# SOME APPLICATIONS OF A SINGLE-DILUTION METHOD OF TITRATING NEUROTROPIC VIRUSES IN ZERO MORTALITY ($D_0$) UNITS

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THE 50 per cent end-point is an article of faith in virology. Practically every quantitative determination, whether of virus titre, estimation of content of neutralizing antibody, or investigation of antiviral chemotherapeutic effect, is based upon an experimental design which involves the determination of a 50 per cent end-point. In view of the inaccuracy, artificial nature and practical disadvantages of the method it is of interest to enquire how its use has become so widespread.

The concept of the LD50 was first introduced by Trevan (1927) in order to improve upon the imprecision of the minimal lethal dose, which had become firmly established in pharmacological investigation as the method of determining toxicity. The occurrence or non-occurrence of death is a quantal response, and each test animal can yield only 2 pieces of information. Any quantitative method which rests upon such a limited basis therefore suffers from inherent lack of precision which cannot be remedied, and this applies not only to the minimal lethal dose but also to the more refined concept of the LD50. The factor which has done more than anything else to establish the 50 per cent end-point as the method of choice in virology was the work of Reed and Muench (1938), which, although it consisted of a hypothetical illustration from serology, has become the most widely quoted source in virological literature. Shortly afterwards Gard (1940) showed that the reciprocal of the incubation period in mice infected with the GD VII and FA strains of mouse encephalomyelitis virus had a linear regression on the logarithm of the virus dilution, and this relation could be used as a means of titrating virus suspensions of unknown infectivity by interpolation from a previously determined response curve. In spite of the many advantages of this method it has not come into general use, and the purpose of the present work is to show that the procedure proposed by Gard is applicable to the titration of many other neurotropic viruses and, in a more refined form which involves measurements in units of zero mortality, possesses many advantages over the determination of the LD50.

## MATERIALS AND METHODS

*Viruses.*—The origin of the viruses used in the present work has been described in an earlier communication (Bauer and Bradley, 1956).

*Mice.*—The work was carried out with commercial Swiss white mice of varying weights obtained from a number of different dealers.

*Dose-response curves.*—Serial decimal dilutions of the virus preparation, which usually consisted of infected mouse brain, were inoculated intracerebrally into groups of 6–12 mice, beginning either at the highest dilution, or at the lowest dilution using a separate syringe

for each dilution. The animals were observed for up to 3 weeks, and the deaths which occurred were recorded at 9 a.m. and 5 p.m. each day ; a mouse found dead in the morning was counted as having lived for an integral number of days, and when found dead in the afternoon for an extra half-day. Mice discarded alive and well at the end of the observation period were considered to have survived for infinite time for the purpose of calculating the mean response.

*Yellow fever vaccine.*—Five-dose ampoules of " Wellcome " brand yellow fever vaccine were selected at random from commercial stocks. The vaccine consists of a freeze-dried suspension of chick embryos infected with the 17D strain of yellow fever virus.

*Yellow fever immune serum.*—The immune serum was prepared by the hyperimmunization of rabbits and was presented by Dr. J. S. Porterfield of the National Institute for Medical Research, London.

## RESULTS

### *Dose-response curve of neurovaccinia virus*

It has previously been shown (Bauer, 1958) that in mice infected intracerebrally with the IHD strain of neurovaccinia virus there is a linear relationship between the reciprocal of the survival time and the logarithm of the dilution of virus inoculated, and that the regularity of the relationship was such that it was

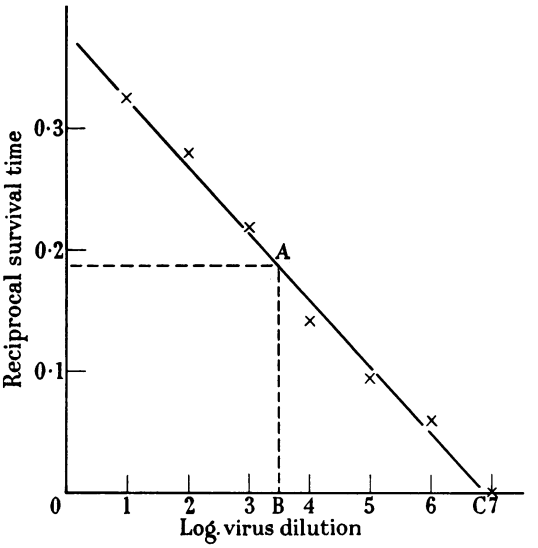


FIG. 1.—Dose-response curve of neurovaccinia virus in mice. The points represent the mean reciprocal survival times of groups of 6 mice.

