# STATISTICAL STUDIES OF THE NATURE OF THE INFECTIOUS UNIT OF VACCINE VIRUS

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An infectious unit of vaccine virus is the smallest amount of the active agent which is capable of initiating infection when inoculated under certain conditions into a specified host. This definition does not depend upon any hypothesis as to the nature of the pathogenic agent, and likewise no assumption is made as to the physical state in which the virus exists. Thus the virus preparation might be a solution of an active protein, and Stanley (1) has shown that this is apparently true for tobacco mosaic virus. On the other hand, the virus might exist in particulate form with the particles distributed at random in the suspension. The inoculation of 1 or of a certain number of these particles might be required to cause infection. Several investigators have presented evidence that vaccine virus is in fact particulate.

Duran-Reynals (2) has observed that the inoculation of very dilute suspensions of vaccine virus intradermally in rabbits may give rise to a group of papules rather than to a single lesion. This occurs more frequently if the suspension contains a "spreading factor" which he has described. This he regards as evidence that the critical concentration of virus required for infection is a "single infectious unit," presumably an individual particle. Doerr (3) points out that when a virus suspension is titrated by inoculating serial dilutions of the stock suspension, it sometimes happens that a lesion follows the inoculation of a given dilution of virus but not of the next lower one which should theoretically contain 10 times as much virus. He shows that this paradox can most easily be explained if one assumes that the virus is particulate, and that a single particle can cause infection. He does not, however, develop the idea further. Burnet (4) recently has advocated the use of the chorio-allantoic membrane of chick embryos for titrating certain viruses, and Keogh (5) has used the technique in quantitative studies of vaccine virus. Suitably inoculated, this virus causes the development of discrete lesions in the chick membrane which may be counted. If a sufficient number of mem-

branes are inoculated with each dilution, and the average number of lesions determined, an arithmetic ratio may be shown to exist between the concentration of virus and number of lesions produced. Thus twofold dilution of the virus suspension leads to the production on inoculation of half as many lesions as were produced by inoculation of the original suspension. These workers consider, therefore, that each lesion arises as the result of the infection of a cell with a single particle of virus.

These observations constitute presumptive evidence that the virus of vaccinia is particulate and that a single particle of virus can give rise to infection. Evidence is presented by other workers that vaccine virus in a suspension is associated with elementary bodies, minute structures which under the proper conditions can be made visible (6). It becomes important, therefore, to determine whether a single elementary body can give rise to vaccinal infection, or whether in order to accomplish this several corpuscles must be introduced simultaneously.

Parker and Rivers (7) prepared suspensions of elementary bodies which were relatively free from other material. The number of infectious units per unit volume was determined by titration in rabbits, the number of particles by counting the particles in a small sample in a calibrated chamber. They showed that there was a high degree of correlation between the number of particles in a suspension and the number of infectious units. These data did not, however, permit conclusions as to the number of particles which composed an infectious unit of virus.

Since it was evidently impracticable to determine by direct observation the number of elementary bodies necessary to cause infection, a statistical method of approach was sought. The most promising of these seemed to be that elaborated by Greenwood and Yule (8) for application to water bacteriology and widely used since that time. The method was employed by Youden, Beale, and Guthrie (9) in studying the virus of tobacco mosaic, and these authors elaborated the formulae in order to take account of the number of sites available for infection.

The problem to be solved is simply a special case of the theory of probability. The principle involved was first clearly stated by Poisson (10) and is generally referred to as Poisson's law of small numbers. He showed that if successive samples were drawn from a universe which consisted of very small particles suspended in a liquid, the proportion of samples which contain 1, 2, 3 . . . $n$ particles was related to the mean number of particles per unit volume in a definite manner.

