ENCEPHALOMYELITIS OF MICE

II. A Method for the Measurement of Virus Activity

By SVEN GARD, M.D.

(From the Laboratories of the International Health Division of The Rockefeller Foundation, New York)

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In the study on the properties of the virus of mouse encephalomyelitis, reported in the first paper of this series, an accurate measurement of virus activity often was of prime importance. As the usual method of titration did not prove entirely satisfactory, an attempt was made to devise new procedures.

The activity of a given virus-containing material is usually expressed in terms of minimal infective (M.I.D.), or lethal, doses. Groups of test animals inoculated with material containing 1 M.I.D. per inoculum will show, on the average, 50 per cent positive results. A titration of material of unknown activity is simply an attempt to find this 50 per cent morbidity rate end point. In most instances the value has to be calculated through interpolation from two observations, preferably by means of the method of Reed and Muench (1). The result is usually given as log M.I.D. per quantity of inoculum.[1]

In titrating a specimen containing an unknown amount of a potent virus, one usually has to try at least six different dilutions. In the calculation of the titer, however, only two, or at most three, of the test groups are of great value. The accuracy of the method varies with the number of test animals used. If each test group consists of eight animals the theoretical error of the titer value obtained is from 0.2 to 0.25 log M.I.D. The result hardly justifies the use of such large numbers of animals.

Horsfall (2), applying the method of Reed and Muench to a series of titrations of influenza virus, found a mean deviation of 0.16 to 0.20 log virus units.

Procedure and Results

In the present study of this problem two highly potent strains of mouse encephalomyelitis virus, GD VII and FA, have been used (3). With the purpose of improving the titration methods, it was attempted to make use of a certain characteristic of the experimental disease.

There exists a definite relationship between the amount of virus injected intracerebrally and the length of the incubation period. This fact is

[1] This is a matter of convenience. It is, no doubt, more appropriate to express the activity in M.I.D. or log M.I.D. per ml. or per gm. of material.

demonstrated in Table I, showing the results of two titrations of infective mouse brain. The records in the table furnish two independent sources of information regarding activity: (*a*) the percentage of sick mice in each group regardless of the incubation period, and (*b*) the average incubation period of a group of mice, in the calculation of which no attention has been paid to the morbidity rate. Both functions attempt to measure the same variable, but it is evident that (*a*) cannot vary at low dilutions, when all mice die; (*b*) breaks down when some mice do not sicken. It should be possible to find a function which will take advantage of more of the information available. Such a function would have a wider range of variation.

TABLE I

*Titration Results Obtained with Two Different Strains of Mouse Encephalomyelitis Virus**

Strain	Concentration of brain	N No. inoculated	n No. sick	No. of mice becoming sick after t days																		Average incubation time	$\frac{\Sigma 1/t}{N}$
				2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	**	
																							days	
GD VII	10^{-2}	18	18			2		5	6	4	1												6.7	0.156
GD VII	10^{-3}	18	18				2	1	2	6	1	3	1	1			1						8.6	0.125
GD VII	10^{-4}	18	18					2	1	1		2	4	4	2	1	1						10.8	0.099
GD VII	10^{-5}	17	15									1	3	4	1	1	2		3			2	13.3	0.069
GD VII	10^{-6}	18	12							1					2	1			6	1	1	6	15.6	0.045
FA	10^{-1}	6	6	1	4	1																	3.0	0.347
FA	10^{-3}	6	6			2	4																4.7	0.217
FA	10^{-5}	6	4				1		1			1						1				2	9.5	0.088
FA	10^{-7}	6	0																			6	—	0.000

* Dose = 0.03 cc.

** Number lived.

The simplest function to fulfill this condition is probably the following expression:

$$\frac{1}{T} = \frac{1/t_1 + 1/t_2 + 1/t_3 + \cdots + 1/t_N}{N} \tag{1}$$

when $t_1 \cdots t_N$ are the individual incubation periods and N the number of mice inoculated. In the case of a mouse remaining well the incubation time is infinite and the reciprocal = 0. The whole expression (1) is actually the reciprocal of the harmonic mean (T) of the incubation period. In the last column of Table I the value of this function has been calculated for each mouse group. In Chart 1 the values are plotted against log con-

centration of virus, given as mouse brain, wet weight. The resulting curves are approximately linear.

