NEUTRALIZATION OF EPIDEMIC INFLUENZA VIRUS

The Linear Relationship between the Quantity of Serum and the Quantity of Virus Neutralized

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It seems generally agreed, among those investigators who have studied the neutralization of epidemic influenza virus by various sera, that the test is quite accurate and that dilution end points are reproducible. In the initial paper describing the isolation of the virus in ferrets from patients with influenza, Smith, Andrewes, and Laidlaw (1) showed that the serum of both human beings and ferrets convalescent from the infection neutralized the virus if mixed with it before the intranasal inoculation of ferrets. Simultaneously with the demonstration of the susceptibility of mice to the viruses of human and swine influenza, Andrewes, Laidlaw, and Smith (2) reported that either virus could be specifically neutralized by its homologous antiserum. Laidlaw, Smith, Andrewes, and Dunkin (3) described the neutralization technique in which serial dilutions of serum were tested against a constant amount of virus. This technique, although it has been modified somewhat, remains the most widely used method. They recorded six neutralization experiments on serum I.H.2 which gave quite consistent results. Andrewes, Laidlaw, and Smith (4) found no difference in neutralization end points when serum dilutions and virus were in contact for 10 minutes at room temperature, or for 2 hours at 37°C. In the case of thirty human sera, with each of which neutralization tests were carried out on more than one occasion, they stated that in every instance consistent results were obtained. Francis and Magill (5) compared the neutralization results they obtained on twenty-three sera with those obtained by Andrewes, Laidlaw, and Smith on the same sera and found the correlation to be good. Francis, Magill, Rickard, and Beck (6), in a large series of neutralization tests, repeatedly titered a human convalescent serum as a standard control, and stated that a surprising constancy occurred in the titer of this serum in different tests.

The fact that there are antigenic differences between various strains of epidemic influenza virus was demonstrated by Magill and Francis (7) using the neutralization technique. The extensive studies of Magill and Francis (8) and Smith and Andrewes (9) upon the antigenic differences between strains of the virus have depended very largely upon neutralization tests in mice. The former investigators have expressed the titers of sera in terms of those dilutions which were capable of neutralizing between 100 and 1000 lethal doses of virus, while the latter investigators have expressed the titers of sera in terms of their relative capacity to neutralize an infected mouse lung filtrate as compared with that of a so called standard serum.

It is evident that the neutralization test in mice is of great importance in the investigation of epidemic influenza. It is also apparent that the more quantitative the test can be made, the more valuable will be the information obtained. Particularly is it important to be able to relate directly the results obtained in one series of tests with those obtained in another. It is very desirable to be able to relate the neutralization titers obtained against a given amount of virus with those obtained when a greater or smaller quantity of virus is used. This may be essential when different strains of virus are tested against the same serum, since it is difficult, and in the case of certain strains almost impossible, to prepare suspensions which contain a predictable number of lethal doses of virus.

With these considerations in mind, a series of studies upon the quantitative relationships of the neutralization of epidemic influenza virus by homologous antiserum was undertaken. The results of these experiments will be reported in this paper, and evidence will be presented which indicates that under certain conditions a linear relationship exists between the quantity of virus neutralized and the quantity of serum.

Methods

Sera.—Ferret A-254 was inoculated intranasally under light ether anesthesia with 1.5 cc. of a 10^{-4} suspension of consolidated lung from the 91st ferret passage of the PR8 strain (10) of epidemic influenza virus. The ferret had a typical attack of experimental influenza. 13 days later this ferret was again inoculated intranasally with 2.0 cc. of a 10^{-2} dilution of the same virus and was found to be solidly immune. 24 days after the first and 11 days after the second inoculation, blood was obtained by intracardiac puncture. The serum was separated and was stored at +4°C. for 3 weeks. Then it was divided into five samples of equal quantity and rapidly frozen at −80°C. It was stored at this temperature during the intervals between experiments.

Rabbit R-72 was injected intraperitoneally with 3.0 cc. of a 10^{-1} suspension in 0.85 per cent NaCl of consolidated lung from the 91st ferret passage of the PR8 strain of epidemic influenza virus. 13 days after the injection, blood was obtained by intracardiac puncture. The serum was separated and was stored at +4°C. for 2 months.