He presented methods, moreover, by which that proportion could be calculated. It follows that if the mean number of particles per unit volume is known, and if an adequate number of samples is drawn, the proportion which will contain no particles, 1 or more, 2 or more, ...$n$ or more particles can be calculated. It has been shown by Greenwood and Yule that the proportion of samples containing no particles can be determined by solving the simple equation $P = e^{-m}$, where $P$ is the proportion of samples containing no particles, $e$ is the base for natural logarithms (2.718), and $m$ is the mean number of particles. The proportion of samples

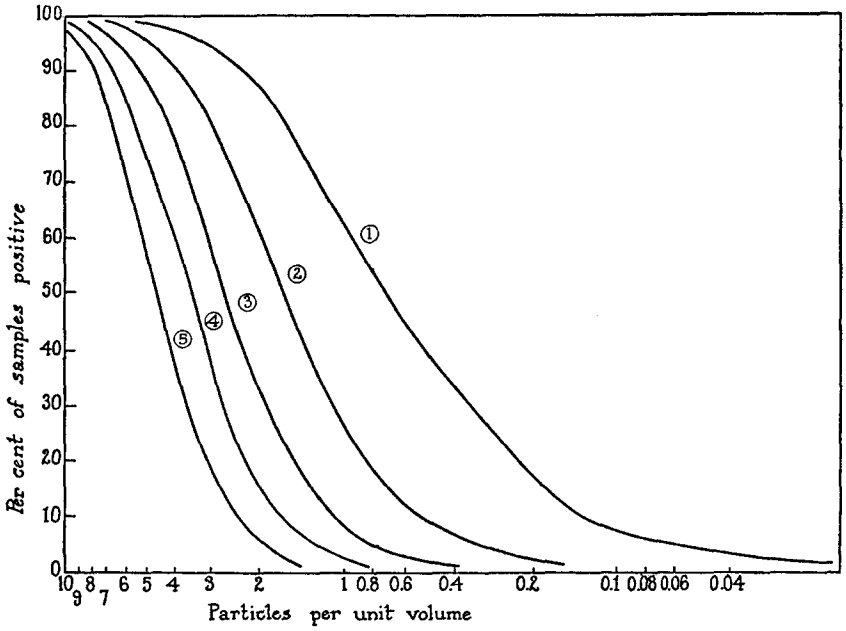


FIG. 1. Theoretical curves. Per cent of samples containing at least 1, at least 2.... particles plotted against mean concentration of particles. Figures on the curves indicate the minimum number of particles per sample required to classify the sample as positive.

containing 1 or more particles is therefore $1 - e^{-m}$. The calculation of the proportion of samples containing 2 or more particles is equally straightforward but laborious and the figures are more easily obtained from published tables of Poisson's exponential limit (11). If the probability of obtaining a sample containing 1 or more, 2 or more, 3 or more particles is plotted against various values of $m$, a characteristic curve is obtained in each case. Such curves are shown in Fig. 1, where it will be seen that as the number of particles required for success is increased the slope of the curve becomes progressively steeper. It is evident that if the conditions of experiment are such as to allow the result of an inoculation to be

interpreted as either positive or negative, experimental curves can be constructed and fitted to these theoretical ones. Other conditions then being suitable, it should be possible directly to determine the virulence of a virus even though one is not able to count the virus particles directly. The application of the principle to a broad range of biological problems has been suggested by Iwaszkiewicz and Neyman (12), who give derivations of their formulae.

## EXPERIMENTAL

The theoretical considerations which have been outlined led us to believe that by the application of relatively simple statistical principles it should be possible to determine the number of particles—elementary bodies—of vaccine virus which are required to initiate infection in the rabbit skin. The present experiments were designed to test this hypothesis and to make use of the method to compare the characteristics of a number of strains of vaccine virus.

### *Materials and Methods*

*Virus.*—Several strains of vaccine virus have been employed. They are the following:

*1. Board of Health Strain.*—This strain is that used by the New York City Board of Health and has been maintained for some time by testicular passages in rabbits. For the past year it has served in our laboratory for the routine production of elementary bodies of vaccinia. Inoculated intradermally in rabbits it causes typical lesions which have a moderate tendency to become hemorrhagic and necrotic. When many heavy inoculations have been given, generalized vaccinia usually results. Generalization does not commonly follow the multiple inoculation of small quantities of virus.

