units.

mixtures before plating or by washing the plates after adsorption of the virus. Both procedures are legitimate since there is practically no reactivation of the neutralized virus upon dilution.

Similar conclusions were found to be valid for the system constituted by the P1 virus and its specific antibody.

Determination of the Kinetics of Virus Neutralization

Virus and antiserum properly diluted and prewarmed were mixed at time zero in the neutralization tube. The medium used for the suspension of the virus-antiserum mixture consisted of equal parts of PBS and of 20% chick embryo extract (1:1) in Earle's saline (ES); the embryo extract was used to diminish the heat inactivation of the virus. The volume of the mixture in the neutralization tube varied in different experiments between 0.2 ml—in a tube of 10 mm diameter—to 2 ml—in a tube of 15 mm diameter. The tubes were stoppered with rubber stoppers, and incubated at 37°. The content of the neutralization tube was diluted at regular time intervals and assayed for unneutralized virus. In some experiments, the incubation lasted up to 6 hours. A control tube, containing virus but no antibody, was treated similarly.

To analyze the results, the logarithm of the ratio: residual virus titer in the neutralization tube/virus titer in the control tube (= *survival*) was plotted against the time of incubation. The resulting curve will be called the *kinetic curve*.

Figure 1 gives an example of a kinetic curve obtained with WEE virus. As may be seen from the figure, the kinetic curve starts at time 0 as a straight line with a negative slope. After a few minutes, the slope decreases and the curve eventually becomes horizontal. For the purpose of discussion, we will therefore distinguish between the *descending part* and the *horizontal part* of the kinetic curve.

The descending part will be considered first. The characteristics of the straight initial part can only be determined in the presence of excess antibody, i.e., under conditions in which the antibody/virus ratio in the neutralization tube is high. A suitable ratio could be obtained, both for WEE and P1 virus, by using equal volumes of stock virus of titer pfu*/ml 10^8 to 10^9 diluted $1:2 \times 10^3$, and serum, diluted $1:10^3$ to $1:10^4$. It will be shown later that under these conditions antibody was present in large excess.

* pfu = plaque-forming units.

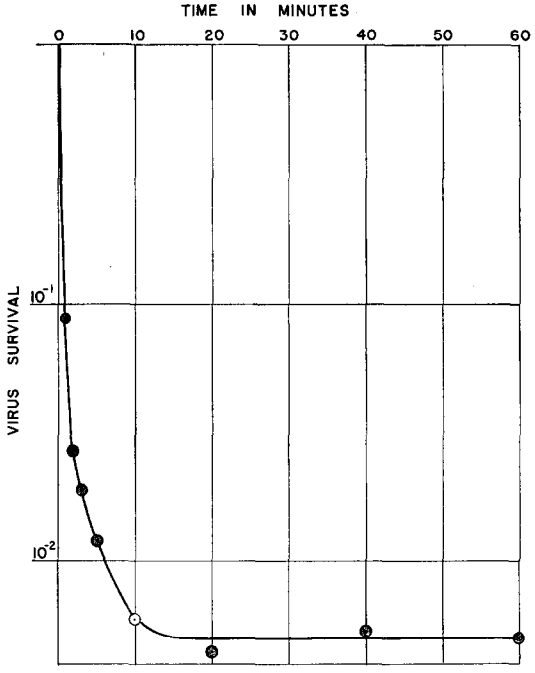

FIG. 1. Kinetic curve of neutralization of WEE virus. Horse antiserum at a concentration 4.75×10^{-3} and virus (5×10^{7} pfu/ml) were prewarmed and mixed at time $t = 0$ and incubated at 37°. At various subsequent times a sample of 0.1 ml of the mixture was blown into a small flask containing 25 ml of cold PBS, to insure correct timing. The contents of the various flasks were then diluted further and assayed for active virus.

Under the conditions described, the descending part of the kinetic curve remains straight to a survival of 10^{-2}, both for WEE and P1 virus. In repeated experiments with both viruses, it was established that the initial part of the curve is not significantly different from a straight line passing through the origin (Fig. 2). A lag which would cause the straight part of the curve to intersect the axis of ordinates at log 1.2 may yet exist; a longer lag is, however, very unlikely.

The descending part of the kinetic curve is thus of first order, to a close approximation. The slope of the straight part of the curve measures the rate of neutralization, i.e., the probability per time unit for a given virus particle to be neutralized in the presence of a given antiserum concentration.

The effect of the concentration of the antibody and of the temperature on the rate of neutralization were determined. The effect of concentration of the antibody was tested at 37° in the range of a serum concentration 10^{-3} to 10^{-4} for WEE virus, and in the range of a serum concentration 3.0×10^{-3} to 3.3×10^{-4} for P1 virus (Fig. 3). At all dilutions of the antiserum tested, the descending part of the kinetic curve maintained its first order character. In the range explored, the rate of neutralization was proportional to the concentration of the antiserum.

The effect of the temperature was tested in the range between 5° and 37° using WEE virus at a concentration 10^{-3} and serum at a concentration 1.7×10^{-4}. The kinetic curves were found to remain straight at the various temperatures (Fig. 4). The Arrhenius plot produced a nearly straight line from which an activation energy of the order of 6000 calories per mole of virus could be calculated.

We shall now consider the horizontal part of the kinetic curve. The

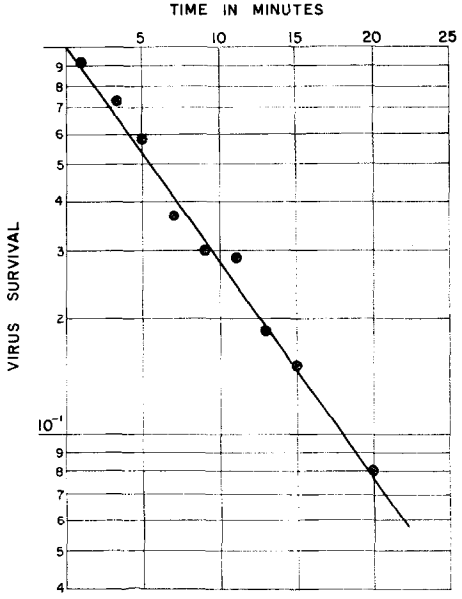

FIG. 2. Detailed initial part of the kinetic curve of neutralization of WEE virus. Horse antiserum at a concentration 3.15×10^{-4} and virus (5×10^5 pfu/ml) were prewarmed, mixed at time $t = 0$ and incubated at 37°. At various subsequent times a sample of 0.1 ml was blown into cold PBS to insure correct timing and then assayed for active virus.

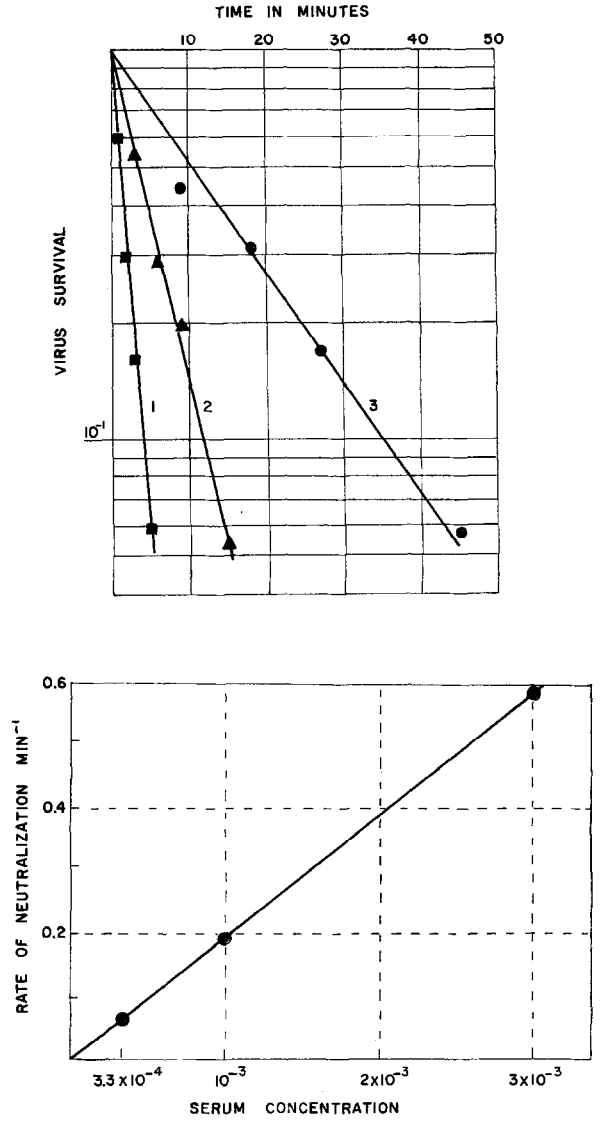

FIG. 3. Dependence of the slope of the kinetic curve on the concentration of the antiserum. The kinetic curves were determined by using Pl virus (Brunhilde wild type) at the concentration of 3.6×10^5 pfu/ml. The general procedure was that described for Fig. 2. The concentration of the antiserum (rabbit) was 3×10^{-3} for curve *1*, 10^{-3} for curve *2* and 3.33×10^{-4} for curve *3*.