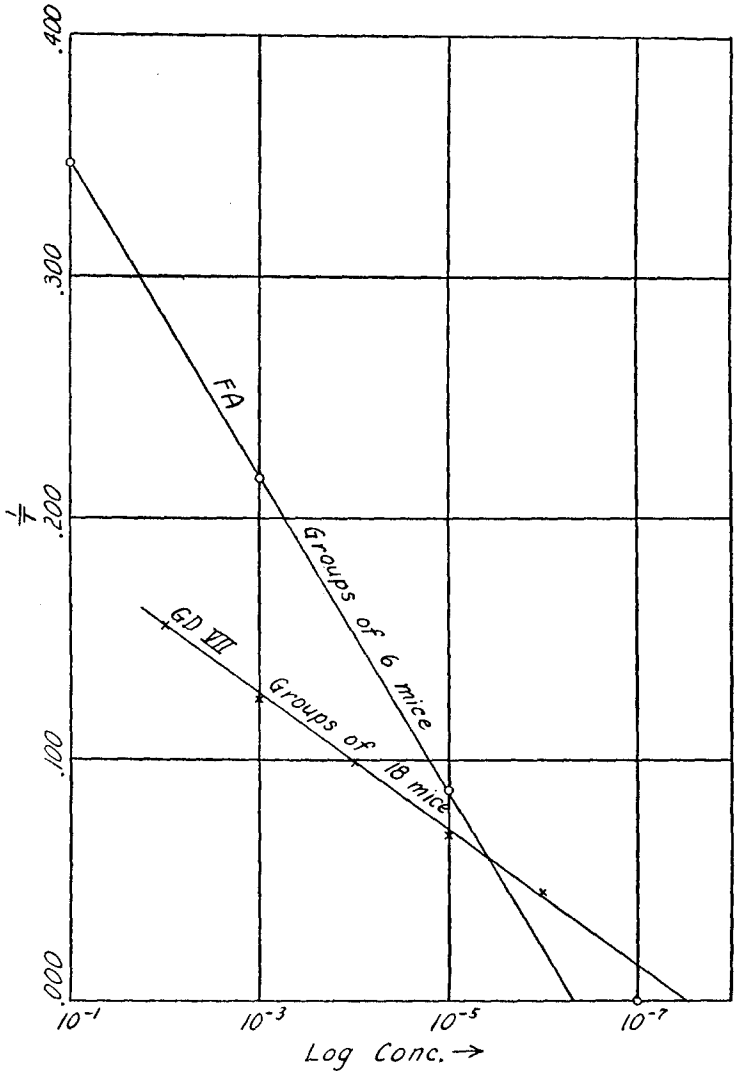

CHART 1. Linear relationship between the reciprocal of the harmonic mean of the incubation periods ($1/T$) and the logarithm of the concentration of virus.

The two titrations in Table I were carried out with two strains of virus, which differ considerably as to the length of the incubation period after intracerebral inoculation. The difference in the slope of the curves is

attributed mainly to this fact. An attempt to find out whether or not the characteristics of the titration curves are constant from one experi-