*2. Cultured Vaccine Virus.*—This strain, derived from the Board of Health strain, has been cultured in a chick embryo-Tyrode solution medium for many generations.[1] A complete description of its history and present characteristics will be found in the publications of Rivers and Ward (13). Suffice it to say that during prolonged cultivation its pathogenicity for the rabbit has altered markedly and it now causes relatively mild lesions in rabbit skin, which rarely become necrotic.

*3. Noguchi Strain.*—This strain was adapted to passage in the testicles of rabbits by Noguchi (14) and since has been maintained in the same manner. It causes generalization more often than the Board of Health strain.

*Titration of Virus.*—The virus was titrated by inoculating 0.25 cc. portions of successive serial dilutions of virus suspension intradermally into rabbits. The animals were observed daily and the presence of lesions recorded. Following

[1] In the laboratory of Dr. T. M. Rivers.

inoculation of Board of Health or of Noguchi virus, little trouble was experienced because of the appearance of "indeterminate" lesions. More difficulty was encountered with cultured virus because of the different character of the papules which followed inoculation of minimal amounts of virus. Many of these had a diameter of but 3 to 4 mm. and few showed evident necrosis. Nevertheless 9 out of 10 inoculations could usually be classified easily as positive or negative. In the remainder an element of judgment was of necessity admitted.

*Source of Animals.*—Rabbits were purchased in the open market and were of many breeds and mixtures. They weighed from 4 to 10 pounds; were selected on the basis only of being in a good state of nutrition and of having minimal areas of coarse skin. No animals which manifested detectable resistance to infection with vaccine virus were encountered.

The Board of Health strain of vaccine virus was chosen for the first experiment because it had been used in making preparations of elementary bodies as routine and because it causes well defined lesions in the rabbit skin.

*Experiment 1.*—The virus was used in the form of elementary bodies suspended in buffer solution. The suspension was centrifuged for 30 minutes at 2000 R.P.M. in order to ensure that the elementary bodies remaining in suspension would be well distributed, and in so far as possible without any clumps of particles being present. A rough titration was done by inoculating a rabbit with serial dilutions of suspension, 4 inoculations being made of each dilution. From this it was calculated that a virus dilution of $10^{-7.4}$ should give an equal number of positive and negative results. An exact titration was done as follows. A quantity of virus was diluted to $10^{-6.7}$ (5 times the concentration giving equal numbers of positive and negative results) in order to ensure that the lowest dilution inoculated would give rise to a high percentage of takes. From this suspension serial dilutions were prepared having a ratio of approximately 1–2 (log 0.3) and as soon as possible rabbits were inoculated therewith. The inoculations were completed within an hour and a half after the suspensions were prepared. Each animal was given an average of 45 inoculations, 5 or 6, therefore, of each dilution. Care was taken to distribute these so that the sites of inoculation of each dilution were distributed at various places over the rabbit. A single set of virus suspensions served for inoculation of all of the animals. The animals were observed daily.

The results of this experiment are presented graphically in Fig. 2. The calculations are collected in Table I. It will be observed that the experimental curve agrees very well with that calculated on the hypothesis that 1 particle is capable of causing infection since a deviation from the theoretical curve as great as the one observed would be expected to occur, owing to chance, in 9 of 10 trials. If