These two sera were repeatedly studied in order to determine their capacity to neutralize varying quantities of the homologous strain of virus.

Normal horse serum 986 was used in the preparation of the virus suspensions and dilutions thereof. This one lot of horse serum was used in all the tests.

Virus.—The PR8 strain of epidemic influenza virus which was isolated by Francis (10) was used throughout these studies. Two stock suspensions of infected mouse lungs from the 331st and 332nd consecutive mouse passages were the source of virus. Suspensions were prepared in the following manner:

Mice were inoculated intranasally under light ether anesthesia with 0.05 cc. of a 10^{-3} suspension of infected mouse lung and were killed on the 4th day after inoculation. Their lungs were removed aseptically, weighed, and ground with crystalline alundum;

and sufficient beef infusion broth containing 20 per cent normal horse serum was added to make a 20 per cent suspension by weight. This suspension was centrifuged for 15 minutes at 2500 R.P.M. The supernatant liquid was then withdrawn and divided into ten samples of equal quantity which were immediately frozen at −80°C. The suspensions were kept continuously at this low temperature for 6 months. When virus was required, one tube containing the frozen suspension was rapidly thawed, and the desired quantity was withdrawn. The thawed specimen was then immediately refrozen at −80°C.

Mice

Albino Swiss mice between 3½ and 4 weeks of age, weighing from 13 to 18 gm., were used. The mice were obtained at weekly intervals from four different mouse breeding farms, which will be designated respectively as A, B, C, and D. Six mice were used as a group, and each group was kept in a separate glass jar. Each mouse in a group was inoculated intranasally under light ether anesthesia with 0.05 cc. of the desired dilution of virus or serum and virus mixture. The mice were checked twice daily, and those which died were removed from the jars and their period of survival recorded. Mice which survived the observation period were killed with chloroform on the 10th or 11th day. Their lungs were removed immediately thereafter and examined for the presence and the extent of pulmonary consolidation. The degree of consolidation was expressed in terms of the relative volume of the total lung consolidated; thus ¼ of the lung consolidated = 1, ½ = 2, ¾ = 3, and complete consolidation = 4.

Virus Titrations

Titrations of the virus suspensions, in order to determine the extent to which they could be diluted and still retain their infectiousness for mice, were carried out as follows:

Serial decimal dilutions of the original 20 per cent suspension were made by serially transferring 0.5 cc. to 4.5 cc. of beef infusion broth containing 20 per cent normal horse serum. 1 cc. of each dilution was then mixed with 1.0 cc. of broth in order that the final dilutions would correspond to the series 10^{-1} to 10^{-9} in 10 per cent normal horse serum. Each of a group of six mice was inoculated intranasally with 0.05 cc. of the desired dilution from 10^{-2} to 10^{-9}.

Neutralization Tests

The sera were inactivated by heating to 56°C. for 30 minutes immediately before they were diluted. Serial twofold or fivefold dilutions of serum were made in 0.85 per cent NaCl, the volumes of the final solutions being 2.0 cc. or more. Serial decimal dilutions of the virus suspension were made in beef infusion broth containing 20 per cent normal horse serum, the final volumes of the various dilutions being 5.0 cc. or more. Immediately after the completion of the dilutions, 0.3 cc. of the desired serum dilution was thoroughly mixed with 0.3 cc. of the various virus dilutions. Each dilution of virus was tested against five or six consecutive dilutions of serum, and the serum dilutions were chosen so that the neutralization end point would fall approximately midway between the most and the least dilute serum. The mixtures were incubated at 37°C. for 30 minutes. Immediately thereafter each mixture was given intranasally to a group of six Swiss mice. A virus titration was done with each neutralization test in the man-

ner described above. The same virus dilutions were used for both the neutralization mixtures and the titration. In order to control the time factor, the latter was performed in the period during which the mixtures were incubated at 37°C.

