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The Neutralization of Influenza and Other Viruses by Homologous Immune Serum. Studies in Roller Tube Tissue Cultures

By

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With 6 Figures

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Introduction

Much work has been done on the quantitative aspects of the neutralization of influenza and other viruses by immune serum. In an earlier paper [*Tyrrell* and *Horsfall* (1)] this was reviewed and it was shown that the host used and the route of inoculation had a profound effect on the amount of serum required to neutralize various dilutions of influenza virus. Since then *Dulbecco* et al. (2) have made a detailed analysis of the kinetics of the neutralization of poliovirus, assessing virus concentration by the production of plaques in monolayers of monkey kidney cells. They give a theoretical treatment of the events occurring when neutralization is done by conventional methods using several dilutions. From this they conclude that a linear relation between log virus used and log serum titre is consistent with their results, and the slope of the line would be steeper in some instances because serum remains near the cells and can neutralize some of the progeny of a particle which escaped neutralization in the original mixture. The more cycles in which this partial neutralization of the progeny occurs the steeper would the slope be.

It has been found that influenza viruses will grow in many types of susceptible cells in roller tube tissue cultures (3, 4, 5). Preliminary observa-

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tions showed that neutralization tests could also be done in such tissue cultures. As these cultures are more readily manipulated than animal hosts, experiments were done to find out how various procedures influenced the serum dilutions required to neutralize large and small amounts of virus. The influenza virus strain used in previous work was used for most experiments. Some experiments with adenoviruses and polioviruses are also described.

Materials

Viruses. Influenza A. The neurotropic WS (NWS) strain of *Francis* and *Moore* (6) contained in allantoic fluids from eggs incubated at 35° C for 48 hours was used. Haemagglutination titres varied from 1000 to 8000.

Poliovirus. The Saukett strain of type III propagated in monkey kidney tissue culture was used.

Adenovirus. A type III strain received from Dr. *H. G. Pereira* was propagated in human liver tissue culture.

All viruses were stored at −70° C in the absence of CO_2 in many small tubes. The contents of the tubes were not used again after they had been thawed.

Antisera. Anti-NWS serum was made in a rabbit as described by *Tyrrell* and *Horsfall* (1).

Anti-poliovirus type III. A rabbit was inoculated intravenously with one ml of infected tissue culture fluid and at the same time intramuscularly with one ml of the same fluid diluted 1 in 2 with oil adjuvants (7). Two weeks later a further 1.5 ml was given intramuscularly and 17 days later the rabbit was bled.

Anti-poliovirus monkey serum was made by inoculating a monkey intramuscularly with one ml of the same antigen with oil adjuvants. This was repeated after 21 days and the monkey was bled 2 and 4½ weeks after the second injection.

Adenovirus immune serum was prepared as described by *Balducci* et al. (8).

All sera were kept at −26° C without preservative.

Tissue Cultures. Media were prepared as described by *Balducci* et al. (8).

Trypsinised chick embryo lung cultures were made as described by *Tyrrell* (3). The maintenance medium consisted of one ml of 5% inactivated horse serum, 5% chick embryo extract, and 90% bovine amniotic fluid.

Trypsin-dispersed monkey and human kidney cultures were made as described by *Balducci* et al. (8). Maintenance medium in experiments with poliovirus consisted of 1.5 ml of 2% calf serum and 0.25% lactalbumin hydrolysate in Hanks' balanced salt solution (Hanks' saline) to which 3 times the normal amount of 1.4% $NaHCO_3$ had been added (i. e. 1.5 ml to 20 ml).

Trypsin-dispersed hamster kidney cultures were made by the method of *Heath* (5). When influenza virus was grown in these or in monkey and human kidney cultures the medium used consisted of one ml of 5% calf serum and 0.25% lactalbumin hydrolysate in Hanks' saline to which twice the normal amount of 1.4% $NaHCO_3$ solution had been added.

Chang's human liver cells (9) were serially propagated. The maintenance medium consisted of 1.5 ml of 5% rabbit serum and 0.25% lactalbumin hydrolysate in Hanks' saline.

In all except a few experiments cultures were incubated at 36° to 37° C in a roller drum.