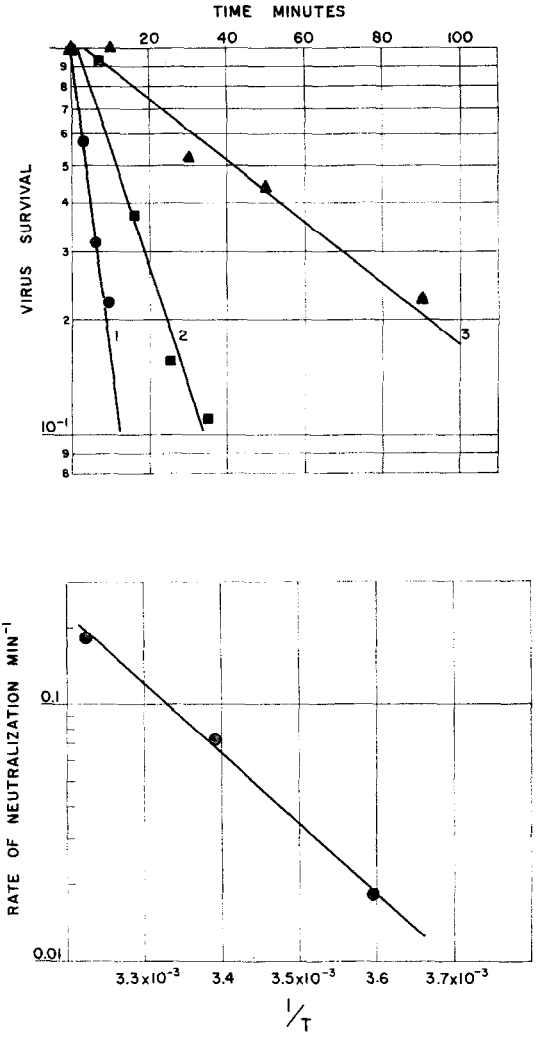

FIG. 4. Dependence of the slope of the kinetic curve on the temperature. The kinetic curves were determined by using WEE virus at the concentration 5×10^5 pfu/ml and rabbit antiserum at the concentration 1.7×10^{-4}. The general procedure was that described for Fig. 2; the serum, the virus, and the mixtures were however kept at different temperatures: for curve *1* at 37°, for curve *2* at 22°, for curve *3* at 5°.

virus survival corresponding to this part of the curve (= *final survival*) was found to be a function of the antibody/virus ratio in the neutralization tube. It decreases when the latter ratio increases; for high values of this ratio, however, the final survival tends to a constant value.

To investigate this dependence more quantitatively, serum-virus mixtures of various composition were incubated for 2 hours at 37° and the final survival was determined.

The results of some experiments with WEE virus are given in Table 2. They show that the final survival depends essentially on the ratio serum/virus in the neutralization tube and is independent of the absolute concentration of the reactants. Thus, aggregation of virus particles is not an important factor in neutralization. If the virus concentration was, however, too low—for example, 1/100 of the stock—less neutralization was observed. It is likely that this is owing to the fact that at low virus concentrations, neutralization was not yet completed after two hours. To avoid this complication only relatively high virus concentrations were used in the experiment of Table 2 and in the experiments that follow.

In these, detailed curves giving the logarithm of the final survival as a function of the serum/virus ratio in the neutralization tube were determined, both for WEE and P1 virus (Figs. 5 and 6). The serum virus ratio was changed by varying the antiserum concentration between 1/2 and 10^{-4}.[1]

Since the serum/virus ratio determined the multiplicity of neutralization, i.e., the number of neutralizing antibody molecules absorbed per virus particle (see Discussion), these curves will be called *multiplicity curves*.

The multiplicity curve resembles the kinetic curve, although it has an entirely different significance. It begins as a straight line at the origin, and then decreases in slope to finally reach a horizontal line. As seen in Figs. 5 and 6, the slope decreases gradually in the case of the WEE virus and more abruptly in the case of P1 virus.

The significance of the multiplicity curve and its use in determining the amount of antibody present in a given serum will be discussed later. We will presently analyze only a special feature of the curve, i.e., its transition into a horizontal line at high serum-virus ratios. Since under

[1] The final survivals of WEE virus in Table 2 and in Fig. 5 differ widely for the same S/V ratio: this discrepancy is introduced by the use of a different serum and of a different virus stock in the two experiments.

TABLE 2

DEPENDENCE OF THE FINAL VIRUS SURVIVAL ON THE SERUM/VIRUS RATIO IN THE NEUTRALIZATION TUBE

WEE virus and horse antiserum were mixed at the indicated concentrations. The virus-serum mixtures were incubated for 2 hours at 37° and then assayed for surviving virus.

Ratio[a] S/V	Virus concentration in the neutralization tubes (pfu/ml)					
	1.3×10^8	6.5×10^7	3.2×10^7	1.6×10^7	8.0×10^6	2.0×10^6
	Final virus survival					
7.7×10^{-12}	4.8×10^{-1}	2.2×10^{-1}				
1.5×10^{-11}	2.6×10^{-1} 1.6×10^{-1}		1.3×10^{-1} 2.3×10^{-1} 2.3×10^{-1}			
3.1×10^{-11}	5.7×10^{-2} 2.7×10^{-2}		6.0×10^{-2}	7.0×10^{-2}		
6.2×10^{-11}	1.1×10^{-2} 1.0×10^{-2}		1.6×10^{-2} 1.2×10^{-2}		1.6×10^{-2}	
2.5×10^{-10}			6.5×10^{-3} 5.0×10^{-3}			4.2×10^{-3}

[a] The ratio S/V is expressed as: $\frac{\text{serum concentration}}{\text{virus concentration (pfu/ml)}}$

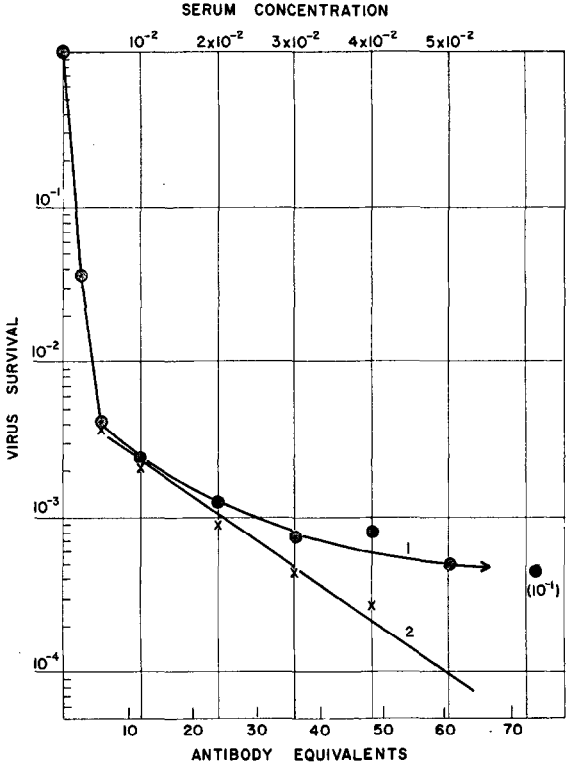

FIG. 5. Multiplicity curve of neutralization of WEE virus. A series of tubes containing WEE virus at a concentration of 5×10^8 pfu/ml and rabbit serum at various concentrations were incubated for 2 hours at 37°.; the mixtures were subsequently diluted and assayed for active virus. Curve *1* = experimental curve; Curve *2* = corrected for a persistent fraction of 4×10^{-4}.

these conditions, a large amount of free antibody could be demonstrated in the supernatant of the neutralization tube after high-speed centrifugation (see Table 10), a fraction of the virus population must escape neutralization.

To get some information on the state of this surviving virus fraction, the following experiment was designed:

A tube containing WEE virus diluted 1:2 and horse antiserum diluted 1:125 was incubated for 2 hours at 37°. The content of the tube was then split into two parts: to the first part, an equal amount of fresh virus was added, to the other part an equal amount of serum diluted 1:125. The

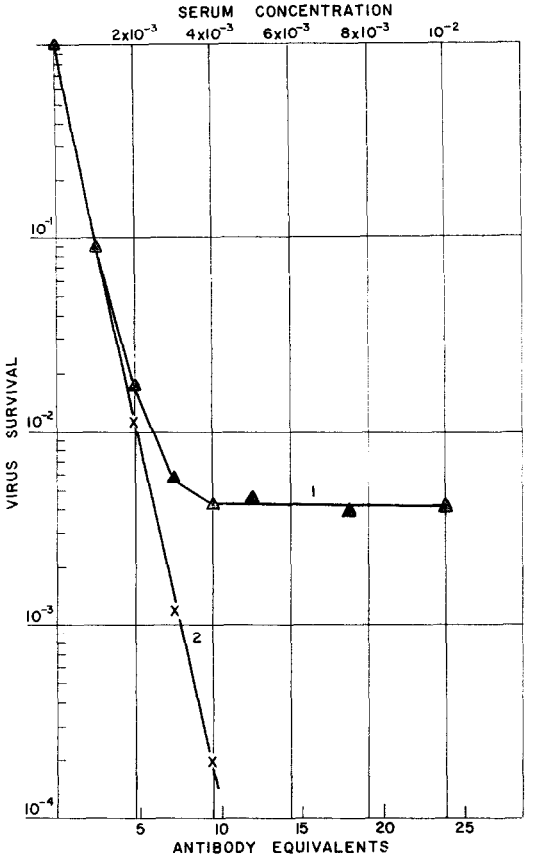

FIG. 6. Multiplicity curve of neutralization of P1 virus. The curve was obtained by a method similar to that given for Fig. 5; P1 (Brunhilde, r 68) virus was used, at a concentration of 4×10^7 pfu/ml; rabbit antiserum. Curve *1* = experimental curve; Curve 2 = corrected for a persistent fraction of 4.2×10^{-3}.

two parts were incubated two additional hours and then assayed for unneutralized virus. Both showed a virus survival almost identical to that of the original tube (Fig. 7).

This experiment shows that the antibody in excess in the neutralization tube was able to neutralize fresh virus whereas the surviving virus fraction could not be inactivated by fresh antibody.

A series of experiments were devised to clarify the conditions giving rise to the horizontal part of the multiplicity curve.

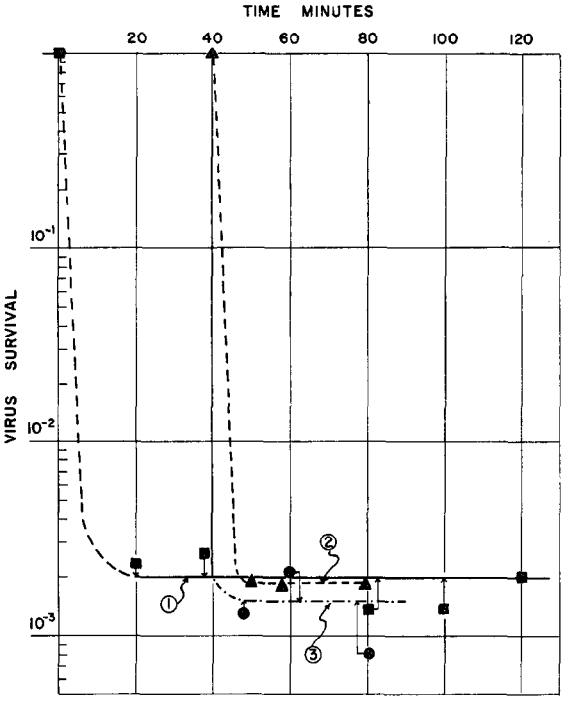

FIG. 7. State of the persistent fraction. Pl virus (Brunhilde, wild type) at a concentration 10^8 pfu/ml and monkey antiserum at a concentration 5.5×10^{-2} were incubated at 37°. Samples were taken and assayed for active virus at various times (curve *1*). After 40 minutes of incubation two aliquots were taken from the mixture, to one of them (curve *2*) new active virus at a concentration 5×10^7 pfu/ml was added; to the other aliquot (curve *3*) new serum, at the concentration 5.5×10^{-2}, was added. Both aliquots were incubated at 37° and from them samples were taken at various intervals and assayed for active virus. The survival of curves *2* and *3* were calculated by taking into account the change in total virus concentration produced by the new additions.