Calculation of Neutralization End Points

In order to determine accurately the highest dilution of the virus suspension which still retained infectiousness for mice, or the highest dilution of serum which was capable of neutralizing a given dilution of the virus suspension, the 50 per cent end point calculation method of Reed and Muench (11) was used. By means of this statistical method it was possible to calculate a number of different end points. For the purposes of this study two end points were chosen, the familiar 50 per cent mortality end point and a new end point which will hereafter be termed the 50 per cent maximum score end point. The latter end point was based upon the sum of the numerical scores obtained for each of the six mice in a group. To obtain a numerical score for each mouse, the following six possible results of the intranasal inoculation of influenza virus were given the indicated values; thus, survival without pulmonary consolidation = 0, survival with ¼ of lungs consolidated = 1, survival with ½ of lungs consolidated = 2, survival with ¾ of lungs consolidated = 3, survival with 4/4 of lungs consolidated = 4, and death with 4/4 of lungs consolidated = 5. In any group of six mice there were then thirty-six possibilities, but the maximum possible score has the numerical value 30 since death with complete pulmonary consolidation is taken to equal 5. The sum of the scores within a group expresses in one term the entire result for the group as a whole; thus, if all six mice die, the sum of their individual scores is $6 \times 5 = 30$; or if all six mice survive and three are found to have consolidations equal to 2, while the three others have consolidations equal to 1, the sum of their scores is $(3 \times 2) + (3 \times 1) = 9$. By means of the 50 per cent end point calculation method it was possible to determine the dilution of virus or of serum and virus mixture which should result in an additive score of 15, or 50 per cent of the maximum score for a group of six mice. The various end points which will be presented, whether in the virus titrations or in the neutralization tests, are in every instance based upon the results obtained in thirty or more mice.

EXPERIMENTAL

Virus Titration

To determine the relative quantity of epidemic influenza virus contained in a suspension of infected mouse lung, almost the only method available at present is the inoculation of mice with serial dilutions of the suspension. Although titrations of this kind may give no indication of the absolute quantity of virus present in the suspension, they do permit a reasonable approximation of the number of infective doses or the number of lethal doses contained in the suspension.

Before attempting to analyze the quantitative aspects of the neutralization of influenza virus by homologous antiserum, it was necessary to determine the accuracy of virulence titrations. A suspension of the PR8 strain of influenza virus which had been kept continuously at −80°C. was

titered at various intervals during a period of 155 days. The suspension was rapidly thawed, a sample withdrawn, and the remainder immediately refrozen at −80°C. Titrations were performed by the method described above.

The results of ten separate titrations on the same suspension are shown in Table I. Both the 50 per cent mortality and the 50 per cent maximum score end points have been calculated, as well as the differences between them and the deviation of each end point from the respective mean. Seven

TABLE I

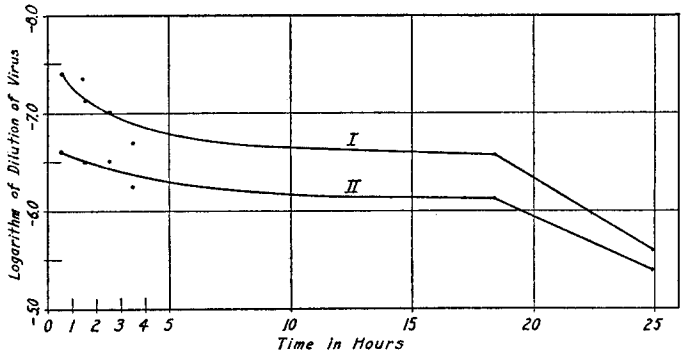
Results of Repeated Titrations on One Suspension of the PR8 Strain of Epidemic Influenza Virus

Titration No.	Time at −80°C.	Mouse breeder	50 per cent mortality end point		50 per cent maximum score end point		Difference between 50 per cent mortality and 50 per cent maximum score end points
			Log	Deviation from mean	Log	Deviation from mean	
	days						
1	2	A	−6.75	+0.26	−7.44	+0.14	−0.69
2	50	A	−6.70	+0.21	−7.96	+0.66	−1.26
3	108	A	−6.37	−0.12	−7.00	−0.30	−0.63
4	108	B	−6.62	+0.13	−7.39	+0.09	−0.77
5	112	C	−6.50	+0.01	−7.20	−0.01	−0.70
6	116	A	−5.83	−0.67	−6.86	−0.44	−1.03
7	121	B	−6.60	+0.11	−7.28	+0.02	−0.68
8	126	A	−6.60	+0.11	−7.37	+0.07	−0.77
9	135	A	−6.50	+0.01	−7.06	−0.24	−0.90
10	155	A	−6.50	+0.01	−7.40	+0.10	−0.90
Mean			−6.49	±0.16	−7.30	±0.20	−0.83