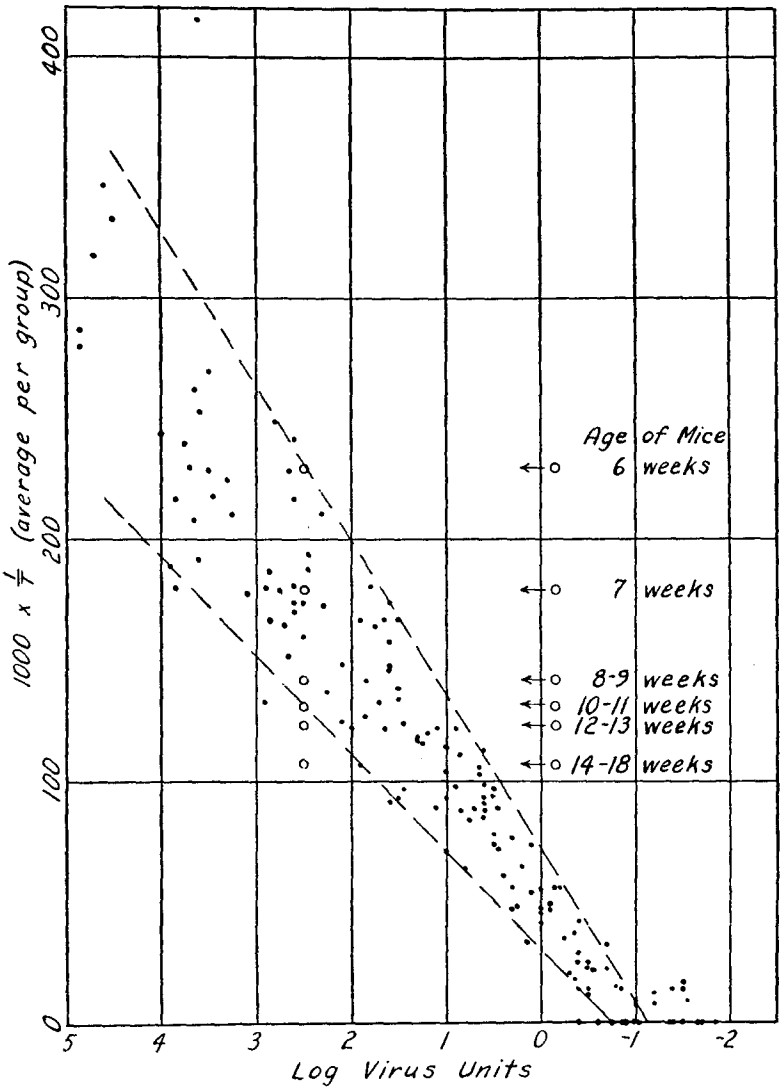

CHART 2. Relationship between the reciprocal of the harmonic mean of the incubation periods ($1/T$) and the logarithm of the quantity of virus in multiple experiments with one strain and the influence of the age of mice upon the incubation period.

ment to another, when one and the same strain of virus is being used, is demonstrated in Chart 2. In thirty-four experiments with the strain FA

the activity was calculated in the usual way in terms of M.I.D./gm. brain, and the values of $1000/T$ plotted against log M.I.D. Each point represents a group of five or more mice. The distribution figure is fan-shaped, converging towards a point on the abscissa corresponding to 10^{-1} M.I.D. This means that the individual titration curves have a different slope but all reach the abscissa at the same point, 10^{-1} M.I.D. (Theoretically one should expect the curves to deviate from a straight line in the vicinity of $1/T = 0$ and approach the abscissa asymptotically. The distribution of the points in that part of the diagram indicates that this is actually the case.)

In an attempt to furnish an explanation of the variations observed, an experiment was carried out in which groups of mice of varying age were inoculated intracerebrally with the same amount of virus. The results

TABLE II

Effect of Age of Mice on Susceptibility to Encephalomyelitis Virus

Age of mice	N No. inoculated	$\frac{\Sigma 1/t}{N}$	Survivors
wks.			*per cent*
6	18	0.230	0
7	18	0.179	0
8–9	37	0.142	8
10–11	34	0.131	9
12–13	34	0.123	9
14–18	77	0.107	19.5

were recorded in the usual way and are summarized in Table II. From this table it can be seen that the resistance of the mice to infection by the intracerebral route rapidly increases with age. The incubation period becomes longer, thus the value $1/T$ smaller, and the mortality rate as well as the morbidity rate decreases. The values of $1/T$ from Table II are superimposed on the distribution diagram in Chart 2. It is obvious that the differences observed in the slope of the titration curves might be satisfactorily explained by a variation of 2 weeks in the age of the mice used for the experiments, and this is probably what has happened. The mice are delivered from the breeder to the laboratory once a week at an age of approximately 6 weeks. They are frequently not used until 1 week after receipt and have sometimes been held over for even longer periods when it has been considered important to use the same lot of mice throughout an experiment.

This observation emphasizes the importance of a uniform supply of animals for quantitative virus work. Obviously two different titration

results are not directly comparable unless all characteristics of the titration curves are identical.