POSSIBLE FACTORS RESPONSIBLE FOR THE HORIZONTAL PART OF THE MULTIPLICITY CURVE

1. Dissociation of the Virus-Antibody Complex

Burnet *et al.* (1937) attributed the flattening of the kinetic curve and its transition into a horizontal line to the existence of an equilibrium between the formation and the dissociation of the virus-antibody complexes. The possible role of such a dissociation in producing the hori-

TABLE 3

CONSTANT VALUE OF THE FINAL VIRUS SURVIVAL AT HIGH SERUM/VIRUS RATIOS

WEE virus and horse antiserum were mixed as indicated. The virus-serum mixtures were incubated for 2 hours at 37°, and then assayed for surviving virus. The assay plates were washed after virus adsorption to the cells to remove the excess antibody.

Neutralization tubes			
Serum concentration	Virus concentration (pfu/ml)	Antibody equivalents[a]	Final virus survival
5.0×10^{-2}	1.5×10^{5}	2×10^{5}	1.8×10^{-3}
1.5×10^{-2}	1.5×10^{5}	6×10^{4}	3.0×10^{-3}
2.0×10^{-3}	1.5×10^{5}	8×10^{3}	2.2×10^{-3}
1.0×10^{-3}	1.5×10^{5}	4×10^{3}	3.4×10^{-3}

[a] Defined on page 164.

zontal part of both the kinetic and the multiplicity curve had therefore to be evaluated.

Dissociation in the neutralization tube. A surviving virus fraction resulting from dissociation in the neutralization tube should be a function of the serum concentration and should—owing to the shifting of the equilibrium of the reaction—tend to zero at high serum concentrations. In the experiment, reported in Table 3, the virus concentration was kept constant whereas the serum concentration was varied from 5×10^{-2} to 10^{-3}. As may be seen from the table, the final virus survival was the same for the four neutralization tubes. A similar result was obtained in other experiments, in which the serum concentration was kept constant and the virus concentration was decreased within limits that would allow complete neutralization within a 2-hour period. Thus, dissociation in the neutralization tube cannot explain the constant final survival at high serum/virus ratios.

Dissociation during the adsorption period. Since the assay of the surviving virus fraction involves a dilution of the virus-serum mixture and a period of adsorption to the cell layer, dissociation could occur during the dilution and adsorption period and thus cause the observed constant final survival. To test this possibility, the following experiment was devised: serum-virus mixtures (WEE virus) containing a constant amount of virus and different concentrations of serum were prepared. In each tube, the serum/virus ratio was such as to fall within the range of the horizontal part of the multiplicity curve. After an incubation

period of 40 minutes, 0.6 ml of each mixture was deposited undiluted onto the cell layer of a plate. The plates were incubated for 40 minutes to allow adsorption of the virus onto the cells, and then washed by four changes of 10 ml of PBS; the plates were subsequently overlaid with agar and incubated for plaque count.

Active virus particles originating from dissociation of antibody-virus complexes during the adsorption period on the plates would be exposed to two competing events: adsorption onto a cell, resulting in plaque formation, or neutralization by combination with a new antibody molecule. While the probability of the first event would be the same on all the plates, the probability of the second event—the neutralization—would vary in the various plates proportionally to the concentration of the antibody. The probability that a virus particle will infect a cell and originate a plaque should tend to zero on the plates with higher serum concentration. As may be seen from Table 4, the surviving virus fraction was, however, the same on all the plates. No dissociation of the virus-antibody complex during the adsorption period could, therefore, be demonstrated.

If the virus-antibody complexes can attach to the cells, it is conceivable that some virus particles may become reactivated upon attachment, by a mechanism analogous to the reactivation of neutralized virus particles upon collision with other virus particles, described in a later section. It seems, however, unlikely, that this phenomenon occurs in the two systems studied here, since the final survival does not depend upon

TABLE 4

TEST FOR DISSOCIATION OF THE VIRUS-ANTIBODY COMPLEX DURING THE TIME OF ADSORPTION

WEE virus and horse antiserum, at the indicated concentration, were incubated for 40 minutes; 0.6 ml from each mixture was then deposited on a monolayer culture and the latter incubated for additional 40 minutes to allow adsorption of the virus. The plates were then washed by 4 changes of 10 ml PBS, overlaid, and incubated for plaque count.

Virus concentration (pfu/ml)	Serum concentration	Number of plaques
5×10^5	5×10^{-2}	9
5×10^5	1.5×10^{-2}	15
5×10^5	5×10^{-3}	27
5×10^5	1×10^{-3}	11
5×10^5	5×10^{-4}	17

the number of antibody molecules attached to the virus particles (see page 172 for the dependence of the reactivation by collision on the multiplicity of neutralization).

Test for the presence of free antibody in a mixture of virus and antibody. A dissociation of the virus-antibody complexes should lead to the presence of free antibody in any mixture of virus and antibody. This was tested as follows:

A mixture of WEE virus, at a concentration of 5×10^8 pfu/ml, and horse antiserum, at a concentration of 5×10^{-3}, was incubated for 2 hours at 37°. The virus-serum mixture was then centrifuged in a preparative Spinco ultracentrifuge at 40,000*g* for one hour. The supernatant was tested for the presence of free antibody by measuring its ability to neutralize fresh virus.

As seen in Table 5, no measurable amount of antibody could be demonstrated in the supernatant (tube A). The same serum, at a corresponding dilution, but without previous exposure to virus, reduced the titer of the test virus by a factor of 10^3 (tube B). At least one hundredth of this amount of serum would have been detected in the test.

Test for reactivation of neutralized virus upon dilution. A dissociation of virus-antibody complexes should lead to reactivation of neutralized virus upon dilution. To test this point, mixtures of WEE virus and antiserum in various proportions were incubated at 37° for 2 hours; after

TABLE 5

TEST FOR THE PRESENCE OF FREE ANTIBODY IN A MIXTURE OF VIRUS AND ANTISERUM

WEE virus and horse antiserum were mixed at the final concentrations of 5×10^8 pfu/ml. and 5×10^{-3}, respectively (6 antibody equivalents). The virus-serum mixture was incubated for 2 hours at 37°, and subsequently centrifuged for 1 hour at 40,000*g*. The supernatant was tested for the presence of free antibody by determining its ability to neutralize fresh virus (tube A). A control tube B was set up to test the neutralizing capacity of the same serum at a corresponding dilution, but without previous exposure to virus.

	Serum concentration	Virus concentration (pfu/ml)	Virus survival	Antibody equivalents[a]
Tube A	supernatant 5×10^{-1}	5×10^7	1.0	0
Tube B	original serum 2.5×10^{-3}	5×10^7	1.0×10^{-3}	30

[a] Defined on page 164.

TABLE 6

TEST FOR REACTIVATION OF NEUTRALIZED WEE VIRUS ON DILUTION

WEE virus and horse antiserum were mixed at the indicated concentrations, incubated for 2 hours at 37°, and assayed for surviving virus; the virus-serum mixtures were then diluted by the indicated factor, incubated for 2 hours, and again assayed.

	Neutralization tubes					
No. of Exp.	Virus concentration (pfu/ml)	Serum concentration	Antibody equivalents[a]	Virus survival before dilution	Dilution factor	Virus survival after dilution
51	4.0×10^{7}	4×10^{-3}	5	1.3×10^{-2}	4	6.0×10^{-3}
					12	1.4×10^{-2}
54	3.5×10^{7}	4×10^{-3}	5	6.0×10^{-3}	4	7.6×10^{-3}
					16	8.4×10^{-3}
					64	1.2×10^{-2}
59	1.0×10^{8}	4×10^{-3}	5	8.0×10^{-3}	10	1.4×10^{-2}
					100	2.7×10^{-2}
					1000	2.1×10^{-2}
60	1.0×10^{8}	2×10^{-3}	2	4.0×10^{-2}	200	6.0×10^{-2}
		1×10^{-3}	1	3.7×10^{-1}	200	2.9×10^{-1}
63	5.0×10^{7}	4×10^{-3}	6	1.7×10^{-2}	1000	1.7×10^{-2}
		2×10^{-3}	3	5.2×10^{-2}	1000	8.0×10^{-2}
		1×10^{-3}	1.5	2.3×10^{-1}	1000	2.7×10^{-1}
64	5.0×10^{7}	4×10^{-3}	6	7.6×10^{-3}	1000	1.2×10^{-2}
		2×10^{-3}	3	1.2×10^{-1}	1000	1.8×10^{-1}
		1×10^{-3}	1.5	2.2×10^{-1}	1000	2.2×10^{-1}

[a] Defined on page 164.

assaying, the mixtures were diluted, then reincubated for 2 hours, and assayed again. The virus survival before dilution and after incubation in the diluted state was compared. In a series of experiments, summarized in Table 6, small differences in the survival before and after dilution were found; the differences were in both directions, those showing an increased survival after dilution being more numerous. A slight, although not definitely proved, amount of reactivation may therefore occur. Again no clear sign of reactivation was found in other experiments, in which the time of incubation of the virus-serum mixture before dilution was varied between 15 minutes and 4 hours.

TABLE 7

TEST FOR REACTIVATION OF NEUTRALIZED P1 VIRUS ON DILUTION

A mixture of P1 virus (Brunhilde, wild type), at the concentration of 2.5×10^7 pfu/ml., and monkey antiserum, at a concentration of 5.5×10^{-2}, was incubated for 40 minutes at 37°. The virus-serum mixture was then diluted $1:10^3$ in PBS, containing 20% chicken embryo extract, and incubated at 37°. At the indicated time intervals, samples of the diluted virus-serum mixture were assayed for virus survival. A virus sample, diluted in the same medium, was incubated at the same time as a control. All survival values are referred to this control tube, and are therefore unaffected by the thermal inactivation of the virus.