Methods

Neutralization tests. Sera were inactivated at 56° C for half an hour. Serial 2-fold dilutions were then made in Hanks' saline. Serial 10-fold dilutions of virus seed were made in Hanks' saline. In experiments with poliovirus both serum and virus dilutions were usually made in medium. Equal volumes (usually 0.5 or 0.6 ml) of virus and serum dilutions were then mixed in screw-capped bottles. The mixtures were allowed to stand at room temperature for 20 minutes in experiments with influenza virus and adenovirus and for one hour in experiments with poliovirus. Two, 3, or 4 tubes were inoculated with each serum/virus mixture. The inoculum was $^1/_5$th the volume of the maintenance medium in experiments with influenza virus and adenovirus and $^1/_3$rd in experiments with poliovirus.

With almost every neutralization test a virus infectivity titration was set up starting from the virus dilutions used in the test and using 10- or 3.2-fold dilutions.

Three and six days after inoculation in experiments with influenza virus, the medium was poured off both the neutralization and infectivity titration tubes and a further one ml of medium added. The medium which had been removed was spot tested for haemagglutinin by adding 0.25 ml of 0.5% chicken red cells to approximately 0.75 ml of medium. The tests were read after settling for one hour. Tubes showing agglutination were considered positive.

With other viruses, the medium was changed when the cultures seemed to require it. This was usually every 5—6 days for monkey kidney and every 2—3 days for human liver.

Whenever the medium was changed, an amount of antiserum equal to that added originally was replaced in each tube. The cultures were maintained for from 6 to 14 days depending on the amount of non-specific degeneration.

The tubes were examined daily under the low power of the microscope and the results considered positive whenever any definite, specific, cytopathogenic effect was seen. The end points were calculated by the method of *Reed* and *Muench* (10) as the dilution of serum in the inoculum (before the addition of virus) at which 50% of the cultures were positive. The end points were usually very clear-cut. In the influenza experiments the haemagglutination end point (which was usually identical with the cytopathogenic end point) was usually taken. Where there was more than a 16-fold difference between the serum concentration at which all the tubes were positive and that at which all the tubes were negative, the results were neglected. The log of the serum dilution end point was plotted against the log of the virus dilution used and regression lines of virus dilution on serum dilution were fitted to the experimental data by the method of least squares. The slope of the line for each experiment was recorded. For each virus/tissue culture system studied, several estimations of the slope were made, at least one with 4—5 points, the others usually with only 2—3 points. There was a 100- to 100,000-fold difference between the largest and smallest amounts of virus used in each experiment. The end points of the virus titrations were calculated by the method of *Reed* and *Muench* (10), and the TCD_{50} of the virus seed calculated at 3, 6, and 9 days for each experiment.

In some experiments, the tubes were washed out 2—3 hours after inoculation. In experiments with influenza virus and adenovirus the medium was poured off; and in experiments with poliovirus it was sucked out of the tubes, a separate pipette being used for each serum/virus mixture. Hanks' saline

was then added to each tube, the tube was shaken, and the process repeated twice. One ml of fresh medium was then added to each tube. In early experiments, one ml of Hanks' saline was added for each of the three washings (partial washing). In later experiments, 4 ml was added (thorough washing). An automatic pipette was used to add the Hanks' saline and medium. This avoided carrying virus over from tube to tube.

Results

Neutralization curves in different systems

Neutralization tests with varying amounts of virus were done using mixtures of influenza virus and immune rabbit serum inoculated into cultures of chick embryo lung, hamster kidney, monkey kidney and human kidney. Similar experiments were done with adenovirus in human liver cells and poliovirus in monkey and human kidney cultures. Figs. 1—5 show graphically the results of one typical experiment with each system.

In almost all cases there appeared to be a linear relation between log virus concentration and log serum concentration at the end point of neutralization. Neutralization of very large amounts of virus was not tested.

For each experiment, the slope of the neutralization line was calculated for readings taken at 3, 6, and sometimes 9 and 12 days. No definite trend in the value of the slope at different days was discovered (see below) so that for each experiment the mean of these slopes was taken. In Table 1 the range of results obtained in individual experiments is given for each system. The slope calculated from the pooled data for all the experiments is given together with its estimated standard deviation. Where a different serum or virus seed was used a correction was made for the displacement of the mean of the distribution and a corresponding reduction was made in the degrees of freedom used when calculating the standard deviation.

In the experiments with poliovirus both rabbit and monkey immune sera were used and similar results were obtained with each.

The effect of varying the tissue culture system

The slopes of neutralization lines obtained with influenza virus in tissue cultures of different cells are shown in Table 1. The steepest slopes were similar to that of the influenza virus neutralization line obtained when using allantoic inoculation of eggs.