possible to use it as the basis of a test of antiviral chemotherapeutic activity. In subsequent work the properties of the dose-response curve have been studied in greater detail. A typical example is given in Fig. 1. It should be noted that the curve does not approach the axis of abscissae asymptotically but gives an unambiguous intercept. At this point the ordinate is zero, since all the mice survive indefinitely, and the point therefore represents the LD 0. The LD 0 obtained by this method is a natural parameter of the dose-response curve, and thus differs radically from the LD 0 of the sigmoid mortality curve, which is indeterminate owing to the asymptotic approach, and in order to avoid confusion it is suggested that the point should be called the $D_0$, the dose of virus

which produces zero mortality. The LD50 is now indeterminate, since an indefinite number of ordinates can be consistent with 50 per cent survival once the survival time is incorporated into the response. The titre in $D_0$ units of the virus preparation used for obtaining the dose-response curve can be calculated from the regression coefficient ($b$) and the ordinate intercept ($c$) obtained by the least squares method, and is then $-b^{-1}c$. The titre of a virus preparation of unknown concentration can be calculated with the aid of the previously determined dose-response curve by a method given below provided that the slope of the curve, *i.e.* the regression coefficient, remains constant.

*Consistency of the slope of the dose-response curve*

The results of a number of determinations of the regression coefficients of the dose-response curves of neurovaccinia and other neurotropic viruses, carried out at intervals over a period of 6 yr., are given in the Table. The determinations were carried out with mice of several different strains, some of unknown origin, and no attempts were made to standardize the conditions, but it will be seen that the value of $b$ for neurovaccinia is reasonably constant, with a standard error of 14 per cent.

*Relationship of the $D_0$ to the LD50*

The data used for constructing the dose-response curve can be used for calculating the LD50 provided that the 50 per cent mortality end-point is covered, and the relationship between the $D_0$ and the LD50 can thus be determined. In the titration of Fig. 1 the $D_0$ end-point was 6·92 log units and the LD50 end-point was 5·67 log units by the moving average method (Thompson, 1947) and approximately 5·75 log units by the Reed and Muench method, giving a mean of 5·71 log units. The LD50 therefore exceeded the $D_0$ by 1·21 log unit. In further experiments a standard preparation of neurovaccinia virus was titrated on 3 separate occasions, 191 mice being used in all, and the $D_0$ and LD50 titres were determined. Values of 7·9, 7·9 and 7·8 log units were obtained for the $D_0$ titre and 6·0, 6·3 and 6·0 for the LD50 titre. The reproducibility of the $D_0$ titration is therefore within acceptable limits, and the results in combination with the preceding experiment give a mean difference between the $D_0$ and the LD50 of 1·65 log unit. A titration of another standard preparation gave a difference of 1·60 log unit. The LD50 of neurovaccinia virus is therefore equivalent to approximately 40 $D_0$, and the titre in $D_0$ units can be converted to the familiar units if required, by subtracting 1·6 log unit.

*Dose-response curves of other viruses*

It has already been shown (Bauer, 1958) that a linear dose-response curve can be obtained with neurotropic ectromelia virus (pseudolymphocytic choriomeningitis), and further examples for other neurotropic viruses are given in the Table. In the present work it has been found that linear dose-response curves can also be obtained with herpes, the 17D strain of yellow fever, the Flury strain of rabies virus, Anopheles A and dengue I (Hawaiian strain) viruses. The appropriate numerical data are given in the Table, and the dose-response curves of the 17D and Flury strains, which as will be shown later, are of practical value

TABLE.—*Regression Coefficients of Dose-Response Curves of Neurotropic Viruses*

| Virus | Number of determinations | Number of mice | Mean or individual value of regression coefficient ($b$) | Standard error of $b$ |
|---|---|---|---|---|
| Neurovaccinia | 28 | 1012 | −0·043 | 0·006 |
| Ectromelia | 8 | 245 | −0·033 | 0·003 |
| Yellow fever (17D) | 5 | 249 | −0·016 | 0·005 |
| Herpes simplex | 7 | 202 | −0·057 | 0·003 |
| Rabies (Flury) | 2 | 84 | −0·031 }* −0·028 } | — |
| Ilhéus | 1 | 61 | −0·036* | — |
| Dengue I | 1 | 36 | −0·017* | — |
| Anopheles A | 1 | 83 | −0·016* | — |

* Individual values.

in the titration of rabies and yellow fever vaccines, are illustrated separately in Fig. 2 and 3.