Even if variations in the mouse stock make the construction of a standard titration curve impracticable, it is possible to devise a simple method for titration on the basis of the fact that the intersection between the curve and the abscissa appears to be constant at 10^{-1} M.I.D. One need only determine two points on the curve to be able to calculate the activity of an unknown material according to the following formula:

$$\log a = \frac{1/T_1 (\log d_1 - \log d_2)}{1/T_1 - 1/T_2} - (\log d_1) \qquad (2)$$

the activity being a (M.I.D./cc.), the test doses d_1 and d_2. $1/T_1$ and $1/T_2$ are to be calculated according to Equation (1).

There is one particular type of experiment in which a simplification of the titration procedure is highly wanted, namely, when a series of activity

TABLE III

Mean Deviation of the Characteristics 1/T

	Virus FA	Virus GD VII
No. of groups	327	83
Mean standard deviation (σ)	0.0432	0.0380
Correlation: σ against $(1/T)$	+0.1234	+0.2807
Regression of σ on $(1/T)$	σ = 0.0397 +0.0289 $(1/T)$	σ = 0.0310 +0.1038 $(1/T)$

determinations is to be carried out simultaneously. An instance is the titration of neutralizing antisera. A procedure that might be applied in such cases is the following:—

The activity control (the untreated virus suspension, the normal serum control, etc.) is tested in a series of tenfold dilutions. In order to simplify the subsequent calculation of the titration curve, an odd number of points is preferable, *i.e.*, five or seven dilutions. Of the specimens to be compared with the standard virus one or, for the sake of greater accuracy, two different dilutions are tested. From the control titration a standard curve is calculated. The activity of the test samples can then be computed by means of interpolation in the standard curve.

The accuracy of this method of titration can be estimated by measuring the scatter of the reciprocal of the incubation period of individual mice around the mean value and from these obtaining a value for the standard deviation (σ) of individual mice. The standard error of the mean of a group of n mice can then be taken as $\sigma/\sqrt{n}$. The results tabulated in Table III show that there is some variation of σ at different levels of $1/T$.

The correlation between σ and $1/T$ is, however, low and not very significant. If the value of σ, for both viruses, is taken as 0.045 a rather conservative estimate is made. Then the standard error of a group of twelve mice is $0.045/\sqrt{12} = 0.013$. With an average slope of the titration curve of 0.050 per 1.0 log M.I.D. (which is true for the FA strain) this corresponds to 0.25 log M.I.D. The error of the value of the activity, calculated as the average from two observations, can be estimated to about 0.18 log M.I.D. For comparison it may be mentioned that the error of the titer value computed as the 50 per cent morbidity end point usually is not below 0.20 log M.I.D.

If accuracy is wanted rather than simplicity, one has to use a larger number of mouse groups. Thus, the error is reduced to 0.13 and 0.106 log M.I.D. if four or six groups respectively are used. In these cases the distribution of the points along the curve might give further information as to the reliability of a particular titration result.

The data in Table I show that the incubation period sometimes has a considerable length. In the case of the FA strain it very seldom exceeds 21 days, but has occasionally reached 25 days. In experiments where the 50 per cent morbidity end point titration method has been used it was, therefore, considered necessary to extend the observation period to 4 weeks, which makes the experiments very time-consuming. The method for titration, outlined above, makes a considerable shortening of the period of observation possible. In a series of experiments with the FA strain, when test groups of twelve mice were used, it happened, completely negative tests excluded, thirty-seven times out of 100 that one or more mice in a group remained well during the first 14 days of observation. In eleven of these thirty-seven groups one or more additional mice became sick after the 14th day. The 14-day value of $1/T$ was calculated as well as the correction that had to be added to this figure to make up to the final value of $1/T$. This correction was found to vary from 0.0045 to 0.016 with an average of 0.0066. The average correction for the whole group of thirty-seven observations was 0.002. The average error of $1/T$ has previously been found to be 0.013 and the correction is, therefore, of an order of magnitude that makes it negligible. This means that a titration experiment can be concluded after 14 days instead of 4 weeks without great loss of accuracy.

In the following example the application of the method is demonstrated.