	Time of incubation of the diluted virus-serum mixture in hours				
	0	8½	20	32½	55
Survival	1.8×10^{-3}	3.2×10^{-3}	1.0×10^{-3}	2.0×10^{-3}	2.7×10^{-3}

Experiments in which the diluted virus-serum mixture was incubated for a longer period were carried out with P1 virus. The results, reported in Table 7, show similarly that no reactivation of neutralized P1 virus occurred after a long incubation time.

All experiments, therefore, seem to concur in showing that in the two systems studied dissociation of the virus-antibody complexes does not play an important role in the production of the horizontal parts of the kinetic or multiplicity curves. It seems likely that the horizontal parts of these curves is due to a fraction of the virus population refractory to neutralization. We shall call this virus fraction the *persistent fraction*.

2. Inhomogeneity of the Virus Population

To test whether the persistent virus fraction was due to virus particles genetically resistant to antibody, the progeny of several surviving virus particles was tested for their antibody resistance. This was done by picking a certain number of plaques from platings of a (P1) virus-serum mixture that had been previously incubated for 2 hours at 37° and was known to give a final survival corresponding to the persistent fraction. Independent virus stocks were grown from each plaque suspension and their sensitivity to antiserum tested by determining the kinetic curve for each stock. No difference was found between the sensitivity of the new stocks and that of the original stock. Thus, the majority of the virus particles constituting the persistent fraction is not genetically resistant to the antibody.

TABLE 8

TEST FOR AN INHIBITOR OF NEUTRALIZATION IN NORMAL MONKEY SERUM

One ml of a mixture of P1 (Brunhilde wild type) virus and monkey antiserum was transferred to 2 tubes A and B: to tube A 0.5 ml of monkey serum, to tube B 0.5 ml of PBS was added. The final concentration of virus in both tubes was 10^8 pfu/ml, that of the antiserum 5.5×10^{-2}. Both neutralization tubes were incubated for 40 min. at 37°, and assayed for virus survival.

Experiment No.	Virus survival in	
	Tube A (+ monkey serum)	Tube B (+ PBS)
76	5.5×10^{-3}	2.1×10^{-3}
78	1.9×10^{-3}	1.9×10^{-3}

3. Inhibitors in the Serum

The persistent fraction may be caused by an inhibitor that combines with the virus and thereby inhibits the attachment of the antibody to the virus particle. This inhibitor would have to combine firmly with the virus since a readily dissociable inhibitor would be replaced by antibody. The latter can be excluded owing to the fact that the persistent fraction was found to remain constant with time, in the case of P1 virus, even after days of incubation.

To test whether normal serum contained this hypothetical inhibitor, two neutralization tubes containing the same amount of P1 virus and antiserum were prepared: normal monkey serum was added to one tube, a corresponding amount of PBS to the other. As shown in Table 8, the virus survival after an incubation period of 40 minutes at 37° was, in two independent experiments, the same for both tubes. Thus, the results do not provide any evidence for the presence of an inhibitor in normal serum. This, however, does not exclude the existence of an inhibitor in immune serum, where it may, for instance, occur under the form of an incomplete antibody.

4. Absence of Cofactors

Another possible factor causing the persistent fraction may be the absence of cofactors required for complete neutralization; hypothetical cofactors present in the blood serum might, in fact, be excessively diluted in our experiments. Fresh normal rabbit serum (as suggested by Dr. Sabin) was therefore added to the neutralization tubes, at a final concentration of 1/2. The effect of this addition varied greatly from experi-

ment to experiment; on the average, the persistent fraction of WEE virus was lowered by a factor of 5. Thus, the absence of cofactors may play a role in the causation of the persistent fraction.

5. Size of the Persistent Fraction of Various Lines of P1 Virus

When comparing the size of the persistent fraction of different lines of P1 virus, slight but consistent differences between certain lines were found. These are summarized in Table 9. It may be seen that the persistent fraction is largest for the r68 line of Brunhilde virus.

In conclusion, it may be seen from the reported experiments that a definite picture as to the nature of the persistent fraction cannot yet be given. The presence of the persistent fraction disturbs the kinetic study of neutralization. To eliminate this disturbance, the experiments to be described will be based on determinations of the virus survival well above that of the persistent fraction.

Determination of the amount of neutralizing antibody that can be absorbed by a virus particle. Before describing the experiments, the following clarification must be made. It has already been mentioned earlier (p. 172) that the multiplicity curve (= plot of the logarithm of the final survival as a function of the serum-virus ratio in the neutralization tube) starts from the origin as a straight line. It can, therefore, be expressed by the equation $V/V_0 = e^{-m}$, m giving the average number of neutralizing antibody molecules per virus particle adsorbed in such a way as to produce

TABLE 9

COMPARISON OF THE PERSISTENT FRACTION OF VARIOUS LINES OF P1 VIRUS

Mixtures of various lines of P1 virus were incubated for 1 hour at 37° with rabbit antiserum at the indicated concentrations. The virus-serum mixtures were assayed for virus survival.

Virus strain	Virus line	Neutralization tubes			Virus survival = persistent fraction
		Virus concentration (pfu/ml)	Serum concentration	Antibody equivalents[a]	
Brunhilde	R 68	1.6×10^{7}	5×10^{-3}	30	5.0×10^{-3}
Mahoney	S	4.0×10^{6}	5×10^{-3}	120	3.8×10^{-3}
Brunhilde	R 30	6.0×10^{6}	5×10^{-3}	80	1.5×10^{-3}
Brunhilde	wild type	2.5×10^{7}	5×10^{-3}	19	1.7×10^{-3}
Brunhilde	wild type	2.5×10^{6}	5×10^{-3}	190	0.8×10^{-3}
Brunhilde	wild type	2.5×10^{5}	5×10^{-3}	1900	0.7×10^{-3}

[a] Defined on page 164.

TABLE 10

DETERMINATION OF THE AMOUNT OF NEUTRALIZING ANTIBODY THAT CAN BE ABSORBED BY A VIRUS PARTICLE

Aliquots of a given virus suspension were mixed with various concentrations of rabbit antiserum and incubated for 2 hours at 37° and overnight at 4° (first neutralization tubes). The virus-serum mixtures—in the case of WEE virus, the high-speed supernatants after a centrifugation at 40,000*g* for 1 hour—were diluted as indicated in column 7 and mixed with aliquots of fresh virus of the same original virus suspension (second neutralization tubes). The tubes were incubated for 2 hours at 37° and assayed for virus survival. A control tube was set up, to which virus was added only during the second incubation period.

The last column gives the number of antibody equivalents absorbed by the virus suspension and, at the same time, the average number of antibody molecules absorbed by each virus particle (compare text).

		First neutralization tube			Second neutralization tube					
Virus	Virus serum mixture	Virus concentration (pfu/ml.)	Serum concentration	Antibody equivalents[a, b]	Virus concentration (pfu/ml)	Dilution of virus-serum mixture	Final virus survival	Antibody equivalents in 2nd tube[c]	Unabsorbed antibody equivalents in 1st tube[d]	Absorbed antibody equivalents in 1st tube[e]
1	2	3	4	5	6	7	8	9	10	11
WEE	A	5×10^8	5×10^{-3}	17.7	5×10^8	10	0.70	0.36	3.6	14.1
	B	5×10^8	1×10^{-2}	35.4	5×10^8	20	0.40	0.92	18.4	17.0
	C	5×10^8	2×10^{-2}	70.8	5×10^8	20	0.06	2.81	56.2	14.6
	D	5×10^8	3×10^{-2}	106.2	5×10^8	20	0.01	4.60	92.0	14.0
	control	—	1×10^{-2}	35.4	5×10^8	20	0.18	1.77		
P1	A	4×10^7	1×10^{-3}	2.4	4×10^7	2	1.0	0	0	(2.4)[f]
	B	4×10^7	2×10^{-3}	4.8	4×10^7	2	1.0	0	0	(4.8)
	C	4×10^7	3×10^{-3}	7.2	4×10^7	2	1.0	0	0	(7.2)
	D	4×10^7	4×10^{-3}	9.6	4×10^7	2	0.43	0.84	1.7	8.4
	E	4×10^7	5×10^{-3}	12.0	4×10^7	2	0.20	1.60	3.2	8.8
	F	4×10^7	7.5×10^{-3}	18.0	4×10^7	2	0.10	2.30	4.6	13.4
	G	4×10^7	1×10^{-2}	24.0	4×10^7	2	0.04	3.20	6.4	17.6
	control	—	1×10^{-3}	2.4	4×10^7	2	0.30	1.20		

[a] Defined on page 164.

[b] Calculated from the antibody equivalents found for the control tube (column 9) by taking into account the various dilution factors.

[c] Calculated from the observed final survival (column 8) according to the equation $V/V_0 = e^{-m}$.

[d] Value obtained in column 9 multiplied by the dilution factor in column 7.

[e] Value in column 10 subtracted from value in column 5.

[f] The values in parentheses indicate that all antibody molecules available in the first tube had been absorbed. The values are, therefore, either smaller than or equal to the maximal number of molecules that can be absorbed by a virus particle.

inactivation. (For further details, see p. 200). The amount of antibody corresponding to a multiplicity $m = 1$ (final survival $e^{-1} = 0.37$) has already been defined as the antibody equivalent of a given virus. A serum concentration leading, for example, to a final survival of 0.05 contains 3 antibody equivalents ($e^{-3} = 0.05$).