*Calculation of titre in $D_0$ units*

Consider the death of a single animal occurring at a time $t$ after infection; $t^{-1}$ then defines a point on the dose-response curve of Fig. 1 corresponding to the abscissa $OB$. The distance of $B$ from $C$, the $D_0$ point, is the content of virus in

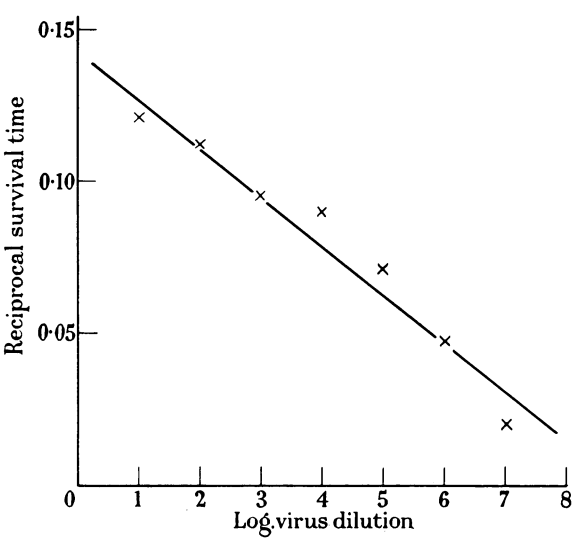


FIG. 2.—Dose-response curve of the 17D strain of yellow fever virus.

$D_0$ units, and is equal to $t^{-1}b^{-1}$. The survival time of a single animal is thus an experimental estimate of the titre, and the survival time of each animal in a group infected with the same dose provides its own independent estimate of the titre. The mean titre can thus be obtained from the mean reciprocal survival time of the group, and data are available for calculating the standard deviation. Moreover, the titre can be determined by the inoculation of a single dilution without previous knowledge of an expected end-point, and the failure often

encountered in LD50 titrations as a result of the use of an inappropriate range of dilutions cannot occur. A numerical example of the calculation of a titre by this method is given below.

*Some applications of the $D_0$ titration*

*Single dilution titrations.*—The method of obtaining the titre from a single dilution is particularly suitable for carrying out large numbers of independent titrations, such as determinations of the potency of batches of live virus vaccines. As an example, an ampoule of yellow fever vaccine was reconstituted to give a $10^{-1}$ dilution of the original material, diluted 1 : 100 to give a final dilution of

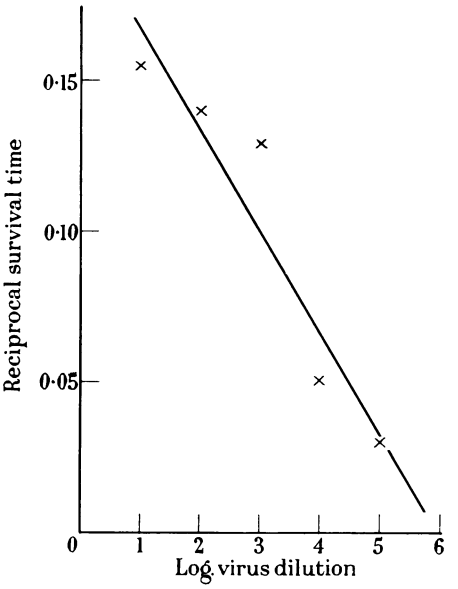


FIG. 3.—Dose-response curve of the Flury strain of rabies virus.

$10^{-3}$, and injected intracerebrally into 5 mice, which died after 8, 9, 11, 11 and 12 days. The mean reciprocal survival time was then 0·0835 which divided by the value of *b* for the 17D strain of yellow fever virus given in the Table (0·016) gave 5·22 as the logarithm of the titre in $D_0$ units.

*Multiple dilution titrations.*—The precision of the estimate of the titre can be increased by inoculating 2 or more dilutions, and the fate of each single animal contributes information, whereas in LD50 titrations only the dilutions enclosing the end-point can be used for calculation, the remaining animals being wasted. The value of *b* for the responses obtained from the titration or previously determined value of *b* can be used to calculate the $D_0$.