In an experiment on the pH stability of the virus, reported in the preceding paper, a series of five samples was set up, each containing the same amount of virus. The pH was adjusted to 2.7, 3.3, 3.75, 4.1, and 8.0, respectively, the last sample serving as a

control. From the control five tenfold dilutions were prepared and each given intracerebrally in 0.03 cc. amounts to one group of twelve mice. The test samples were kept in the incubator. After 6 hours 1 cc. samples were drawn and immediately diluted with 9 cc. of cooled buffer of pH 8. Two tenfold dilutions of each test sample were given to groups of twelve mice. The animals were observed for 21 days and the onset of symptoms was recorded. For each group the value of $1/T$ was calculated. The result is shown in Table IV.

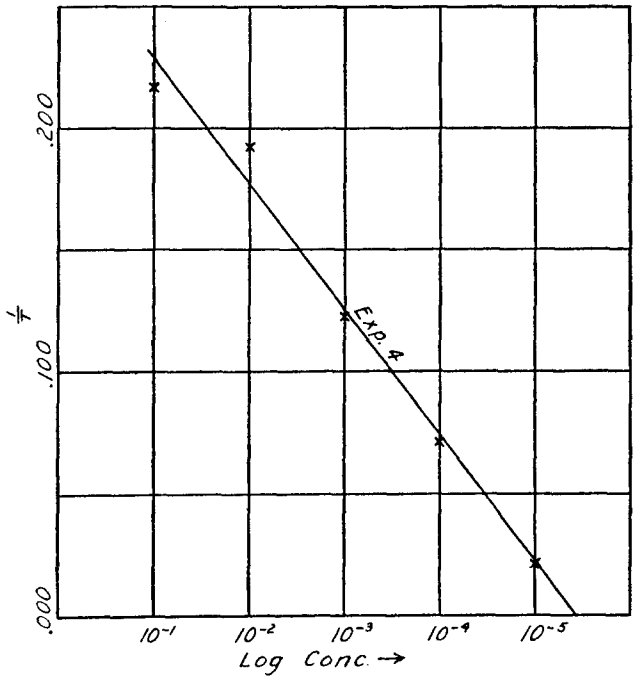

CHART 3. Linear relationship between the reciprocal of the harmonic mean of the incubation periods ($1/T$) and the logarithm of the concentration of virus. Observation period limited to 21 days.

The titration curve of the control is shown in Chart 3. The loss of activity in the test samples can be computed by means of interpolation of the respective $1/T$ values in the curve. The figures, in terms of log M.I.D. thus obtained, are shown in Table V. The theoretical error of an individual value should not exceed 0.25 log M.I.D. As shown in the table, the differences between the two series of observations are well within these limits.

The titration method now described has so far been applied exclusively in experiments with mouse encephalomyelitis virus. It is possible, however, that its field of application is much wider as a similar connection

between test dose and incubation period is known to exist in several experimental virus diseases as well as bacterial infections. A simplified method should be of particular value in experiments with viruses of low

TABLE IV

Titration Results from a pH Stability Experiment Expressed in Terms of 1/T

pH	Time	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
	hrs.					
8.0 (control)	0	0.218	0.194	0.124	0.072	0.022
2.7	6	0.088	0.035			
3.3	6	0.216	0.153			
3.75	6	0.177	0.138			
4.1	6	0.119	0.062			

TABLE V

Calculated Loss of Activity in the pH Stability Experiment

Dilution	pH 2.7	3.3	3.75	4.1
10^{-1}	2.75	0.25	1.00	2.15
10^{-2}	2.75	0.50	0.80	2.25
Average.....	2.75	0.4	0.9	2.2

stability, where a prolonged technical procedure affects the reliability of the results.

SUMMARY

In a study of mouse encephalomyelitis the following observations were made:—

1. A definite relationship exists between the amount of virus inoculated intracerebrally and the length of the incubation period.

2. The reciprocal of the incubation period was found to be approximately proportionate to the logarithm of the amount of virus inoculated. The relationship can, therefore, be given a simple mathematical form.

3. This fact provides a basis for a new method for measurement of the activity of mouse encephalomyelitis virus.

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