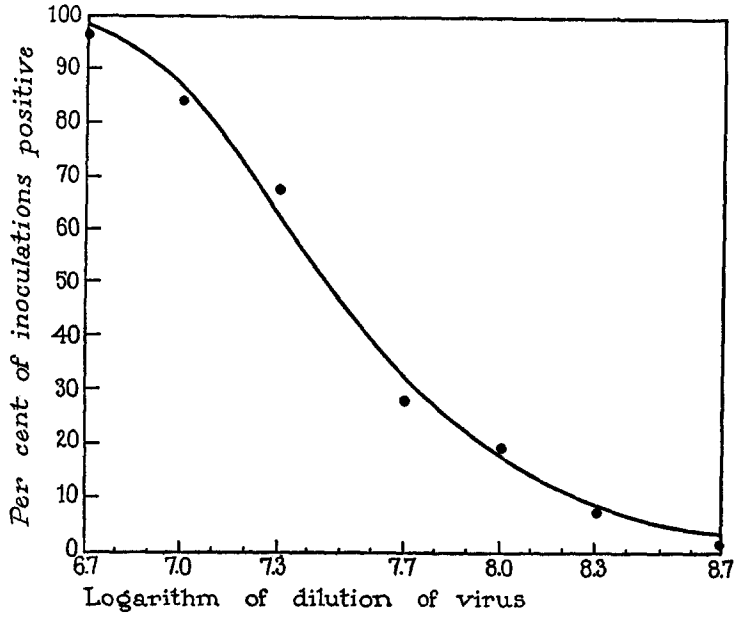


FIG. 2. Titration of Board of Health virus. Solid line represents theoretical curve, calculated on hypothesis that a single particle causes infection. Dots are experimentally determined points.

TABLE I

*Experiment 1. Titration of Board of Health Virus. Calculations of Goodness of Fit of Experimental and Theoretical Curves*

| Logarithm of virus dilution | Results of experiment | | If 1 particle initiates infection | | If 2 particles initiate infection | | If 3 particles initiate infection | |
|---|---|---|---|---|---|---|---|---|
| | Number of inoculations | Number positive ($x$) | Number expected positive ($\bar{x}$) | $\frac{(x-\bar{x})^2}{\bar{x}}$ | Number expected positive | $\frac{(x-\bar{x})^2}{\bar{x}}$ | Number expected positive | $\frac{(x-\bar{x})^2}{\bar{x}}$ |
| 6.7 | 69 | 67 | 67.5 | 0.004 | 68.3 | 0.025 | 69.0 | 0.06 |
| 7.0 | 69 | 58 | 59.3 | 0.028 | 57.7 | 0.002 | 67.5 | 1.36 |
| 7.3 | 72 | 49 | 44.6 | 0.433 | 48.0 | 0.208 | 50.2 | 0.03 |
| 7.7 | 71 | 20 | 22.8 | 0.345 | 16.1 | 0.947 | 13.6 | 3.02 |
| 8.0 | 72 | 14 | 13.0 | 0.076 | 6.5 | 8.650 | 2.5 | 49.80 |
| 8.3 | 75 | 6 | 6.7 | 0.073 | 1.3 | 15.00 | 0.6 | 48.70 |
| 8.7 | 76 | 1 | 3.0 | 1.330 | 0.5 | 0.500 | 0.2 | 3.20 |
| | | | $\chi^2$ | 2.28 | | 23.33 | | 106.2 |
| | | | $P$ | 0.9 | | | | |

we assume that 2 particles are required for infection the agreement is much worse, the value for chi square being 10 times as great.

This experiment confirmed the opinion that the statistical method outlined could be used for determining the virulence of vaccine virus. Therefore, since we possessed a method for determining the number of virus particles which are probably required to cause infection, we decided to apply it to other strains. The cultured strain of virus was studied next.

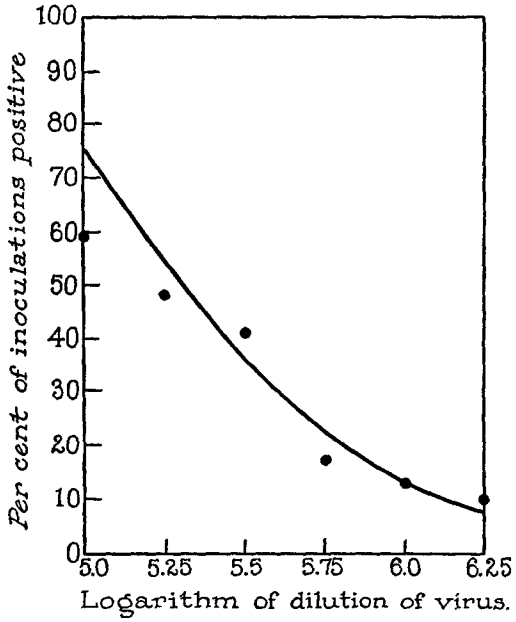


FIG. 3. Titration of cultured vaccine virus. Solid line represents theoretical curve, calculated on hypothesis that a single particle causes infection. Dots are experimentally determined points.