The slope of the influenza virus neutralization line obtained using chick embryo cultures prepared as explants in plasma clot (3.31) was similar to that obtained using chick embryo lung cultures prepared by trypsin treatment.

It was found that in tissue cultures kept stationary at a low temperature the appearance of cytopathogenic effect was delayed by several days and

Table 1. The slopes of neutralization lines in different systems

Virus	Tissue Culture	Mean TCD_{50} /ml seed (log_{10})	Range of slopes obtained in different experiments	Slopes calculated from pooled data.		Number expts.	Number points
				Slope	Standard deviation.		
Influenza	Eggs via allantois:	10.0		5.24	0.61	1	5
	Chick embryo lung:	7.85	3.07—4.43	3.72*	0.37*	4	10
	Hamster kidney:	7.29	3.28—3.92	3.64*	0.23*	3	10
	Monkey kidney:	6.87	2.55—2.57	2.54*	0.04*	2	8
Poliovirus	Monkey kidney:	6.7	1.49—3.32	2.00*	0.07*	5	15
	Human kidney:	6.3	2.77—3.93	3.25	0.35	2	8
Adenovirus	Human liver:	6.45	1.95—1.97	1.94	0.06	2	7

* Different lots of serum or virus used and displacement of neutralization line allowed for in calculation.

virus infectivity titres were reduced. The slope of the neutralization line of influenza virus in monkey kidney was 1.45 and 2.99 in two experiments done at 30° C, and 0.99 in an experiment done at 26° C. The slope was therefore lower in these cultures than in experiments where the cultures were rolled and kept at 37° C.

It was concluded that the slope of the neutralization line varied when different tissue cultures were used just as it does when different animal host systems are used (1). With influenza virus the slopes seemed to be higher in systems where the virus titre was high and cytopathic effect appeared quickly.

The effect of serum remaining in the culture tubes

The above experiments showed that in many cases the serum neutralization end point was almost unaffected by very large differences in the amount of virus used. This might be because the serum added to the culture in the inoculum was able to neutralize the progeny of virus particles which somehow escaped neutralization when the virus/serum mixture was made initially (2).

Neutralization curves were repeated in all the systems, washing out the serum 2 hours after inoculating the mixtures (see above). The serum was not replaced when the medium was changed. Some experiments were done with partial washing and some with thorough washing.

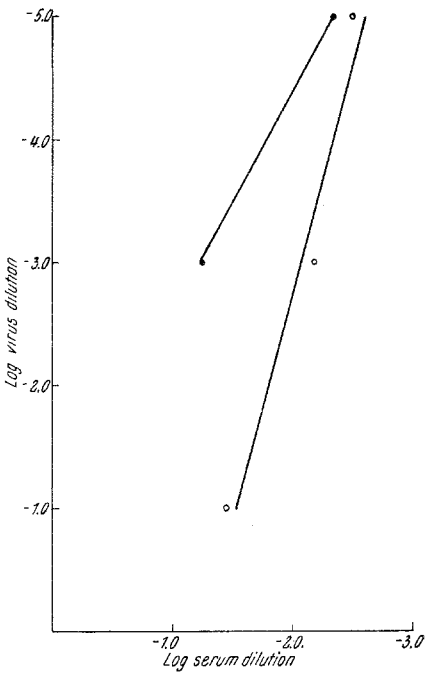

Fig. 1. Neutralization curves of influenza virus in chick embryo lung cultures at 3 days
●——● = Curve using washed cultures. Slope 1.82
o——o = Curve using unwashed cultures. Slope 3.58

Figs. 1—5 each show one typical neutralization line obtained in washed cultures. The end point with a small amount of virus is similar to that with unwashed cultures, but after washing, much larger amounts of serum are needed to neutralize large amounts of virus.

The slopes are shown in Table 2. All slopes obtained in these experiments were lower than the lowest slopes found using unwashed cultures (Table 1).