The following procedure was adopted to measure the maximal amount of neutralizing antibody that can be absorbed by a virus particle. A given virus suspension was mixed with aliquots of various antiserum concentrations, such as to give a high S/V ratio corresponding to a high input multiplicity of neutralization (column 5, Table 10). After an incubation of 2 hours at 37° and an overnight incubation at 4°, a high-speed supernatant of the virus-serum mixtures (in the case of WEE virus) or the mixtures themselves (in the case of P1 virus) were exposed at an appropriate dilution to a fresh suspension of the same virus. After a further incubation of 2 hours, the final virus survival was determined. Due to the relationship $V/V_0 = e^{-m}$, the number of antibody equivalents remaining in the second neutralization tubes could be determined (column 9). This number, multiplied by the dilution factor (column 7), gave the number of antibody equivalents that had remained unabsorbed during the first incubation of the virus-serum mixtures (column 10). The number of antibody equivalents originally present in the different neutralization tubes was calculated from the final survival in a control tube, not previously exposed to virus (column 8). To obtain the number of antibody equivalents absorbed by the virus suspension (column 11), the values in column 9, multiplied by the dilution factor, were subtracted from those of column 5. Since the same virus suspension was used for all determinations, the number of antibody equivalents absorbed by the whole suspension is proportional to the average number of neutralizing antibody molecules adsorbed by each virus particle (see p. 183). It may be seen from column 11 that, for WEE virus, the number of absorbed antibody equivalents is approximately constant (average = 14.9) over a range of 18–106 antibody equivalents in the neutralization tube. In the case of P1 virus, the value found varies with the input multiplicity between 8.6 and 17.6. A more detailed evaluation of these results will be made in the discussion.

It was mentioned that, in the case of WEE virus, only the high-speed supernatants of the virus-serum mixtures were tested for free antibody. As will be seen from the next two sections, the elimination of the neutralized virus from the mixtures was required since neutralized virus particles of WEE virus can be reactivated in the presence of fresh virus.

TABLE 11

REDISTRIBUTION OF ABSORBED ANTIBODY MOLECULES UPON ADDITION OF FRESH VIRUS TO A VIRUS-SERUM MIXTURE

WEE virus was mixed with various amounts of horse antiserum and incubated at 37° for 2 hours. Fresh virus at the same concentration was then added to the mixtures and the tubes were incubated a second time for a 2-hour period. The mixtures were then assayed for virus survival.

First incubation		Second incubation	Expected survival		
Antibody equivalents	Unabsorbed antibody equivalents[a]	Antibody equivalents[b]	From unabsorbed equivalents	Upon complete redistribution of antibody	Observed survival
9.6	0	6.4	1	1.3×10^{-3}	1.8×10^{-2}
4.8	0	3.2	1	4.0×10^{-2}	6.0×10^{-2}
2.4	0	1.6	1	2.0×10^{-1}	2.5×10^{-1}
1.2	0	0.8	1	4.5×10^{-1}	4.7×10^{-1}
0.6	0	0.4	1	6.7×10^{-1}	5.8×10^{-1}

[a] On the basis of data in Table 10.

[b] From the first incubation, both absorbed and unabsorbed.

The centrifugation step could be omitted with P1 virus where a similar reactivation did not take place.

Redistribution of absorbed antibody molecules upon adding of fresh virus to a virus-serum mixture. Samples of WEE virus were mixed with antiserum at a concentration varying between 0.6 and 9.6 antibody equivalents, an amount of antibody known from the results in Table 10 to be completely absorbed by the virus. After an incubation period of 2 hours at 37°, fresh virus was added at the same concentration, and the mixtures were again incubated at 37° for 2 hours. As shown in Table 11, an assay of the final survival showed that the virus added later had been partly neutralized, in spite of the fact that no free antibody had been present in the virus-serum mixture after the first incubation. It could be calculated that nearly the same final survival would have been obtained if both virus samples had originally been pooled together and then exposed to antibody. The result described above shows, therefore, that antibody molecules can be exchanged between virus-antibody complexes and free virus particles. This result is in apparent contradiction with the very limited spontaneous dissociability of the virus-antibody complexes demonstrated in a previous section. An hypothesis that resolves this apparent contradiction will be presented in the discussion.

No evidence for a redistribution of absorbed antibody molecules upon

adding of fresh virus could be obtained in an analogous experiment with P1 virus.

Reactivation of neutralized WEE virus by adding an excess of ultraviolet inactivated virus. Aliquots of WEE virus and antiserum at various concentrations (see Table 12) were incubated for 2 hours at 37°. The mixtures were then diluted into a suspension of undiluted virus previously inactivated by ultraviolet light to a survival of 2×10^{-4}, and again incubated at 37° for 2 hours. The virus survival at the end of the first and second incubation periods, respectively, is shown in Table 12. Both values represent the survival of the initial virus inoculum, since the contribution of the ultraviolet-inactivated virus to the second value is negligible. As can be seen from the table, all virus-serum mixtures showed a higher final survival after the second incubation period. This higher survival can be expressed as a decrease of the multiplicity of neutralization (as calculated from the virus survival, see p. 165) due to reactivation. The multiplicity of neutralization was decreased by a nearly constant factor (average = 1.4) independent of the multiplicity in the first neutralization tube. The possible meaning of this result will be discussed later.

In similar experiments with P1 virus, no reactivation could be demonstrated. It should, however, be pointed out that, owing to the existence of the persistent fraction, an amount of reactivation smaller than the persistent fraction cannot be excluded.

Rate of adsorption to the cells of the surviving virus particles in a virus-antiserum mixture. In the following experiments, it was determined whether the virus particles that escape neutralization after exposure to antibody are adsorbed onto the cells at a normal rate. This was done to test the hypothesis that the observed inactivation by antibody is only apparent and due to a decrease in the rate of adsorption of all virus particles present in the virus-antiserum mixture. This hypothesis has to be considered, since under the usual plating conditions, the period allowed for adsorption is restricted to 30 minutes. Most of the virus particles that remain unadsorbed during this period—approximately 20–30 % of the virus particles in the case of WEE virus—get thermally inactivated before they reach the cell layer by diffusion through the agar and are thus lost as plaque-formers.[2] Any decrease in the adsorption rate of the virus particles owing to an interaction between virus and antibody

[2] Due to the greater thermal stability of P1 virus, these considerations apply to a lesser degree of P1 virus.

TABLE 12

REACTIVATION OF NEUTRALIZED WEE VIRUS BY ADDING AN EXCESS OF ULTRAVIOLET-INACTIVATED VIRUS

Aliquots of WEE virus and horse antiserum were mixed at the concentrations indicated, incubated for 2 hours at 37°, and assayed for virus survival (first neutralization tubes). Samples from each tube were then diluted 1:20 into a suspension of undiluted virus, inactivated by ultraviolet light to a final survival of 2×10^{-4} (second neutralization tubes). The tubes were again incubated for 2 hours at 37° and assayed for virus survival.

	First neutralization tube				Second neutralization tube				
Experiment No.	Virus concentration (pfu/ml)	Serum concentration	Virus survival, 1	Multiplicity, 1[a] (m_1)	Dilution of virus serum mixture	Original conc. of UV-irr. virus (pfu/ml)	Virus survival, 2	Multiplicity, 2[b] (m_2)	Factor of decrease of multiplicity m_1/m_2
1	5×10^7	4×10^{-3}	1.7×10^{-2}	4.1	20	2.5×10^7	9.5×10^{-2}	2.3	1.6
	5×10^7	2×10^{-3}	5.2×10^{-2}	2.9	20	2.5×10^7	1.2×10^{-1}	2.1	1.4
	5×10^7	1×10^{-3}	2.3×10^{-1}	1.4	20	2.5×10^7	3.1×10^{-1}	1.2	1.2
2	5×10^7	4×10^{-3}	1.0×10^{-2}	4.6	20	2.5×10^7	4.5×10^{-2}	3.1	1.5
	5×10^7	1×10^{-3}	2.2×10^{-1}	1.5	20	2.5×10^7	3.3×10^{-1}	1.1	1.4

[a] Calculated from survival 1.

[b] Calculated from survival 2.

would, therefore, reflect itself in a reduced number of plaques on the assay plates and be indistinguishable from a true inactivation. To distinguish between both possibilities, the following two experiments were carried out with WEE virus.

In the first experiment, a series of cultures was divided into four batches of four plates each. Two batches were infected with enough normal virus to give approximately 60 plaques per plate; the two remaining batches were infected with virus that had been exposed to antiserum for 2 hours and again gave about 60 plaques from its surviving fraction. One batch of each kind was incubated for 30 minutes, the other two batches for 90 minutes. At the end of the incubation period, the plates were washed and overlaid with agar. The number of plaques counted on the four batches of plates are given in Table 13. The data show that the number of plaques produced by the virus-antiserum mixture increased less than that of the normal virus when the time of adsorption was increased, a result opposite to that expected if the adsorption rate of the survivors in the mixture had been reduced.

In the second experiment, two batches of four plates each were infected with normal virus and the virus-antiserum mixture, respectively, as above. After an adsorption period of 50 minutes, the unadsorbed virus, present in the supernatant, was removed from each culture; the cultures were then washed and overlaid with agar. The removed supernatants were assayed for nonadsorbed virus by plating them onto new plates.

TABLE 13

ADSORPTION RATE OF THE SURVIVING VIRUS PARTICLES IN A VIRUS-ANTISERUM MIXTURE

Table 13a. Two batches (A1, A2) of 4 plates were infected with normal WEE virus, two other batches (B1, B2) were infected with WEE virus that had been exposed for 2 hours to horse antiserum at a concentration of 4×10^{-3}. Batches A1 and B1 were incubated at 37° for 30 minutes, batches A2 and B2 for 90 minutes. At the end of the incubation periods, the plates were washed and overlaid with agar.

Batch	Virus	Time of incubation in minutes	No. of plaques (on 4 plates)
A1	normal	30	210
A2	normal	90	305
B1	virus-antiserum	30	231
B2	mixture	90	243

Table 13b. Two batches of 4 plates were infected with normal virus and with the virus-antiserum mixture, respectively, as above. After an incubation period of 50 minutes at 37°, the supernatants were withdrawn and replated onto two new batches of 4 plates which were incubated for 55 minutes. After incubation, all plates were washed and overlaid with agar.

Batch	Virus	Plaques on 1st batch of plates	Plaques produced by supernatants on 2nd batch of plates	Ratio: adsorbed/to unadsorbed virus
C	normal	269	70	3.8
D	virus-antiserum mixture	230	37	6.2

As may be seen from Table 13b, the ratio of adsorbed/unadsorbed virus particles is somewhat larger for the survivors of the virus-antiserum mixture, again indicating a slightly increased adsorption rate of the survivors over the control. The possible significance of this slight increase in the adsorption rate of the survivors in a virus-antiserum mixture over control virus will be discussed later. It should be mentioned that this result cannot be caused by inactivation of the unadsorbed virus by carried-over antibody, since the virus-serum mixture was sufficiently diluted before infecting the plates. Both experiments show, therefore, that the hypothesis assuming as the mechanism of neutralization a decreased adsorption rate of all virus particles in the mixture can be excluded.