*Determination of antiviral chemotherapeutic effect.*—It has already been shown (Bauer, 1958) that treatment of infected animals with an agent possessing antiviral chemotherapeutic activity will displace the regression line to lower response levels. This results in an increase in the value of the $D_0$, and the extent of the increase in log units can be used as a measure of antiviral chemotherapeutic

activity. The method is then exactly comparable to a conventional 2 $n$-point pharmacological assay, where $n$ is the number of dilutions used for inoculation.

*Determinations of virus growth curves.*—A commonly occurring requirement in research is the determination of virus titre in an infected organ at successive intervals of time after infection ; if the LD50 end-point is used the procedure is very cumbersome, requires a large number of mice, and does not give very accurate results. The $D_0$ method affords a much simpler method of obtaining the required information by the process of transformation of regression coefficients. A number of mice are infected intracerebrally with a constant dose of virus ; on successive days after infection 1 or more mice are killed, and the brains removed, pooled and prepared as a 10 per cent suspension. Each suspension thus prepared is inoculated intracerebrally into a group of mice, the size of the group depending upon the degree of accuracy required, and the survival times are recorded and converted into reciprocals. The coefficient of regression ($b_1$) of the reciprocal survival times on time in days after infection is then determined by the method of least squares and divided by $b$, the regression coefficient of the virus dose-response curve, giving $b_2$, the regression coefficient of the virus titre growth curve. This follows from the fact that the reciprocal survival times ($t^{-1}$) are converted into titres in $D_0$ units when divided by $b$ ; since the $t^{-1}$ occur in both terms of the numerator of the expression for $b_1$, replacement of each response by $t^{-1}b^{-1}$ gives $b_1b^{-1}$, whence $b_2 = b_1b^{-1}$, where $b_2$ is the regression coefficient of the virus growth curve. The actual growth curve is obtained by adding 1 log unit to each transformed ordinate to allow for the initial $10^{-1}$ dilution.

Since the $D_0$ bears a constant relationship to the LD50 the growth curve in LD50 units, if required, can be obtained by subtracting a previously determined constant from each ordinate. An example of the method is shown in Fig. 4, in which the growth curve for neurovaccinia virus is given together with the reciprocal survival time regression line from which it was derived. The mean titres for each day were obtained by dividing the corresponding mean reciprocal survival times by $b$. The results afford the necessary data for determining the standard error of the regression coefficient and carrying out tests of linearity.

*Investigation of the kinetics of virus inactivation.*—In studies of the preservative effect of various diluents, or of the inactivating effect of physical and chemical agents, it is necessary to carry out serial determinations of titre at frequent intervals in order to obtain an inactivation curve. This can be done most easily by injecting the undiluted virus preparation into a group of animals as often as is necessary and determining the $D_0$ by the method given above. If inactivation is proceeding by a first-order reaction the velocity coefficient can be determined directly by the method of regression coefficient transformation. The occurrence of inactivation can be inferred from a calculation of the probability of the deviation of $b$ from zero, and the significance of the difference in velocity coefficients obtained under different experimental conditions can be determined in a similar manner. The responses obtained are also suitable for carrying out an analysis of variance and test of linearity. An illustration of the method is given in Fig. 5, which illustrates the inactivation curve obtained with an ampoule of commercial yellow fever vaccine reconstituted with saline in accordance with the instructions for use and left at room temperature (16°). The material was inoculated into groups of 11–13 mice 5, 10, 20 and 40 min. after reconstitution, 47 mice being

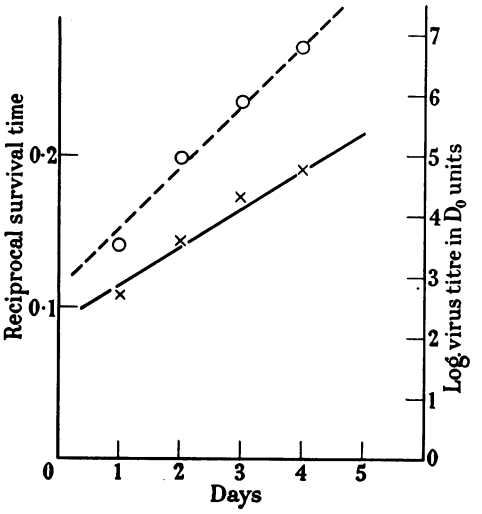


FIG. 4.—Growth curve of neurovaccinia virus in mouse brain. Upper line; regression of responses on time (see text). Lower line; growth curve of virus titre obtained by transformation of the response line. The points represent the mean responses and the mean titres obtained by transformation. *Ordinates*; left-hand, reciprocal survival time; right-hand, virus titre in $D_0$ units. *Abscissa*; time in days after infection.