of the titrations were done in mice from breeder A, two from breeder B, and one from breeder C. Although the suspension was stored at −80°C. for 155 days and during this period was thawed and refrozen nine separate times, it is obvious from the results obtained that no appreciable alteration in the infectiousness of the virus originally contained in it had occurred during the interval. Of the ten titrations, only No. 6 was significantly different from the other nine, and the reasons for this single discrepancy are unknown. In the ten titrations the mean value for the 50 per cent mortality end point was 0.05 cc. of a $10^{-6.49}$ dilution, or $5.0 \times 10^{-8.49}$ gm. of infected mouse lung, and the mean deviation of the exponent from this value was ± 0.16. Similarly, the mean value for the 50 per cent maximum score end point was 0.05 cc. of a $10^{-7.30}$ dilution, or $5.0 \times 10^{-9.30}$ gm. of infected

mouse lung, and the mean deviation of the exponent from this value was ± 0.20.

Comparisons between the two different end points indicate, as was to be expected, that the 50 per cent maximum score end point represents a greater dilution of virus than the 50 per cent mortality end point. In the ten titrations the mean difference between the exponents of the two different end points is −0.83. On the basis of this series of repeated titrations on one suspension of virus, it seems evident that consistent results can be obtained



TEXT-FIG. 1. Virus titration end points obtained with a single series of dilutions of the PR8 strain of epidemic influenza virus after varying intervals at 22°C. Line I = 50 per cent maximum score end points. Line II = 50 per cent mortality end points.

by the calculation of either of the two end points and that corresponding end points in two separate titrations are quite accurately reproducible.

Effect of Time and Temperature upon Virus Titration End Points

Since it seemed possible under the conditions described to achieve quite reproducible dilution end points for the infectiousness of a suspension of virus stored for 5 months at −80°C., it was of interest to determine as accurately as possible the decrease in infectiousness which occurs in a suspension maintained at higher temperatures. To determine the decrease in titer which occurs at room temperature, six consecutive titrations were performed on one series of decimal dilutions of virus which were kept at approximately 22°C. for 25 hours. The first titration was done 33 minutes after the dilutions had been prepared, and the last 25 hours later. The results of these six titrations are shown in Text-fig. 1. Both the 50 per cent mortality and the 50 per cent maximum score end points have been calculated, and the difference between them is graphically shown. It will be observed that both end points show a progressive decrease with increas-

ing time and that the 50 per cent maximum score end point decreases slightly more rapidly than does the 50 per cent mortality end point.

To determine the decrease in titer which occurs at 37°C., four separate pairs of titrations were performed on the same suspension of virus. The initial titration in each pair was done immediately after the dilutions had been prepared, and the second was done after the dilutions had been heated to 37°C. for 30 minutes in a water bath. The 50 per cent mortality end point had in the four initial titrations a mean value of $5.0 \times 10^{-8.36}$ gm. of nfected mouse lung, while in the four second titrations it had a mean value

TABLE II

Mortality Rates Resulting from the Inoculation of Mice with Varying Amounts of the PR8 Strain of Epidemic Influenza Virus

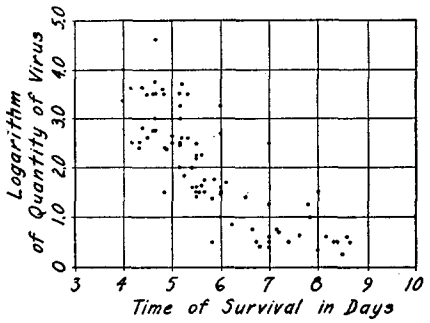
Lethal doses of virus		Number of mice			Mortality ratio	Survival time for individual mice		
Log	Number	Total	Died	Survived		Shortest	Longest	Mean variation within a group
						days	*days*	*days*
3.5–3.9	3160–7940	66	66	0	1.000	4	7	1.73
3.0–3.4	1000–2510	30	30	0	1.000	4	7	1.40
2.5–2.9	316–794	83	83	0	1.000	4	9	2.00
2.0–2.4	100–251	48	46	2	0.959	4	7	1.25
1.5–1.9	32–79	83	83	0	1.000	5	10	1.73
1.0–1.4	10–25	47	46	1	0.979	5	10	2.25
0.5–0.9	3–8	84	80	4	0.952	5	10	2.07
0.1–0.4	1.2–2.5	35	26	9	0.743	6	9	2.20