Independence of the multiplicity curve from the cell type used for the assay. It is known from experiments of neutralization of animal viruses carried out by the usual end-point technique that both the neutralizing titer and the slope of the neutralization curve depend on the cell type used for the test. As will be pointed out in the discussion, this is most likely a consequence of the technique used. As seen from the following experiments, it does not hold for the technique adopted in the present work.

Multiplicity curves, giving the logarithm of the survival as a function of the serum/virus ratio in the neutralization tube, were determined for P1 virus, as described on p. 183, with the only difference that the surviving virus fraction was assayed in parallel on monkey kidney cultures and on cultures of HeLa cells. The results of one such experiment are given in Fig. 8. It is clearly to be seen that the shape and the slope of the two curves are equal and that the number of antibody equivalents

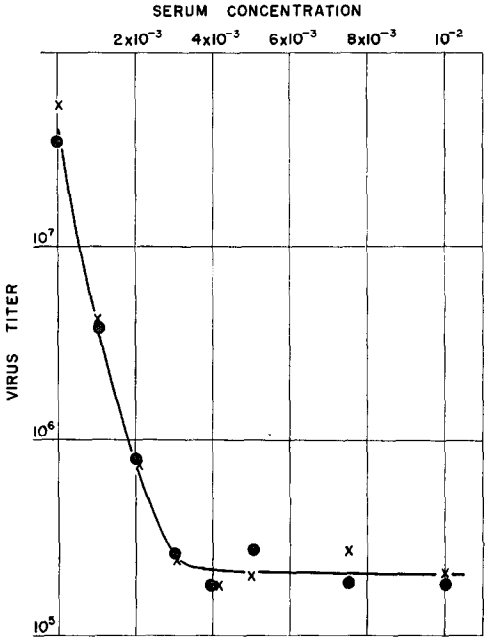

FIG. 8. Multiplicity curve of the neutralization of P1 virus by using two different types of cells. Multiplicity curves of P1 (Brunhilde, r 68) virus were obtained as indicated under Fig. 6. The virus survival was determined in parallel assays on cultures of monkey kidney cells (dots) and on cultures of HeLa cells (crosses).

determined from the two curves is the same. To demonstrate that the results obtainable by this technique are generally independent of the type of cells, extensive experiments should, however, be carried out, by employing cells widely different in their susceptibility to the virus.

DISCUSSION

The process of inactivation of two animal viruses by antiserum, commonly called neutralization, was studied by a method which allowed a separation between the process of combination between virus and antibody and the measurement of the resulting virus inactivation. This approach was followed to avoid the difficulties arising from the classical method in the study of neutralization of animal viruses, in which a considerable amount of antibody is carried into the measuring system. Because of its complexity, the latter method cannot provide a clear picture of the neutralization process. This will be shown later in the dis-

cussion, where a hypothesis that accounts for certain results obtained with the classical method will be developed.

1. Theory of Neutralization

The main experimental results obtained in the present work can be summarized as follows: (1) Neutralization is a direct consequence of the combination of the virus particle with antibody molecules. Indirect mechanisms, such as agglutination of the virus particles, do not play any important role, as shown by the independence of neutralization of the concentration of the virus. (2) The kinetics of neutralization under conditions of antibody excess is of first order. (3) The rate of neutralization, i.e., the probability for a virus particle to be neutralized per unit time is linearly dependent on the concentration of the antibody. Results (2) and (3) show that the attachment of a single molecule of neutralizing antibody is sufficient to inactivate a virus particle. (4) Under the conditions used, the virus-antibody complexes formed were very stable. (5) A virus particle is able to combine with more than one molecule of neutralizing antibody. In the presence of large excess of antibody, both WEE and P1 virus particles could adsorb up to 15 equivalents of neutralizing antibody. (6) The characteristics of the neutralization process are independent of the cell system used for assaying the surviving virus.

On the basis of these findings, a simple model of neutralization can be developed. It involves the following assumptions: (1) Each virus particle possesses on its surface a number n of antigenic sites, each of which is able to undergo combination with one neutralizing antibody molecule. The stability of this combination may vary for different sera; since it was stable in the experiments reported in this article, only the case of an irreversible combination will be considered in this theory. (2) The combination of any one site with a neutralizing antibody molecule leads to inactivation of the virus particle. The antigenic sites fulfilling conditions (1) and (2) will be called *critical sites.*

The model, thus, involves the following reaction:

$$A + Q \xrightarrow{k_1} (AQ), \tag{1}$$

where

A = concentration of the free neutralizing antibody molecules in the neutralization tube at time t;

Q = concentration of the free critical sites in the neutralization tube at time t;

(AQ) = concentration of combined antibody-site complexes.

In addition, the following relationship holds: $A = A_0 - (Q_0 - Q)$, where A_0 and Q_0 are the values of A and Q, respectively, at time $t = 0$ (i.e., the moment of mixing the virus and the antibody in the neutralization tube).

According to this model, the kinetics of disappearance of the free critical sites is then given by the following equation:

$$dQ/dt = -k_1 Q\,[A_0 - (Q_0 - Q)]$$

the solution of which is:

$$\frac{Q(t)}{Q_0} = \frac{1 - A_0/Q_0}{1 - \frac{A_0}{Q_0} e^{-k_1 Q_0 (1 - A_0/Q_0)t}} = \frac{1 - m_0/n}{1 - \frac{m_0}{n_0} e^{-k_1 V_0 n (1 - m_0/n)t}} \tag{3}$$

where:

$Q_0 = V_0 n$, V_0 being the concentration of virus particles in the neutralization tube at time $t = 0$; and,

$m_0 = A_0/V_0$ = ratio of number of neutralizing antibody molecules to number of virus particles in the neutralization tube or input multiplicity.

Due to assumption (2), only those virus particles which have all their n critical sites free are active. Thus, the kinetics of disappearance of active virus particles is described by the equation:

$$V(t)/V_0 = [Q(t)/Q_0]^n \tag{4}$$

Equation (4) can be written in two forms:

$$\ln \frac{V(t)}{V_0} = n \ln \frac{(m_0/n - 1)e^{-k_1 V_0 n (m_0/n - 1)t}}{\frac{m_0}{n} - e^{-k_1 V_0 n (m_0/n - 1)t}}, \qquad \text{valid for } \frac{m_0}{n} > 1, \tag{4a}$$

and,

$$\ln \frac{V(t)}{V_0} = n \ln \frac{1 - m_0/n}{1 - \frac{m_0}{n_0} e^{-k_1 V_0 n (1 - m_0/n)t}}, \qquad \text{valid for } \frac{m_0}{n} < 1. \tag{4b}$$

From these equations, the following predictions can be derived: In the case of a large antibody excess, that is, if m_0/n becomes large as compared to 1, so that $m_0/n - 1 \approx m_0/n$, equation 4a transforms into:

$$\ln \frac{V(t)}{V_0} = -nk_1 A_0 t \tag{5}$$

The kinetic curve is, thus, at high concentrations of antibody, a straight line with slope equal to $-nk_1A_0$. At smaller values of m_0/n, the kinetic curve bends progressively upward; its initial slope remains, however, the same as shown by the fact that

$$\frac{d\left(\ln \frac{V}{V_0}\right)}{dt} = -nk_1A_0 \quad \text{for} \quad t \to 0 \tag{6}$$

The first part of the theoretical curve is, therefore, for all values of m_0/n, of first order, its slope being proportional to the concentration of antibody.

In the case of excess of antigen (critical sites) ($m_0/n < 1$), equation 4b transforms, for $t \to \infty$, into the equation of the multiplicity curve:

$$\ln \frac{V}{V_0} = n \ln \left(1 - \frac{m}{n}\right) \tag{7}$$

where m, the multiplicity of neutralization, stands for the number of antibody molecules absorbed (to critical sites) per virus particle; m is equal to m_0 at time $t \to \infty$. For $n \to \infty$, equation (7) becomes:

$$V/V_0 = e^{-m} \tag{7a}$$

The initial slope of the multiplicity curve (7), as well as the slope of the curve represented by equation (7a), are given by:

$$\frac{d\left(\ln \frac{V}{V_0}\right)}{dm} = -1 \tag{8}$$

The theoretical multiplicity curve begins, therefore, as a straight line of slope equal to -1, and bends progressively downwards, unless n is large (see curve on Fig. 9, calculated for $n = 8$). For values of m/n smaller than 0.4 and $n \geq 8$, the theoretical curve (7) and the curve $V/V_0 = e^{-m}$ (7a) can be considered—for all practical purposes—as identical. For instance, for $m/n = 0.3$ and $n = 8$, the multiplicity calculated from equation (7a) is 16% larger than that calculated from equation (7); the difference decreases for larger n. The use of equation (7a) to calculate the average number of neutralizing antibody molecules absorbed to critical sites per virus particle (see p. 193) is, therefore, justified.

The simple model of neutralization discussed until now predicts thus

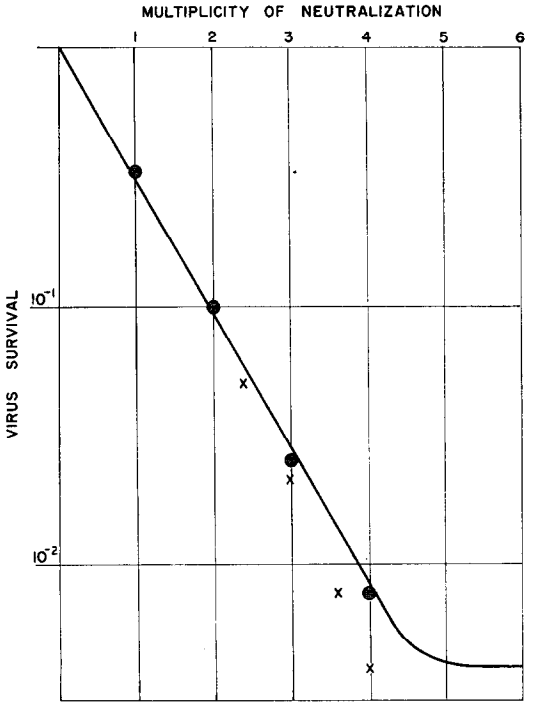

FIG. 9. Theoretical multiplicity curves. Crosses: curves $1n(V/V_0) = n\ln(1 - m/n)$. Dots: same curve, corrected for a persistent fraction of 4×10^{-3}. Line: straight line fitted to the dots.

the main experimental results, if we disregard the later part of the kinetic and multiplicity curves. These show an upward bending not predicted by the theory. The upward bending is partly caused by a virus fraction that escapes neutralization in the presence of excess antibody, and which was called persistent fraction.