On the whole the end points after washing were rather uneven. Many more experiments with washed cultures were done which could not be included in Table 2 since with some of the end points on which the slopes were based there was more than a 16-fold difference between the serum concentration at which all the tubes were positive and that at which all the tubes were negative. However, the slopes were very similar to those in Table 2. Three of these experiments excluded from Table 2 using influenza virus in chick embryo lung and partially washed tubes had slopes of 1.33, 1.79, and 2.22. One experiment with influenza virus in hamster kidney cultures which were partially washed had a slope of 1.21. Two experiments with

Table 2. The slopes of neutralization lines in different culture systems in which the cultures were washed 2 hours after inoculation

Virus	Tissue Culture	Slope* after washing:	
		Partial	Thorough.
Influenza	Chick embryo lung	1.82	
Influenza	Hamster kidney	1.03	1.25
Influenza	Monkey kidney	1.56	1.22
Adenovirus	Human liver	1.59	

* Based on 2 to 5 experimental points in each case.

influenza virus in monkey kidney cultures which were thoroughly washed had slopes of 1.11 and 1.76. One experiment with poliovirus in monkey kidney cultures which were partially washed had a slope of 1.33, and three such experiments in which the cultures were thoroughly washed had slopes of 0.79, 0.80, and 0.98.

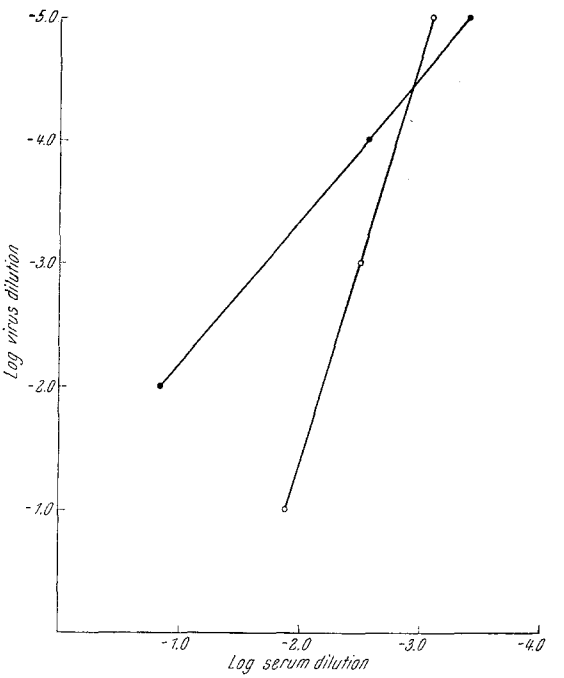

Fig. 2. Neutralization curves of influenza virus in hamster kidney cultures at 6 days
●——● = Curve using washed cultures. Slope 1.17
○——○ = Curve using unwashed cultures. Slope 3.62

In one experiment with influenza virus in hamster kidney the tubes were thoroughly washed out and then the serum was replaced. The slope was 3.28 and therefore similar to slopes using unwashed tubes. The smallness of the slopes in the experiments with washed cultures was therefore due to the removal of serum rather than to other changes produced by the washing process.

Further experiments were carried out to determine the time at which residual serum produced its effect. In experiments with influenza virus in chick embryo lung cultures, the serum dilutions were added to the cultures 2 hours after the virus (i. e. probably after most of

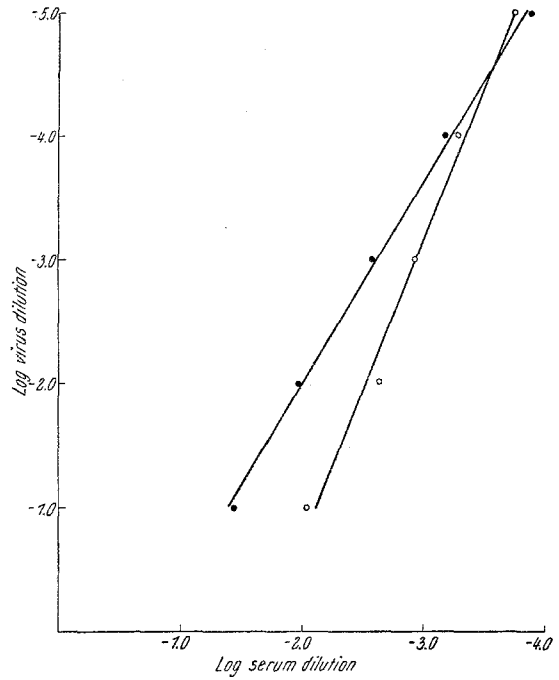

Fig. 3. Neutralization curves of influenza virus in monkey kidney cultures at 3 days
●——● = Curve using washed cultures. Slope 1.66
○——○ = Curve using unwashed cultures. Slope 2.53