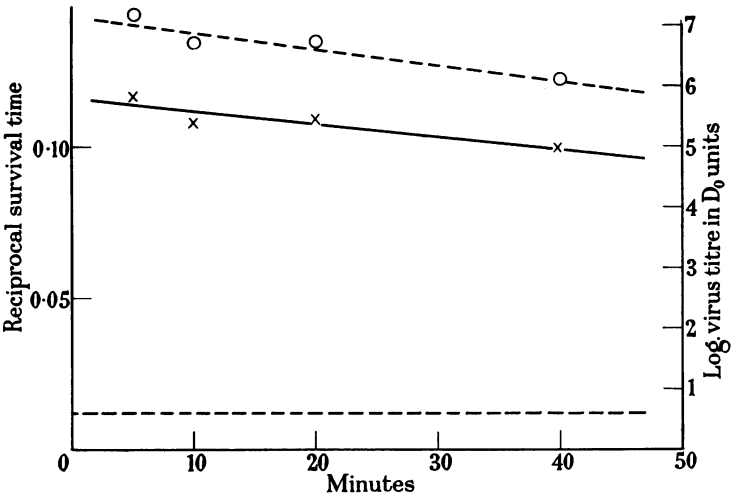


FIG. 5.—Thermal inactivation of yellow fever vaccine. Lower line; regression of responses on time. Upper line; regression of virus titre on time obtained by transformation. *Ordinates*; left-hand, reciprocal survival time; right-hand, virus titre in $D_0$ units. *Abscissa*; time of exposure in minutes. Dotted line; minimum permissible virus titre.

used in all. $b_1$ was found to be $-4{\cdot}409 \times 10^{-4}$, and the regression of the response on time is shown in the lower curve of Fig. 5. The variance of $b_1$ was $1{\cdot}532 \times 10^{-8}$ and the probability of the difference of $b_1$ from zero was then $< 0{\cdot}001$, 43 degrees of freedom being available for determining this quantity. The high level of significance shows that inactivation of the virus was taking place. The regression of virus titre in $D_0$ units on time ($b_2$) was then obtained by dividing $b_1$ and the means of the responses by $-0{\cdot}016$, the mean value of $b$ for the 17D strain of yellow fever virus given in the Table, and is illustrated in the upper curve of Fig. 5. It can be seen that inactivation of virus to the extent of 0·8 log unit (in both $D_0$ and LD50 units) took place over 30 min., the recommended period of use for the reconstituted vaccine. The minimum permissible titre as required by inter-

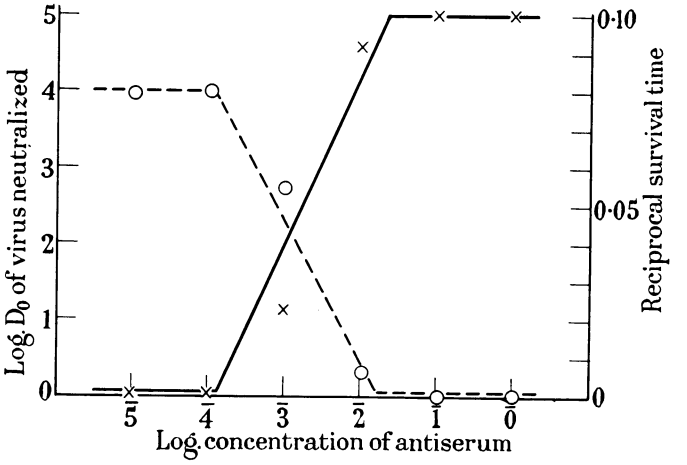


FIG. 6.—Neutralization curve of the 17D strain of yellow fever virus. *Ordinates*; left-hand, logarithm of amount of virus neutralized in $D_0$ units; right-hand, reciprocal survival time. *Abscissa*; logarithm of concentration of antibody in arbitrary units.

○ Mean Responses.
× Mean amounts of virus neutralized, obtained by transformation of the responses.

national regulations is given by the dotted line of Fig. 5, from which it can be seen that there is still a considerable excess of virus present in spite of the inactivation which has taken place.

The rate of loss of infectivity of chemically inactivated vaccines could also be determined by the same method.