That the persistent fraction is, however, not wholly responsible for the deviations at high multiplicities, is seen from a comparison of the experimental and theoretical curve, after both have been corrected for the persistent fraction. Whereas the theoretical curve bends downwards at higher multiplicities (crosses in Fig. 9), the curve of P1 virus continues with the same slope (Fig. 6), that of WEE virus shows a sharp change in slope in the opposite direction (Fig. 5). These deviations from the predicted curve can be explained, if it is assumed that the same virus stock contains classes of virus particles that differ in their average

multiplicity of neutralization. The presence, in the same virus stock, of a small fraction of virus particles with a smaller average multiplicity would cause a decrease in the slope of the later part of the multiplicity curve.

Different multiplicities of neutralization may be due to a difference in the number of critical sites per virus particle, or to an unequal distribution, among the virus particles, of sites of various antigenic configuration. In the first case, the virus particles having a larger number of critical sites would tend to have a higher multiplicity of neutralization. In the second case, the antiserum against a virus preparation would contain antibody of different specificity, of which one would be the most active in producing neutralization. The virus particles containing the largest number of critical sites combining with this especially active antibody would have the highest multiplicity of neutralization.

These deviations from the predicted multiplicity curve are due to nonhereditary properties of the virus particles. Nonhereditary differences in the antigenic constitution of the particles of a clone could be explained by the hypothesis that accounts for the phenomenon of phenotypic mixing in bacteriophage (Streisinger, personal communication). According to this hypothesis, the viral protein is made up of subunits which are synthesized separately in each infected cell and assembled more or less at random. In the present case, one may assume that some of these subunits contain a critical site, whereas others lack it, and that the critical sites may vary for different subunits. The relative proportions of the various types of subunits synthesized within the cell would be determined by the genetic constitution of the virus, whereas the assembling of the various subunits would occur at random.

Under this picture, a fraction of virus particles could be built up entirely by protein subunits lacking critical antigenic sites; these virus particles may correspond to the persistent fraction. That the virus particles constituting the persistent fraction actually differ in their surface from the remaining virus population was shown experimentally for WEE virus: their rate of attachment to the cells was found to be higher than that of the average virus population.

The same picture does not exclude that particles of the persistent fraction bind antibody to noncritical antigenic sites. Whereas, under the standard experimental conditions, a combination between a noncritical site and antibody does not lead to inactivation of the virus particle, it is conceivable that, in the presence of special cofactors or

of a special ionic environment, the same combination may lead to inactivation of the particle. The decrease of the persistent fraction in the presence of undiluted fresh rabbit serum may be due to such a mechanism.

As already discussed in the experimental part, other factors may be responsible for the persistent fraction. One such factor may be the presence, in the antiserum, of molecules capable of combining with the virus particle in such a way as to inhibit the attachment of antibody without interfering with the infectivity of the virus particle. Another factor may be the reactivation of some neutralized virus particles upon adsorption to the cell. No experimental evidence was obtained in favor of these two factors.

The mechanism by which a single antibody molecule inactivates a virus particle is not explained by the present experiments. One possible mechanism could be ruled out, i.e., that the antibody molecule acts by hindering sterically the attachment to the cells of a part of the virus surface without interfering with the normal attachment of the remaining parts. Such a mechanism of inactivation would decrease the probability that a virus particle infects the cell layer during the time usually allowed for adsorption, and thus would lead to an apparent inactivation of first order kinetics. By increasing the time for the adsorption of the virus to the cells, this apparent inactivation should be reversible, an effect which was not observed experimentally.

2. The Problem of the Dissociability of the Virus-Antibody Complex

A number of experiments in this work were devoted to the study of the reversibility of the combination between virus particle and its neutralizing antibody. In a variety of tests (performed at pH 7.5 and at physiological ionic strength), no measurable dissociability of the P1 virus-antibody complexes and only a slight dissociability of the WEE virus-antibody complexes could be demonstrated.

This result is contrary to conclusions reported in the literature. This discrepancy may be explained in certain cases by the use of different antigens or of antisera prepared in a different way. It was, for example, shown by Jerne and Avegno (1956) that antisera obtained after short immunization may produce more readily dissociable complexes than those obtained after prolonged immunization. In many cases, however, the reactivation observed in the past may have been spurious and a consequence of the assay method, which did not prevent a further

interaction between carried-over free antibody and virus in the measuring system. Since this interaction would decrease at higher dilutions of the virus-serum mixture, it would lead to a larger survival at higher dilutions, and may, thus, have simulated a reactivation.

In the present work, it was shown that neutralized WEE virus is reactivated to a significantly greater extent if mixed with a high concentration of unneutralized, ultraviolet-inactivated virus. A similar phenomenon was described for influenza virus (Hultin and McKee, 1952). In the latter case, it is, however, uncertain whether this reactivation was not only apparent for the same reasons discussed in the preceding paragraph.

A phenomenon probably related to the reactivation by ultraviolet-inactivated virus was observed when a sample of fresh (active) WEE-virus at high concentration was added to a previously incubated virus serum mixture. In spite of the fact that no free antibody had been present in the virus-serum mixture at the moment at which the fresh virus was added, the latter became partly inactivated after further incubation.

It is likely that the two last effects were produced by the same mechanism, i.e., by an exchange of antibody molecules between virus-antibody complexes and free virus particles. Since the low spontaneous dissociability of the virus-antibody complexes could not account for the amount of exchange observed, it may be assumed, that an exchange took place whenever a virus particle collided with a virus-antibody complex. That the collision frequency would be sufficient to account for the observed effect, may be seen from the following calculation:

The number of collisions, c, per particle per second in a suspension can be calculated from Smoluchowski's formula:

$c = 8\pi D\ r\ n$, where

D = diffusion coefficient of the virus particles, assumed to be equal to 1.67×10^{-7} cm^2 sec^{-1} for WEE virus,

r = distance between the centers of the two colliding particles assumed to be 4×10^{-6} cm for the same virus,

n = number of virus particles per cm^3. Since the WEE virus stocks used had titers from 2 to 5×10^8 pfu/ml, each plaque-forming unit containing at least 14 physical particles countable in the electron microscope (Williams, personal communication), n varied from 2.8 to 7.0×10^9 particles per cm^3.

Entering the values into the equation, we obtain for c a value of 4.7×10^{-2} to 1.2×10^{-1}. The proportion of reactivated particles after one hour was 10%. The number of collisions was, therefore, adequate to account for the observed reactivation.

Since after reactivation by collision the multiplicity of neutralization is reduced by a constant factor, it is likely that a constant fraction of the antibody molecules adsorbed to a virus particle is transferred at each collision.

3. Determination of the Parameters of Neutralization

The results of the present work suggest the use of certain parameters as basis for a quantitative description of the process of neutralization. These parameters are: (1) m: the multiplicity of neutralization, i.e., the average number of neutralizing antibody molecules combined with the critical sites of a virus particle; (2) B_c: the concentration of neutralizing antibody in a given antiserum, expressed as antibody equivalents of a given virus; (3) n: the average number of critical antigenic sites per virus particle; (4) K: the initial slope of the kinetic curve multiplied by the dilution factor D of the antiserum. Since the preceding considerations suggest that the surface of a virus particle is made up of a mosaic of antigens which possess a different antibody-eliciting ability—both in respect to the quantity and kind of antibody elicited—the parameters (1) to (3) will give an over-all value over all the antigen-antibody systems involved, whereas parameter (4) will be essentially determined for the antigen-antibody system having the highest velocity of neutralization, i.e., the highest product $k_1 c\, A_0$.

The parameters can be determined in the following way:

Parameter (1), the multiplicity of neutralization, m, over all the critical sites can be determined from the initial part of the multiplicity curve (antigen in excess), using the equation $V/V_0 \approx e^{-m}$. The value obtained for m is absolute.

The knowledge of m allows the calculation of *parameter (2)*.

Due to the relationship:

$$m = c\, A_0/(V_a + V_i), \tag{8}$$

where

A_0 = concentration (per milliliter) of neutralizing antibody molecules, of any kind, in the neutralization tube;

c = fraction of the neutralizing antibody molecules absorbed to the critical sites after a long period of incubation. This ratio depends upon the ratio of critical to noncritical sites on the virus surface, and may vary for different virus stocks.

V_a = concentration (per milliliter) of active (plaque-forming) virus particles in the neutralization tube;

V_i = concentration (per milliliter) of inactive (non-plaque-forming) virus particles in the neutralization tube;

we obtain: $$A_0 = m\,(V_a + V_i)/c.$$

$V_a + V_i$ may be determined by counting the virus particles in the electron microscope; c cannot be determined. Thus, an absolute value for A_0 cannot be obtained.

However, by comparing different antisera with the same virus, $(V_a + V_i)$ and c being constant, a value proportional to A_0 can be determined. If we define an amount of antibody giving a multiplicity of neutralization $m = 1$ (final virus survival $e^{-1} = 0.37$) with a given virus, as antibody equivalent (B_e) of this virus, the concentration of neutralizing antibody may be expressed by the number of antibody equivalents present in one milliliter of antiserum.

Since an antibody titer expressed in antibody equivalents depends also on the titer of the virus used, it is for certain cases advantageous to refer the equivalent to the plaque-forming unit. An antibody equivalent of a given virus contains as many antibody equivalents of a plaque-forming unit (B_{pfe}) as there are plaque-forming particles in the virus against which the equivalent was determined. The antibody equivalent of a plaque-forming unit measures a constant amount of antibody, if the ratio of active and inactive antigenic particles in the virus stocks is constant. To insure the reproducibility of this ratio, virus stocks of the same line and produced by a standard technique should be used. We shall call the amount of virus antigen (active and inactive) contained in one plaque-forming unit of standard virus the antigen equivalent of the plaque-forming unit (G_{pfe}). We have thus: (1) The plaque-forming unit, i.e., the amount of virus producing, as average, one plaque; (2) the antigen equivalent of the plaque-forming unit (G_{pfe}), i.e., the amount of virus antigen (active and inactive) that is contained in one plaque-forming unit of the standard virus; and, (3) the antibody equivalent of the plaque-forming unit (B_{pfe}), i.e., the amount of antibody that gives a multiplicity $m = 1$ with one plaque-forming unit of the standard virus.