of $5.0 \times 10^{-7.84}$ gm., representing a decrease in the exponent of -0.52. The 50 per cent maximum score end point had in the four initial titrations a mean value of $5.0 \times 10^{-8.89}$ gm., while in the four second titrations it had a mean value of $5.0 \times 10^{-8.43}$ gm., representing a decrease of -0.43 in the exponent. Both end points indicate that a definite decrease in virus titer occurs in 30 minutes at 37°C.

Susceptibility of Swiss Mice

In order to assess the relative susceptibility of Swiss mice to comparable numbers of lethal doses of influenza virus, the 50 per cent mortality end point in each titration was taken as equal to one lethal dose, and from this the various numbers of lethal doses each group of mice had received were determined. As has already been indicated in Table I, no detectable difference in susceptibility to the PR8 strain of influenza virus was shown by

Swiss mice received from three separate breeders as determined by repeated dilution end points on the same suspension of the virus. This conclusion was also borne out by the results obtained when mortality ratios were calculated for various mice which received known numbers of lethal doses of the PR8 strain. In Table II are shown the results obtained in 462 mice which received various amounts of the PR8 strain of influenza virus. These mice were obtained from four separate breeding farms. It will be seen that the mortality rates were very high in the case of all mice which received three or more lethal doses. Even in the case of 84 mice which received only from three to eight lethal doses, 80 mice died, a mortality rate of 0.952, and of the four mice which survived three had +++ lesions when autopsied. It is apparent from these results that the mice tested were quite uniformly susceptible, even to very small amounts of the virus.



TEXT-FIG. 2. Mean survival time of groups of six Swiss mice inoculated intranasally with varying numbers of lethal doses of the PR8 strain of epidemic influenza virus.

Relation between Amount of Virus Inoculated and Time of Survival

It is well known that mice which receive small amounts of influenza virus survive for a longer period than do mice which receive large amounts. It was of interest to determine whether the period of survival was directly related to the number of lethal doses inoculated and whether it could be used as an indication of the amount of virus received by a group of mice.

As in the case of the calculation of mortality rates, one lethal dose was taken to be that dilution of virus of which 0.05 cc. would cause death of 50 per cent of the inoculated mice. In Text-fig. 2 are shown the results obtained in a total of 476 Swiss mice which received various amounts of influenza virus. The log of the number of lethal doses inoculated has been plotted against the mean survival time in days for each group of six mice. It is obvious that the results are widely scattered, and not only may groups of six mice receiving comparable numbers of lethal doses vary by $\pm$ 1.5 days in mean survival time, but also those which survive for equal periods may have been inoculated with a number of lethal doses varying by $\pm$ log 1.5. The period of survival, therefore, cannot be taken as more than a very crude indication of the amount of virus inoculated. The results tend

to confirm the impression that 10 days is a sufficient period to prolong observation of mice inoculated with influenza virus, and it will be noted that the longest mean survival time shown in Text-fig. 2 is 8.65 days, and that these mice received only three lethal doses of virus. It may also be worth pointing out that no group of six mice, however much virus they received, had a mean survival time of less than 4 days. The shortest and the longest survival time of individual mice inoculated with comparable numbers of lethal doses of virus has also been determined and is shown in Table II. The mean difference between the shortest and the longest individual survival time within each group of six mice is also given.