*Neutralization tests.*—Virus neutralization tests are carried out by determining the LD50 end-point of a virus preparation in the presence and in the absence of immune serum, and it is therefore of interest to investigate whether the $D_0$ method can be used for the same purpose. Decimal dilutions of a rabbit yellow fever hyperimmune serum were mixed with equal volumes of a suspension of mouse brain infected with the 17D strain of yellow fever virus, and the mixtures were inoculated immediately into groups of 11–13 mice by the intracerebral route. The reciprocal survival times in the separate groups were plotted against serum dilution, with the result shown in Fig. 6. With the lowest dilution of serum ($10^{-1}$) all the virus was neutralized, giving a reciprocal survival time of

zero ; over the $10^{-2}$–$10^{-4}$ range of dilutions the reciprocal survival time increased progressively, and between $10^{-4}$ and $10^{-6}$ did not differ from the mean value obtained from 2 control groups inoculated with virus and normal serum at the beginning and at the end of the inoculations of the test group. The rising part of the curve thus represents the amount of virus neutralized by varying amounts of antiserum, and is also shown in Fig. 6 in its more familiar form, obtained by transforming the regression coefficient as described above and subtracting the ordinate from the control values. The results thus give a preliminary indication that the $D_0$ method can be used as the basis of a single-dilution neutralization test and is worthy of further investigation for this purpose.

## DISCUSSION

Gard (1940) stated that " theoretically one should expect the (reciprocal survival time) curves to deviate from a straight line in the vicinity of $1/T = 0$ and approach the abscissa asymptotically. The distribution of points in that part of the diagram indicates that this is actually the case ", although the figure given lends little support to this and he has previously pointed out that replicate curves give an unambiguous abscissa intercept of 0·1 minimal infective dose. In the present work, in which more than 100 dose-response curves have been studied, an asymptotic approach has never been observed. The only deviation which may occur is an occasional increase in steepness at high dilutions, due to the survival of animals inoculated with a small amount of virus which develop symptoms and recover.

The use of the incubation period (but not the reciprocal) was suggested by Bryan and Beard (1941) for titrating antibody to rabbit papilloma virus, but the method does not seem to have come into general use. The results obtained in the present work with the 17D strain of yellow fever virus, although only of a preliminary nature, indicate that it should be possible to design a valid neutralization test on the basis of the $D_0$ single-dilution method which should be more accurate and less cumbersome than the LD50 methods which are standard practice at the present time.

The essential weakness of the LD50 method of titration of viruses is that it rejects most of the information content of the titration procedure. The survival time and its reciprocal are continuous in the mathematical sense, in that they may assume an infinitely great number of values, whereas if only occurrence or non-occurrence of death is recorded the information contained in the survival time is rejected in favour of the more limited information obtained from a non-continuous or quantal response, and the linear response is transformed into the much less convenient sigmoid mortality curve. In practice mice cannot conveniently be subjected to continuous observation, and deaths are commonly recorded to the nearest half-day. As death from infection with a constant dose of virus may occur over a range of 3–4 days, each animal may yield 9 responses, *i.e.* death after 8 possible periods measured in half-days, or indefinite survival. In general, the number of responses which $n$ animals can yield is $n(2t + 1)$, where $t$ is the duration of the symptom period in days, whereas if death or survival is taken as the response, $n$ mice can only yield $n + 1$ responses, *i.e.* the death of successive numbers of mice from zero to $n$. This disadvantage, together with the practical disadvantage that several virus dilutions must be

inoculated, the responses from which cannot be used in evaluating the LD50, would seem to indicate that the LD50 is not the method of choice for titrating viruses, although it is so firmly established that it will undoubtedly be used for many years to come.

SUMMARY

The reciprocal survival time of mice infected intracerebrally with neurovaccinia, ectromelia, dengue I, rabies and certain other neurotropic viruses has a linear regression upon the logarithm of the dilution of virus in the infecting dose. The dose-response curves thus obtained have an unambiguous abscissa intercept which corresponds to the dose of virus causing zero mortality ($D_0$). The regression coefficient of the dose-response curve is constant and characteristic of the particular virus. The mean reciprocal survival time of a group of mice inoculated with a single dilution of virus divided by the regression coefficient gives the titre of the preparation immediately in $D_0$ units. The $D_0$ bears a fixed relationship to the LD50, and in the case of neurovaccinia the LD50 is equivalent to 40 $D_0$. The method of single-dilution titration in $D_0$ units facilitates the carrying out of procedures in which serial titrations are required, such as virus growth curves and thermal inactivation curves, and has many advantages over the usual method of LD50 titration. The $D_0$ method can also be used for the titration of yellow fever neutralizing antibody and for the determination of antiviral chemotherapeutic activity.

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