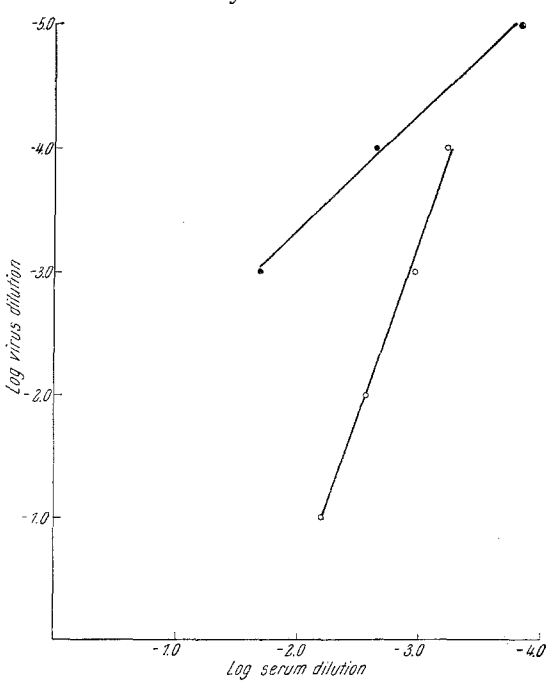

Fig. 4. Neutralization curves of poliovirus in monkey kidney cultures at 6 days
●——● = Curve using washed cultures. Slope 0.95 (This curve was based on very uneven end points)
○——○ = Curve using unwashed cultures. Slope 2.79

the virus was inside the cells). The neutralization end points obtained were 2—3 times lower than in the control, but the slope was 3.33, i. e. similar to that found in unwashed cultures. In two experiments with influenza virus in chick embryo lung cultures the serum dilutions were added for 2-hour periods between 2 and 8 hours after the virus was inoculated and were then washed out again. No neutralization was detected and the titre was reduced at least 16-fold below that obtained when serum was present between 0 and 2 hours. These experiments indicate that in the experiments with the washed cultures the serum is apparently acting on the virus before its adsorption to the host cells but not immediately after adsorption i. e. 2—8 hours after inoculation.

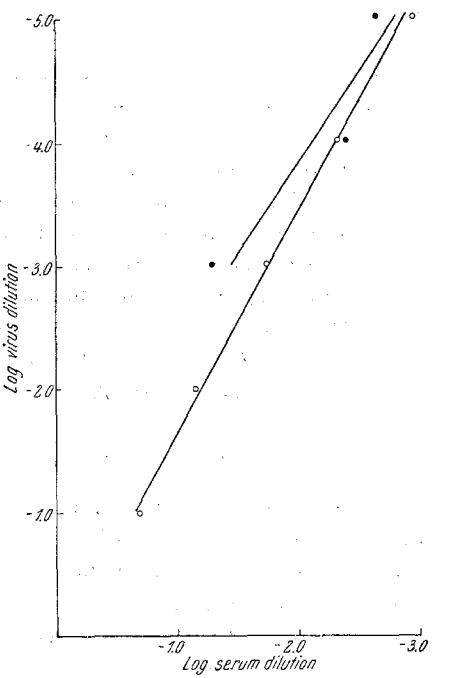

Fig. 5. Neutralization curves of adenovirus in human liver cultures at 9 days
●——● = Curve using washed cultures. Slope 1.3
○——○ = Curve using unwashed cultures. Slope 1.75

The effect of serum components other than antibody

Morgan (11) showed that inactivation of antiserum or storage at 4° C reduced the neutralizing capacity of concentrated antiserum against western equine encephalitis virus, although dilute serum was unaffected. The neutralizing capacity of serum kept at 4° C could be restored by the addition of fresh guinea pig serum.

Dulbecco et al. (2) found that the unneutralizable fraction of western equine encephalitis virus was reduced by the addition of a 1 in 2 dilution of fresh normal rabbit serum to the neutralization tubes.

It was thought that the particular level of serum persisting in the culture which was needed to produce neutralization in unwashed cultures might be due to a limiting concentration of some component other than antibody which could combine with the virus-antibody complex. In one experiment, the effect of removing this hypothetical complement-like substance from the antiserum was noted. 0.5 ml of inactivated anti-NWS serum was mixed with 0.5 ml of inactivated anti-Lee serum and one ml

of infective, undiluted influenza B (Lee) virus (allantoic fluid) and one ml of normal allantoic fluid. For the control, 0.5 ml of inactivated anti-NWS serum was mixed with 0.5 ml anti-Lee serum and 2.0 ml of normal allantoic fluid. Both were shaken and kept at room temperature for one hour before serum dilutions were made as usual in Hanks' saline. A standard neutralization test was then done with influenza (NWS) virus in chick embryo lung cultures. The end point in the test and control series were identical (slope = 4.43 in both).