Neutralization of Epidemic Influenza Virus by Homologous Antiserum

With the aid of the information gained in the attempts to determine as accurately as possible some of the quantitative limits of influenza virus titrations, as well as the decrease in titration end points which occurs with increasing time at both 22°C. and 37°C., and the mortality rates which result from the inoculation of varying amounts of virus, it seemed that it should be possible to assess the relationship between the quantity of virus and quantity of antiserum in the neutralization test. The detailed technique of the neutralization test used in these studies has been described above, and for the purposes of quantitative analysis every effort was made to carry out the various duplicate tests in an identical manner.

Four separate series of neutralization tests were performed with the convalescent ferret serum A-254. In three of the series a suspension of the PR8 strain of virus from the 331st mouse passage was used, while in the fourth the suspension was made from the 332nd mouse passage. Both the quantity of serum and the quantity of virus were varied with respect to each other in order that the quantity of serum required to neutralize each amount of virus could be determined. The 50 per cent mortality and the 50 per cent maximum score end points were both calculated in order that a comparison could be made between the results obtained.

Separate virus titrations were carried out with each series of neutralization tests in order that the quantity of virus initially present in the mixtures could be accurately determined. The quantity of virus neutralized by the various amounts of serum in a particular series of tests was calculated from the virus titration which was done simultaneously with the series. Thus, if the virus contained in $5 \times 10^{-8.5}$ gm. of infected mouse lung was found to cause the death of 50 per cent of mice, and if 5×10^{-4} cc. of serum was just capable of protecting 50 per cent of mice against the virus contained in $5 \times 10^{-4.5}$ gm. of infected mouse lung, this quantity of serum had neu-

tralized $\frac{5 \times 10^{-4.5}}{5 \times 10^{-8.5}} = 10^4$, or 10,000 lethal doses of virus. In Text-fig. 3 the logarithm of the amount of virus neutralized in these four experiments has been plotted against the logarithm of the quantity of serum which was just capable of neutralizing this quantity of virus. The 50 per cent mortality end points are shown in the lower graph and the 50 per cent maximum score end points in the upper graph.

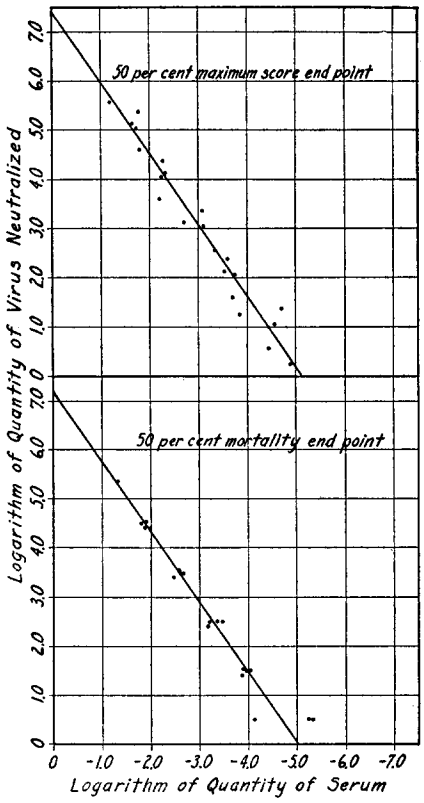
Two straight lines, which on inspection appear best to fit the corresponding end points, have been drawn through the experimentally determined points in Text-fig. 3. The correspondence between the experimental points and straight lines is fairly close, and there is no systematic deviation from the lines which would suggest that they are an erroneous generalization of the results.

The 50 per cent mortality end point appears to be somewhat more consistently reproducible than does the 50 per cent maximum score end point. In those instances in which very small amounts of virus are neutralized by very small quantities of serum neither end point is accurate. It has been impossible to obtain consistent 50 per cent mortality end points in tests in which less than $10^{1.4}$ or twenty-five lethal doses of virus were neutralized. Likewise, when less than $10^{1.6}$ or forty 50 per cent maximum score doses of virus were neutralized, the 50 per cent maximum score end points have been irregular.