*Experiment 2.*—A suspension of cultured virus was prepared as follows: Chick embryo-Tyrode solution medium contained in several flasks was inoculated with virus. After 5 days the contents of all the flasks was pooled and the mixture centrifuged for 10 minutes at 1500 R.P.M. in order to throw down the tissue fragments. The sediment was removed from the tube, ground with sand, and the supernatant fluid from centrifugation was mixed with the ground sediment. The reconstituted virus suspension was then centrifuged at 3000 R.P.M. for 15 minutes in the horizontal centrifuge, the supernatant fluid pipetted off and again centrifuged at 3000 R.P.M. for 15 minutes. This was done in order to remove, as far as

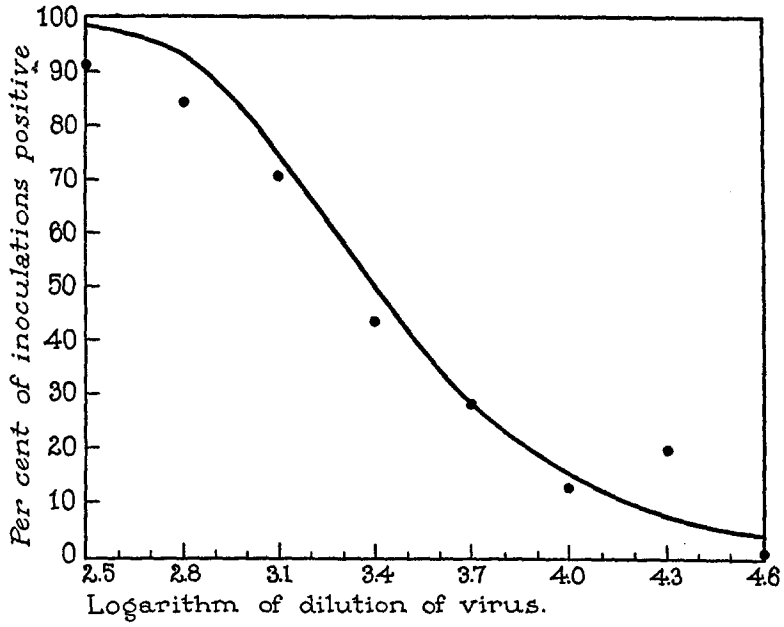


FIG. 4. Titration of cultured vaccine virus. Solid line represents theoretical curve, calculated on hypothesis that a single particle causes infection. Dots are experimentally determined points.

TABLE II

*Experiment 2. Titration of Cultured Vaccine Virus. Calculations of Goodness of Fit of Experimental and Theoretical Curves*

| Logarithm of virus dilution | Results of experiment | | If 1 particle initiates infection | | If 2 particles initiate infection | | If 3 particles initiate infection | |
|---|---|---|---|---|---|---|---|---|
| | Number of inoculations | Number positive ($x$) | Number expected positive ($\bar{x}$) | $\frac{(x-\bar{x})^2}{\bar{x}}$ | Number expected positive | $\frac{(x-\bar{x})^2}{\bar{x}}$ | Number expected positive | $\frac{(x-\bar{x})^2}{\bar{x}}$ |
| 5.00 | 22 | 13 | 15.7 | 0.47 | 19.2 | 2.00 | 19.8 | 2.36 |
| 5.25 | 27 | 13 | 14.7 | 0.20 | 15.1 | 0.29 | 15.6 | 0.43 |
| 5.50 | 27 | 11 | 9.7 | 0.18 | 8.6 | 0.67 | 6.6 | 2.94 |
| 5.75 | 29 | 5 | 6.4 | 0.31 | 5.2 | 0.01 | 2.3 | 1.26 |
| 6.00 | 30 | 4 | 3.9 | 0.03 | 1.5 | 4.17 | 0.6 | 19.30 |
| 6.25 | 30 | 3 | 2.3 | 0.21 | 0.6 | 9.58 | 0.3 | 24.40 |
| | | | $\chi^2$ | 1.40 | | 16.72 | | 50.69 |
| | | | $P$ | 0.93 | | | | |