The effect of adding extra serum components was tested with influenza virus by making the serum dilutions in a $^1/_{20}$ dilution of normal rabbit serum. The end points in monkey kidney cultures using both high and low concentrations of virus were the same as in parallel titrations in which dilutions were made in Hanks' saline.

The effect of centrifuging the virus seed

Black and *Clarke* (12) found that the easily sedimentable fraction of Bwamba virus was more resistant to neutralization than the less easily sedimentable fraction, probably because it represented aggregates of virus particles which the antiserum could not penetrate. Two experiments were done to see if this occurred with influenza virus. The influenza virus seed was centrifuged at 3000 r. p. m. for 40 minutes to remove large particles, the supernatant being used for the neutralization test in chick embryo lung. In both, the end points were in the usual range and the slopes were 5.28 and 4.87. Aggregated virus particles therefore do not increase the slope in these experiments.

The relation of the number of cycles of multiplication to the slope of the neutralization line

We carried out experiments to see if three predictions made by *Dulbecco's* formula (2) could be confirmed.

Firstly a slope of near unity should be found when there is no second cycle of multiplication. Adenovirus type III does not propagate in rabbit kidney cells but causes a toxic effect [*Kelly* and *Pereira* (13)] and neutralization tests can be done in this system (8). This neutralization of the toxic effect should therefore show a slope of 1.0. We found a slope of 0.73 when adenovirus type III was neutralized in rabbit kidney cells. From the results of *Grayston* et al. (14) in which they used toxic doses of adenovirus on HeLa cells [*Pereira* and *Kelly* (15)], one can calculate that the slope was less than 1.0 in that system.

The second prediction is that in a neutralization test performed in a tissue culture where the serum remains in the medium, the slope should

be larger the longer the time between inoculation and the reading of the test. In many experiments there were definite, though rarely large, differences in slope depending on which day the experiments were read. Of 18 experiments using unwashed tubes, in 4 the slope was higher and in 2 the slope was lower in later readings of the test. In 12 there was no change. Of 14 experiments with washed tubes, in one the slope was higher, in 6 it was lower, and in 7 there was no change in later readings of the test. These findings did not therefore support *Dulbecco's* theory.

The third prediction is that if the time of reading were kept constant then the slope would be smaller when tests were done in cultures in which virus multiplied slowly. This was apparently confirmed in experiments described in the section on the effect of varying the tissue culture system.

Effect of mixing virus and serum in small and large volume

Vaccinia virus (16), western equine encephalitis virus (17), and adenovirus (18) are better neutralized when virus and serum react in concentrated solutions than when they react in dilute solutions. We tried to demonstrate this using influenza virus in monkey kidney cultures and eliminating the possible effect on virus multiplication of antiserum remaining in the cultures.

In one experiment (small volume experiment) the virus/serum mixture was made ten times more concentrated than in the standard method and diluted immediately before inoculation. In the other experiment (large volume experiment) the mixture was made 6 times more dilute than in the standard method, the inoculum equalling in composition and volume the standard inoculum plus the standard maintenance medium for one tube. Therefore the final concentration of virus and serum in the cultures was the same in both series, but there was a 60-fold difference in the concentration at which they had interacted in the test tube. All cultures were partially washed two hours after inoculation.

At the end point the serum dilution in the tissue culture tube was at least 6 times higher in the small volume experiment than that in the large volume experiment when a large amount of virus was neutralized. It was at least 50 times higher when a small amount of virus was neutralized.

In three similar experiments with influenza virus in which the cultures were not washed no difference was observed, probably because of the continued action of antiserum.

Reactivation Experiments

Reactivation of virus/serum mixtures on dilution has been demonstrated with fowl plague (19), vaccinia (20), papilloma virus (21), *Theiler's*

virus (22) and influenza (1, 23, 24). It is possible that the apparent reactivation found by these workers was due to the continued action of serum in the undiluted mixtures, making them appear neutral when in fact they were not. *Dulbecco* et al. (2), who washed their cultures after inoculation, were able to demonstrate no reactivation on dilution of mixtures of immune serum and poliovirus and only a little when using western equine encephalitis virus. *Rubin* and *Franklin* (25), using the same technique, showed that $^1/_{100}$th to $^1/_{1000}$th of neutralized Newcastle disease virus particles could be reactivated on dilution. *Kjellén* (18) found a reactivation phenomenon with adenovirus, but could not decide whether or not this was true reactivation.