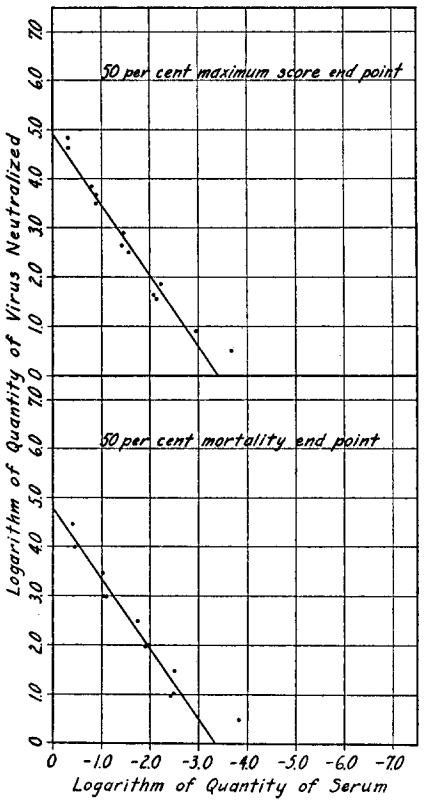
On the basis of the results presented in Text-fig. 3, it appears that the neutralization of the mouse lung passage PR8 strain of epidemic influenza virus by the serum of a ferret convalescent from infection with the homologous ferret passage strain of virus approximates a straight line when logarithms of the respective quantities are used in plotting both variables.

In order to determine whether antiserum resulting from the immunization of a non-susceptible animal would neutralize influenza virus in a manner comparable to the serum of a susceptible animal convalescent from infection by the virus, the serum of an immunized rabbit was studied in an identical manner. Three separate series of neutralization experiments were performed with rabbit antiserum R-72. In one series the 331st and in the other two the 332nd mouse passages of the PR8 strain were the source of virus. Both the amount of virus and the quantity of serum were varied with respect to each other. The 50 per cent mortality and the 50 per cent maximum score end points were both calculated as in the previous experiments, and separate virus titrations were carried out with each of the neutralization series. In Text-fig. 4 the logarithm of the amount of virus neutralized in the three experiments has been plotted against the logarithm of

the quantity of serum which achieved this result. The 50 per cent mortality end points are shown in the lower graph and the 50 per cent maximum score end points in the upper graph. Two straight lines have been drawn



TEXT-FIG. 3



TEXT-FIG. 4

TEXT-FIG. 3. Results of repeated neutralization tests with convalescent ferret serum A-254 and the PR8 strain of epidemic influenza virus. Each end point was determined by the results obtained in thirty or more mice.

TEXT-FIG. 4. Results of repeated neutralization tests with rabbit antiserum R-72 and the PR8 strain of epidemic influenza virus. Each end point was determined by the results obtained in thirty or more mice.

through the experimentally determined points in Text-fig. 4. These lines appear to fit the experimental data rather closely except in those instances in which very small amounts of virus were neutralized. It was previously shown that reproducible end points could not be obtained with the ferret serum when similarly small quantities of virus were neutralized.

From the results presented in Text-fig. 4, it seems probable that the neutralization of the mouse lung passage PR8 strain of influenza virus by the serum of a rabbit immunized with the homologous ferret passage strain of virus also approximates a straight line when logarithms of the respective quantities are plotted along both axes.

Certain characteristics of the four neutralization lines shown in Text-figs. 3 and 4 require special emphasis. The slopes of all four lines are identical even though two different neutralization end points, two entirely different sera, and two suspensions of virus were used in the experiments upon which the lines are based. The neutralization lines do not intersect the two axes of the graphs at points equidistant from zero, but have a much higher value at the point of intersection with the virus axis than at the point of intersection with the serum axis.

DISCUSSION

The variation of both the quantity of epidemic influenza virus and the quantity of homologous antiserum with respect to each other in repeated neutralization tests on the same sera has revealed a definite relationship between the quantity of virus neutralized and the quantity of serum. When the logarithm of the amount of virus neutralized is plotted against the logarithm of the quantity of serum, the neutralization of virus appears to approximate a straight line. This relationship has been found to be the same for the serum of a ferret convalescent from experimental influenza and the serum of a rabbit immunized with the virus. Calculation of both the 50 per cent mortality end point and the so called 50 per cent maximum score end point has shown the relationship to be independent of the end point chosen.