TABLE III

*Experiment 3. Titration of Cultured Vaccine Virus. Calculations of Goodness of Fit of Experimental and Theoretical Curves*

| Logarithm of virus dilution | Results of experiment | | If 1 particle initiates infection | | If 2 particles initiate infection | |
|---|---|---|---|---|---|---|
| | Number of inoculations | Number positive ($x$) | Number expected positive ($\bar{x}$) | $\frac{(x-\bar{x})^2}{\bar{x}}$ | Number expected positive | $\frac{(x-\bar{x})^2}{\bar{x}}$ |
| 2.5 | 32 | 29 | 31.9 | 0.27 | 32.0 | 0.28 |
| 2.8 | 32 | 27 | 30.2 | 0.34 | 31.9 | 0.56 |
| 3.1 | 32 | 23 | 24.0 | 0.04 | 26.3 | 0.42 |
| 3.4 | 32 | 14 | 16.0 | 0.25 | 16.0 | 0.25 |
| 3.7 | 31 | 9 | 9.0 | 0.00 | 6.5 | 0.96 |
| 4.0 | 36 | 5 | 5.8 | 0.11 | 2.5 | 2.50 |
| 4.3 | 36 | 8 | 2.9 | 9.00 | 0.7 | 75.62 |
| 4.6 | 36 | 0 | 1.4 | 1.96 | 0.2 | 0.04 |
| | | | $\chi^2$ | 11.97 | | 80.63 |
| | | | $P$ | 0.10 | | |

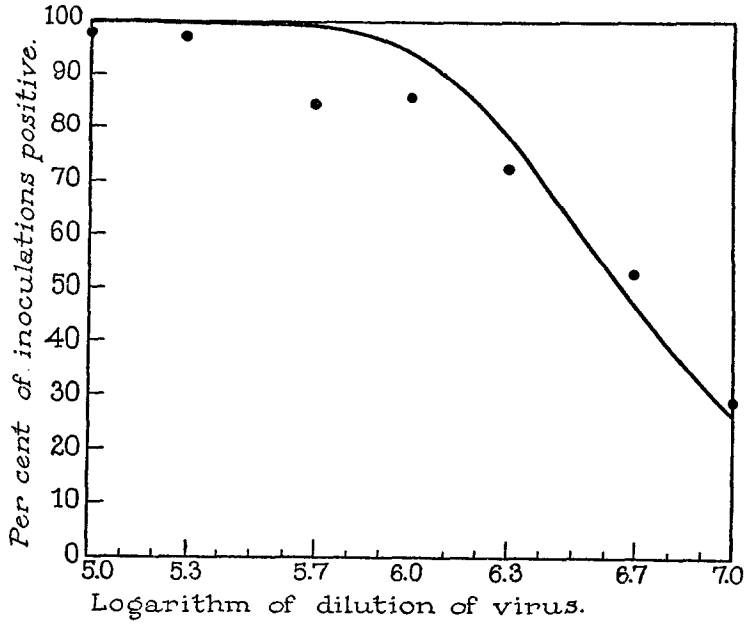


FIG. 5. Titration of Noguchi strain of vaccine virus. Solid line represents theoretical curve, calculated on hypothesis that a single particle causes infection. Dots are experimentally determined points.

possible, tissue debris and clumps of elementary bodies. An accurate titration of this virus suspension was carried out in the manner described in Experiment 1.

*Experiment 3.*—A second experiment with cultured virus was done, with a suspension prepared in a different manner. After centrifuging the virus culture in the horizontal centrifuge at 3000 R.P.M. for 15 minutes to remove debris, the virus particles remaining in the supernatant fluid were sedimented in the angle centrifuge, and then resuspended in buffer solution. This suspension was then subjected to two 15 minute periods of centrifugation in the horizontal centrifuge, the supernatant fluid being saved each time. It was then titrated in the manner which has been described.