Using poliovirus, in which true reactivation does not occur, we attempted to show apparent reactivation of virus/serum mixtures on dilution. Virus/serum mixtures which were known to be just neutral were allowed to stand at room temperature for 1 hour. 10-fold dilutions were then made in Hanks' saline and the dilutions allowed to stand at room temperature for a further hour. Monkey kidney tissue culture tubes were then inoculated with each dilution. Apparent reactivation occurred (Table 3. Mixture 1 and 2). The experiment was repeated with influenza virus washing out the cultures thoroughly after two hours in order to demonstrate, if possible, true reactivation. Just positive virus/serum mixtures were made. After standing at room temperature for 20 minutes these were shaken up with 20% fresh, washed, chicken red cells and then centrifuged. The supernatant was then diluted 3.2-fold in medium. After a further 20 minutes at 4° C, 0.2 ml of each dilution and of each undiluted mixture was inoculated into tissue culture tubes. Two hours later the tubes were washed. To exclude crossinfection during the washing process many control tubes were included. None became infected.

In all cases where the absorbed, undiluted mixtures were negative, no reactivation occurred. Where the absorbed, undiluted mixtures infected a proportion of the tubes inoculated (Table 3. Mixtures 3 and 4), the dilution end points were more uneven then the end points of virus titrations which had been thoroughly washed out (Table 3). Both the mixtures and the virus titrations are the pooled results of several experiments. In some experiments the same batch of tissue cultures was used for the dilution of mixtures and for the virus titration. The results are shown graphically in Figure 6, in which the proportion of positive tubes found is compared with the proportion found in a corresponding portion of the virus titration carried out in similarly washed tubes. The results of the virus titration correspond closely with those calculated from the Poisson distribution on the assumption that one virus particle causes infection of a culture and that the cultures are uniformly sensitive. There

Table 3. Attempted reactivation on dilution

Virus	Tubes Washed	Mixture	Undiluted	Dilution ($\log_{10}$)						
				−0.5	−1.0	−1.5	−2.0	−2.5	−3.0	−4.0
Poliovirus	No	1	0/2		2/2		2/2		1/2	0/2
	No	2	0/2		2/2		0/2		1/2	
Influenza	Yes	3	8/20	6/12	4/12	3/12	0/12			
	Yes	4	9/22	8/14	7/14	5/14	3/14	1/4	0/2	
Influenza	Yes	Virus		−4.0	−4.5	−5.0	−5.5	−6.0	−6.5	
		titration		11/13	10/13	4/13	4/13	0/13	0/13	

The numerator represents the number of positive tubes and the denominator the number inoculated.

is clearly an excess of virus in the dilutions of the mixtures. We believe this excess has been released by complexes of virus and antibody, pre-

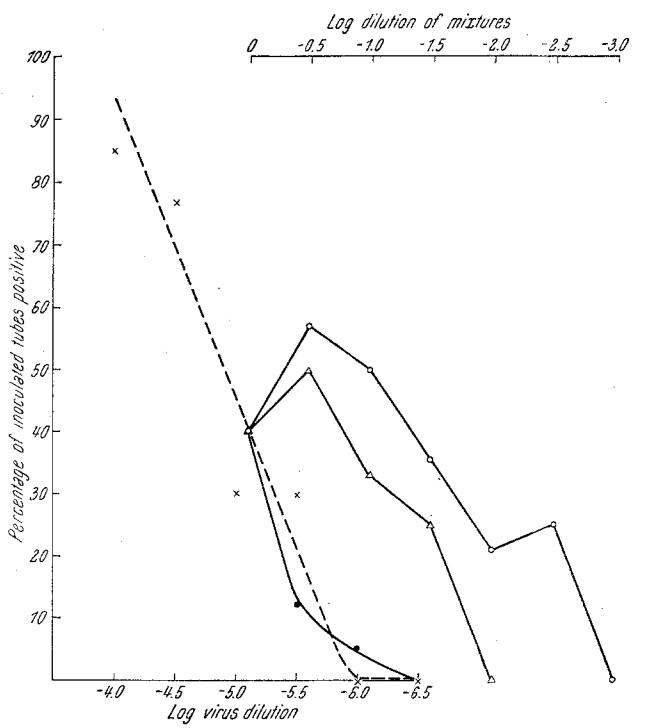

Fig. 6. Reactivation of virus/serum mixtures on dilution
×——× = virus titration
●——● = expected proportion of positive tubes calculated from the proportion positive in the undiluted mixture using the first term of the Poisson distribution
△——△ = dilutions of virus/serum mixture No. 3
○——○ = dilutions of virus/serum mixture No. 4