Because functions of the type $y = bx^a$, in which a and b are constants, give straight lines when plotted with logarithms along both axes, it has seemed reasonable to treat these results mathematically. If in the equation $y = bx^a$, y is taken as equal to the quantity of virus neutralized and x is taken as equal to the quantity of serum, it is not difficult to determine the values of the constants a and b. The intercept on the y axis, which is the constant b, is obviously a characteristic of the particular antiserum and is influenced to some extent by the end point used in the calculations. The exponent a is determined by the slope of the neutralization line, and since the slopes of all four lines shown in Text-figs. 3 and 4 appear to be the same, the exponent a is not only constant for each antiserum but is the same for both sera, irrespective of the end point used. It is possible that any considerable variation of the technique, and particularly in the period of contact between virus and antiserum before inoculation, might alter the slope of the neutralization line. This possibility has not yet been accurately determined. The exponent a has the value 1.44 for both the sera studied, and, therefore, it is apparent

that both the sera become progressively less efficient in their ability to neutralize virus as they are diluted. Since these antisera appear to be most efficient in the undiluted state, it has seemed reasonable to assign to them a neutralizing value determined by extrapolating the neutralization line to zero serum dilution. This happens to be b, the intercept on the y axis, which is the second constant in the equation. If, therefore, b is once determined, and if future investigation indicates that a is actually constant, it is possible that the whole range of neutralizing capacity of a given serum can be determined in a relatively simple manner.

Under the conditions of these experiments the intersection of the neutralization line with the axis, along which has been plotted the logarithm of the quantity of virus neutralized, appears to be quite accurately reproducible. It is suggested that this point can be taken as a fixed value for the neutralizing capacity of a serum since it indicates the greatest quantity of virus which the serum can neutralize in the undiluted state.

This value for the virus-neutralizing capacity of a serum has an entirely different significance than has a serum dilution end point. In the case of the latter value a determined dilution of serum which will neutralize a constant amount of virus is usually compared with that dilution of another serum which will neutralize the same amount of virus. Were the relationship between the quantity of virus neutralized and the quantity of serum one of multiple proportions, the serum dilution end point method could be expected to give neutralization capacity values comparable to those obtained by extrapolation of the neutralization line to zero serum dilution. However, as has been indicated above, the relationship is exponential rather than one of multiple proportions, and a given serum becomes progressively less efficient in its capacity to neutralize virus in proportion to the extent to which it is diluted. Because of this exponential relationship, comparisons between various dilutions of different sera which have been found to neutralize a constant amount of virus are quite different from comparisons of the quantity of virus which constant amounts of sera can neutralize. Only in the case that two sera have identical virus-neutralizing capacities will the comparison of serum dilution end points correspond to the comparison between the quantities of virus either can neutralize. As the difference between the virus-neutralizing capacities of two sera is more and more dissimilar, the discrepancy between the ratio of the serum dilutions capable of neutralizing a constant amount of virus and the ratio of the quantity of virus which a constant quantity of serum can neutralize becomes greater and greater. Thus, it will be seen from comparisons between the neutralization lines shown in Text-figs. 3 and 4 that the ferret serum can be diluted $10^{1.66}$ or 46 times more than the rabbit serum when dilutions of both

are tested against any constant amount of virus. On this basis, therefore, the ferret serum seems to be 46 times more effective than the rabbit serum. Actually, however, the ferret serum as a virus-neutralizing agent is 270 times more effective than the rabbit serum since in the undiluted state, or at any constant dilution, it can neutralize $10^{2.43}$ or 270 times more virus than can the rabbit serum. The discrepancy between the ratios of the neutralizing capacities of these two sera, as determined by the constant virus method and the constant serum method, respectively, is, therefore, 270/46 = 5.87. As an approximation, and for the purposes of rapid calculation, it can be stated that for either of the two antisera studied a fivefold decrease in the quantity of serum results in a tenfold decrease in the amount of virus neutralized, or *vice versa*.

CONCLUSIONS

A linear relationship exists between the logarithm of the quantity of epidemic influenza virus neutralized and the logarithm of the quantity of antiserum which is capable of achieving this result. This relationship is the same for the serum of a ferret convalescent from experimental influenza as for the serum of a rabbit immunized with the virus. By means of the linear relationship between virus and antiserum it is possible to determine a fixed, rather than a relative, value for the neutralizing capacity of a serum.

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