TABLE IV

*Experiment 4. Titration of Noguchi Strain of Vaccine Virus. Calculation of Goodness of Fit of Experimental and Theoretical Curves*

| Logarithm of virus dilution | Results of experiment | | If 1 particle initiates infection | | If 2 particles initiate infection | | If 3 particles initiate infection | |
|---|---|---|---|---|---|---|---|---|
| | Number of inoculations | Number positive ($x$) | Number expected positive ($\bar{x}$) | $\frac{(x-\bar{x})^2}{\bar{x}}$ | Number expected positive | $\frac{(x-\bar{x})^2}{\bar{x}}$ | Number expected positive | $\frac{(x-\bar{x})^2}{\bar{x}}$ |
| 5.0 | 32 | 31 | 32.0 | 0.031 | 32.0 | 0.031 | 32.0 | 0.031 |
| 5.3 | 32 | 31 | 32.0 | 0.031 | 32.0 | 0.031 | 32.0 | 0.031 |
| 5.7 | 32 | 27 | 32.0 | 0.785 | 32.0 | 0.781 | 32.0 | 0.781 |
| 6.0 | 36 | 31 | 34.2 | 0.095 | 36.0 | 0.695 | 36.0 | 0.695 |
| 6.3 | 33 | 24 | 26.1 | 0.170 | 30.4 | 1.350 | 32.4 | 2.190 |
| 6.7 | 32 | 17 | 14.7 | 0.360 | 16.0 | 0.063 | 16.0 | 0.063 |
| 7.0 | 40 | 11 | 10.6 | 0.015 | 8.4 | 0.806 | 6.8 | 2.60 |
| | | | $\chi^2$ | 1.48 | | 3.71 | | 6.59 |
| | | | $P$ | 0.96 | | 0.70 | | 0.40 |

The results of titration of these two samples of cultured vaccine virus are portrayed in Figs. 3 and 4. The data and calculations of goodness of fit of the titration curves are given in Tables II and III. It is seen that the results of the 2 experiments are essentially similar, and that the curves agree well with the calculated ones although the agreement is not quite so close as is the case with the Board of Health strain. This is probably due to two factors, *viz.*, greater difficulty in classifying the results of inoculations in a few instances, and the use of a smaller number of animals with a consequent increase in the standard deviation of each determination. Nevertheless, it is evident that by far the best fit is obtained by using the curve calculated for 1 or more particles causing infection.

A final experiment was performed using the Noguchi strain of virus.

The virus was used in the form of a suspension of elementary bodies, which was prepared in the usual way from the rabbit dermal eruption. The final suspension was twice centrifuged in the horizontal centrifuge in order to ensure that only discrete particles remained in suspension. An exact titration of the virus was carried out as in the preceding experiments, after determining the approximate infective titer of the suspension.

The results of this experiment are portrayed in Fig. 5, and the calculations presented in Table IV. Again it is seen that the best agreement obtains between the experimental curve and the curve postulated on the basis that a single particle gives rise to infection.

## DISCUSSION

Several of the viruses infecting animals are distributed in suspension in particulate form, and some of them appear to be associated with elementary bodies, which under proper conditions can be made visible with the microscope. A group of related problems at once present themselves. Is 1 of these particles, properly introduced into the tissues of the host, capable of setting up infection? Or is it necessary, in order to overcome the natural resistance of the host to infection, to introduce several of the particles? Do highly virulent invasive strains of virus differ from less virulent ones in the number of virus particles which are required in order to initiate infection? The experiments described were undertaken to answer these questions as applied to the virus of vaccinia.