viously present, separating into their components. We estimate that of the $10^{3.5}$ TCD_{50} originally present in the tube before serum was added, between 10 and 100 doses were reactivated by dilution of each mixture. It is not known how much virus had been adsorbed onto the red cells, but the proportion of reactivated virus was at least as much as that observed by *Rubin* using Newcastle disease virus and rather different techniques.

Discussion

All results on the neutralization of influenza virus, poliovirus, and adenovirus in tissue culture, like those of other workers (26), indicated a steep slope to the neutralization line. The slope obtained using adenovirus was steeper than that obtained by *Ginsberg* (27), who found a slope of 1.0 with type III adenovirus. The reason for this difference is not clear, for he did not use amounts of virus large enough to obtain a toxic effect and he allowed the immune serum to remain in the culture tubes. The experiments on washing cultures showed that the serum titre in unwashed cultures is almost as high when large amounts of virus are used as it is when small amounts of virus are used because the serum left in the tube suppresses the growth of the virus which was present in the mixtures of concentrated virus and serum. It does this apparently by neutralizing the new virus which emerges as the progeny of the originally unneutralized particles. This was the case in experiments with influenza virus and poliovirus. One of us suggested previously (28) that the phenomenon was due to the successive additions of antibody molecules (before the virus encountered the susceptible cells) to give neutralization by successively more sensitive host cells. The theory of *Dulbecco* et al. (2) fits the facts better. They concluded that the steep slope of the neutralization end point curve was due to the action of antibody on successive cycles of virus production. This theory and our results in tissue cultures are also in agreement with the results of neutralization experiments on influenza virus in animals [*Tyrrell* and *Horsfall* (1)]. A slope near unity was found when neutralization tests were done in the mouse brain and lung and in this case excess antiserum is probably carried away rapidly by the circulation from the site of inoculation. Inoculation onto the chorio-allantoic membrane allows the serum to influence the early stages of virus multiplication but not the later stages when the virus spreads to the embryo, and the neutralization curve found was steeper than in the mouse. However when mixtures are inoculated into the allantoic cavity each new crop of virus particles is exposed to serum in the allantoic fluid, exactly as in a test tube tissue culture, and so the neutralization curve was very steep. Using the same membrane cells in a tissue culture in which little virus multiplication occurred the slope was near unity because successive cycles

of cell growth probably did not occur. This result is comparable with our experiments using a toxic effect to detect the presence of unneutralized virus. The slope of near unity obtained on washed tubes when neutralizing poliovirus is also to be expected from this theory, as is the diminished slope found in slowly-growing cultures.

By washing cultures, we have obtained evidence that true reactivation may occur on dilution of mixtures of influenza virus and serum. This reactivation was demonstrated only when an incompletely neutralized mixture was tested. If 5-fold more serum were used, the mixture contained no free virus and no active virus appeared when it was diluted.

Further quantitative data could be obtained by measuring precisely and in detail the concentrations of virus, using large numbers of tissue cultures, or better still, by developing a plaque technique as sensitive as that of the embryonated egg. We were unable to carry out such experiments and therefore present the data in its present incomplete form.

Summary

Neutralization tests using influenza virus, poliovirus, and adenovirus in various tissue cultures showed a linear relationship between the log virus concentration and log serum concentration at the end point of neutralization, the slope of the neutralization line being steep. The effect on this slope of variables in the serum, virus seed, and tissue cultures used and in the method of performing the test was observed.

The neutralization slope was reduced in all the systems when the serum was washed out of the culture tubes 2 hours after inoculating the mixtures.

When influenza virus and antiserum reacted in a concentrated solution more neutralization occurred than when they reacted in a dilute solution.

A small amount of true reactivation on dilution of influenza virus/serum mixtures was possibly demonstrated.

It was concluded that many of the results supported *Dulbecco's* theory of neutralization.

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