The relation between plaque-forming equivalents and antibody equivalent is given by the equation: $(B_{pfe}) = (G_{pfe}) \times B_e$.

Given an antiserum with a known number of antibody equivalents (B_{pfe}), the above relation can in turn be used to determine the number of antigen equivalents (G_{pfe}) present in a given virus sample. If the virus is inactive, active virus at low concentration must be added as indicator

for the determination of B_e. This principle has been applied by Krech (1955) to the determination of the antigenic power of poliomyelitis virus inactivated by formaldehyde.

Parameter (3), the average number, n, of critical antigenic sites—of any kind—per virus particle, can be determined from the maximum amount of antibody bound by a virus preparation after a long period of incubation, according to the equation:

$$n = \frac{(cA_0)_{max}}{V_a + V_i} \tag{9}$$

Since, according to (8), $(c\ A_0)_{max} = m_{max}(V_a + V_i)$, (9) reduces to $n = m_{max}$, if the same virus is used for the determination of m and n. The determination of n is, therefore, absolute.

Parameter (4), or K, can be determined with considerable accuracy from the slope of the initial part of the kinetic curve (antibody in excess). The following relationship holds:

$$\ln (V/V_0) = -k_1 n\ c\ A_0 t = -(K/D)t \tag{10}$$

Assuming that k_1, n, and c do not vary appreciably from one virus preparation to another, the parameter K can be used for comparing the antibody concentrations of similar antisera—a procedure commonly used in phage work (Delbrück, 1945). This method for determining relative antibody concentrations does not depend on the concentration of the virus, and requires only a large excess of antibody.

The parameters of the WEE and P1 virus-antibody systems will now be calculated.

The horse serum contained 2.1×10^{11} plaque-forming equivalents of antibody per ml and had a K of 390. Thus the ratio of the potency of the two sera is nearly the same by using either determination. *P1 virus* (rabbit serum). From the multiplicity curve in Fig. 6, obtained with a virus containing 4×10^7 pfu/ml, we find a multiplicity $m = 1$ at an antiserum concentration of 4×10^{-4}, which gives 2.5×10^3 antibody equivalents (B_e) and 10^{11} plaque-forming equivalents (B_{pfe}) of antibody per milliliter of undiluted antiserum. Since electron microscope counts of the virus used are not available, the minimum number of neutralizing antibody molecules cannot be calculated in this system.

From the slope of the kinetic curve at 37° (Fig. 3), we derive: $K/D = 0.066$ min^{-1} at a serum concentration of 3.3×10^{-4}, or $K = 200$.

WEE virus (rabbit serum). From the multiplicity curve of Fig. 5, we derive that one antibody equivalent of the virus ($m = 1$) was contained in 1 ml of antiserum at a concentration of 8×10^{-4}, which corresponds to 1.25×10^3 antibody equivalents (B_e) per ml of undiluted antiserum. Since the virus titer was 5×10^8

pfu/ml, the antiserum contained 6.25×10^{11} plaque-forming equivalents of antibody (B_{pfe}) per ml. As already mentioned, the number of physical virus particles was at least 14 times the number of plaque-forming particles; therefore, the minimum number of neutralizing antibody molecules in the antiserum was 8.7×10^{12} molecules per ml. The actual number may have been considerably higher, if a large proportion of neutralizing antibody molecules were adsorbed to noncritical sites of the virus particles.

From the slope of the kinetic curve at 37° (Fig. 4), we obtain: $K/D = 0.180$ min^{-1} at a serum concentration of 1.7×10^{-4}, or $K = 1080$.

4. Analysis of Certain Results Obtained by the End-point Technique

An hypothesis will now be discussed which may explain two features of neutralization that have been observed with the end-point technique, namely, the influence of the cell system on the antibody titer of a serum, and the influence of the cell system on the slope of the "neutralization curve" (Horsfall, 1939; Walker and Horsfall, 1949; Tyrrell and Horsfall; 1953). The hypothesis is based upon the consideration that free antibody from the virus-antiserum mixture is carried over into the test system. An antibody titration by the end-point technique involves, therefore, an interaction in the measuring system among three components: active virus particles—either belonging to the persistent fraction or not yet neutralized by the antibody—antibody, and cells susceptible to the virus. A number of the active virus particles are neutralized, the remaining fraction attach to the cells and escape neutralization. The infected cells release progeny virus, which is in turn partly inactivated and partly attaches to other cells. A series of growth cycles thus takes place in the culture. The multiplication factor of the virus at each step is a function of the antibody concentration and is smaller when the antibody concentration is higher. This slow viral growth is interrupted at a certain point, either, because the cells lose their ability to support virus growth, or, because a constant time limit is given for the virus growth. In neither case does the "cut-off" time depend on the concentration of the antibody. The decision whether the culture is considered affected by the virus or not depends on whether the number of cells affected by the virus is above or below a certain threshold number. Thus, the variation in the number of the affected cells, which is a continuous function of the antibody concentration, is arbitrarily transformed into an all or none response.

In a system of this kind, the result depends on the state of the cells. With cells which support well the growth of the virus—i.e., have a high virus yield, a short latent period, and are infected more promptly—the

determined antibody titer would tend to be lower than with cells which support virus growth less well. Thus, a dependence of the antibody titer on the type of cells is to be expected.

The effect of the cells on the slope of the "neutralization curve" may be calculated as follows: The multiplication factor of the virus at each growth cycle in the virus-antibody-cell system is, in a first approximation, inversely proportional to the concentration of the antibody, an active particle being exposed to the two competing events: adsorption to a cell with a constant probability per unit time, and combination with antibody with a probability, per unit time, proportional to the antibody concentration A/D, the latter probability being generally larger than the former. The culture is considered infected when the number of cells affected by the virus reaches the threshold size V_T at the "cut-off" time. Thus,

$$V_0 M^c (DK/A)^{c+1} = V_t \quad \text{(or larger), where} \tag{11}$$

V_0 = virus concentration at time zero
M = multiplication factor of the virus at each cycle in the absence of antibody
A = antibody concentration in the antiserum
D = dilution of the antiserum
K = constant related to the velocity of the virus-antibody combination and to the velocity of the attachment of the virus to the cells
c = number of cycles of viral growth taking place before the "cut-off" time; c depends upon both the virus and the cells used.

From (11), we obtain:

$$\log V_0 = \log V_t (A^{c+1}/M^c K^{c+1}) - (c + 1) \log D \tag{12}$$

A plot of $\log V_0$ versus $\log D$ yields a straight line with slope equal to $-(c + 1)$. The slope should be, therefore, cell dependent. The absolute value of the slope should increase when more susceptible cells are present. This result was obtained experimentally for influenza and NDV viruses when the allantoic cavity, the chorionic membrane of the chicken embryo, the mouse lung and mouse brain were compared for their characteristics of neutralization (Tyrrell and Horsfall, 1953).

If no viral growth takes place ($c = 0$), the slope should be equal to -1. This value was found for the hemagglutination-inhibition test of influenza and NDV viruses, and for the neutralization of these viruses

in cell systems of poor susceptibility to the virus (Walker and Horsfall, 1949; Tyrrell and Horsfall, 1953).

This short analysis shows thus that the hypothesis put forward to explain the various features of neutralization accounts satisfactorily for the results of neutralization experiments carried out by the end-point technique.

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REFERENCES

BURNET, F. M., KEOGH, E. V., and LUSH, D. (1937). The immunological reactions of the filterable viruses. *Australian J. Exptl. Biol. Med. Sci.* **15,** 226–368.

DELBRÜCK, M. (1945). Effects of specific antisera on the growth of bacterial viruses (bacteriophages). *J. Bacteriol.* **50,** 137–150.

DULBECCO, R., and VOGT, M. (1954*a*). Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exptl. Med.* **99,** 167–182.

DULBECCO, R., and VOGT, M. (1954*b*). One-step growth curve of Western equine encephalomyelitis virus on chicken embryo cells grown *in vitro* and analysis of virus yields from single cells. *J. Exptl. Med.* **99,** 183–199.

HORSFALL, F. L. (1939). Neutralization of epidemic influenza virus. The linear relationship between the quantity of serum and the quantity of virus neutralized. *J. Exptl. Med.* **70,** 209–222.

HULTIN, J. V., and MCKEE, A. P. (1952). Fixation of "neutralized" influenza virus by susceptible cells. *J. Bacteriol.*, **63,** 437–449.

JERNE, N. K., and AVEGNO, P. (1956). The development of the phage inactivating properties of serum during the course of specific immunization of an animal; reversible and irreversible inactivation. *J. Immunol.* In press.

KRECH, V. (1955). The antigenic potency of non-infectious poliomyelitis virus as determined by its liberating effect on active virus neutralized by immune serum. *J. Exptl. Med.* **101,** 331–340.

MANDELL, J. D. (1955). Inactivation of bacteriophage T4r by specific antiserum. Ph.D. Thesis, California Institute of Technology.

SMOLUCHOWSKI, V. M. (1917). Versuch einer mathematishen Theorie der Koagulationskinetik kolloider Lösungen. *Z. physik. Chem.* **92,** 129–168.

TYRRELL, D. A., and HORSFALL, F. L. (1953). Neutralization of viruses by homologous immune serum. I. Quantitative studies on factors which affect the neutralization reaction with Newcastle disease, influenza A, and bacterial virus T_3. *J. Exptl. Med.* **97,** 845–861.

WALKER, D. L., and HORSFALL, F. L. (1949). Lack of identity in neutralizing and hemagglutination-inhibiting antibodies against influenza viruses. *J. Exptl. Med.* **91,** 65–86.

YOUNGNER, J. S. (1954). Monolayer tissue cultures, I. Preparation and standardization of suspensions of trypsin-dispersed monkey kidney cells. *Proc. Soc. Exptl. Biol. Med.* **85,** 202–205.