Aside from their application in studies of virulence of the virus of vaccinia, the results of the experiments suggest important considerations with regard to the titration of virus suspensions. It is evident that if a single virus particle gives rise to infection, the determination of the number of particles present in a suspension by the inoculation of a single series of serial tenfold dilutions into animals will be attended by a very large experimental error. In order to determine more accurately the number of infectious particles present it is necessary to perform several series of inoculations of the suspension, which has been suitably diluted. The results must then be subjected to some kind of statistical analysis. Three methods for doing this are

available. (*a*) Several series of inoculations may be performed and each series regarded as an individual titration. The end point of each may be recorded and an arithmetic mean of the end points calculated. Accuracy is increased but in a small series a single result differing widely from the mean will unduly influence the result. The difficulty is not solved by discarding the discrepant result and taking the "2 results which agree." (*b*) If infection can be shown to follow inoculation of a single particle, tables of probability can be set up, so that from the results of a series of inoculations the most probable concentration of infectious particles in the original suspension can be derived. The use of that method is greatly facilitated by the extensive tables provided by Halvorson and Ziegler (15). (*c*) The dilution of suspension may be determined, which would be expected on

TABLE V

*Calculation of 50 Per Cent End Point in Titration of Virus*

| Logarithm of virus dilution | Inoculations positive | Inoculations negative | Accumulation, positive | Accumulation, negative | Positive |
|---|---|---|---|---|---|
| | | | | | *per cent* |
| 3 | 4 | 0 | 12 | 0 | 100 |
| 4 | 4 | 0 | 8 | 0 | 100 |
| 5 | 3 | 1 | 4 | 1 | 80 |
| 6 | 1 | 3 | 1 | 4 | 20 |
| 7 | 0 | 4 | 0 | 8 | 0 |

inoculation to give rise to an equal number of positive and negative results (16). A simple method for calculating this[2] was given in our previous paper (7).[3] Of the 3 methods of reduction of data, the third

[2] Suggested to us by Dr. Muench.

[3] This method of computing the strength of a virus suspension has been in use for several years in the Yellow Fever Laboratories of the International Health Division of The Rockefeller Foundation. Its use was described in a previous paper (7), but for convenience will again be described here.

A series of tenfold dilutions of the infective agent is prepared, and a number (at least 4 and preferably a larger number) of inoculations are made of each dilution. The results are recorded as in columns 2 and 3 of Table V. (This is the protocol of an actual titration.) Then the positive and negative results, respectively, are accumulated, the direction in which the accumulation is made being of primary importance. Each column is added, beginning with the smaller end. The various

appears to us to be the best. It is based on sound principles, is easy of performance, and yields an accurate estimate of the concentration of infectious particles. Since it presumes only that the titration curve is a symmetrical one, it does not require for its use the assumption that a single particle gives rise to infection, a prerequisite to the use of the second method. If, however, it is known that a single particle will infect, the exact number of infectious particles per unit volume may be derived easily from the 50 per cent end point should that information be desired.

## SUMMARY

A method has been described by which it is possible to estimate the number of particles of vaccine virus which are required to cause infection in the rabbit skin. The method consists essentially in performing a series of intradermal inoculations in rabbits of suitably diluted virus suspensions. The percentage of inoculations at each dilution giving rise to lesions is observed, and the data are subjected to appropriate statistical analysis. Several strains of vaccine virus, differing in their characteristics, have been studied with the following results. Infection with the New York City Board of Health virus appears to follow the injection of a single particle of virus. The same is true for the strain derived from it but cultured in a chick embryo-Tyrode solution medium for a prolonged period. This strain, as has been noted, has largely lost its ability to cause extensive necrosis in the rabbit skin, and causes generalized infection only exceptionally. From the results here reported, it appears that other factors are responsible for the altered character of the lesion than the ability of the virus to establish a foothold in the animal organism. In this respect the cultured appears to be the equal of the original passage virus. Similarly the Noguchi strain of virus is apparently capable of infecting, if a single virus particle is properly introduced.

sums in each column then represent the number of inoculations positive at that dilution and higher dilutions, or the number negative at that dilution and lower dilutions (columns 4 and 5). The percentage of positives at each dilution and higher dilutions is then calculated from the data of the summary columns (column 6). In this case there are 80 per cent positive reactions at $10^{-5}$ and 20 per cent at $10^{-6}$. 50 per cent then would be 30/60 or 0.5 of the distance from $10^{-5}$ to $10^{-6}$ or $10^{-5.5}$.

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