

APPLICATION OF STATISTICS TO PROBLEMS IN BACTERIOLOGY

I. A MEANS OF DETERMINING BACTERIAL POPULATION BY THE DILUTION METHOD

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INTRODUCTION

Some time ago the authors had occasion to study the effect of bacteria on the curing of meat. As a part of the work it was necessary to determine the number of bacteria present in the pickle at different stages of the cure. When plates were made of the pickle in the usual manner, surprisingly low counts were obtained, as compared with the number estimated by direct microscopic observations of wet preparations. While the latter indicated from 500,000 to 1,000,000 organisms per cubic centimeter, the plate count obtained from the same material was 10,000 or less. The low values obtained with plating were at first attributed to an unfavorable medium; consequently a solid medium was made by adding agar to clarified pickle. The counts obtained with the use of this medium were approximately the same as those obtained with use of standard beef extract agar. These results led us to believe that a considerable portion of the usual flora of curing pickle could not develop on solid media. Attempts were then made to determine the number of bacteria present by means of the direct count. For this purpose a modification of the Breed and Brew (1925) method was used. This was not satisfactory because of the high salt content of the pickle. The salt not only brought about a precipitation of the dye, but also, in crystallizing, obscured many of the organisms. Furthermore, the salt prevented proper fixation of bacteria on the slide, so that

attempts to remove the salt by washing resulted in the removal of most of the bacteria. Because of the great variety of organisms present, many of which were actively motile, and because of the presence of débris, methods making use of a counting chamber could not be used.

These difficulties forced us to consider the dilution method as a means of evaluating the bacterial population.

We were at once confronted with the following problems: (1) calculation of the number of bacteria present from the number of tubes showing growth in the various dilutions, (2) evaluation of the accuracy of the data thus obtained, and (3), determination of the variation in accuracy with the number of tubes used in each dilution. A careful review of the literature was made in an attempt to find answers to the questions involved. We found that many investigators had advanced equations that would enable us to calculate the most probable number of organisms from the number of tubes that showed growth, and some had published tables to aid in the calculation. We were, however, unable to find any satisfactory solution of the problem of variation in accuracy with the number of tubes used in each dilution, or, what would have been more desirable, a method for the determination of the number of tubes that must be used to get a specified accuracy. We therefore deemed it necessary to reconsider the entire problem. This is the first of a series of articles dealing with the various problems involved in the dilution method of determining a bacterial population.

HISTORY

The use of dilution methods in bacteriology dates back to the early days of the science. About 1875 Pasteur obtained pure cultures of bacteria by diluting the original inoculum during several successive transfers to a suitable culture medium. Later Miquel, Brefeld, and Lister (Kolle, Kraus and Uhlenhuth (1930)) obtained pure cultures by inoculating small amounts of diluted bacterial suspension into a series of tubes of medium.

For many years bacteriologists have been using dilution methods to give some idea of the number of organisms in the material

examined. This method consists in diluting the material to be examined, usually in powers of 10, and inoculating equal volumes of the diluted material into liquid media. If growth occurs from the inoculation of 1 cc. of a 1:100 dilution and not from a 1:1000 dilution, the number of organisms present in the original material is said to be between 100 and 1000 per cubic centimeter. On the basis of chance, however, it might be possible to have only 50 organisms per cubic centimeter, or more than 1000 per cubic centimeter in the original material and still get growth from the 1:100 dilution and not above that in a single test. A presumptive coli test, utilizing varying amounts of inoculum, has been used in a similar way in the bacteriological analysis of water to give an approximate idea of the quality of the water.

This was essentially the basis of the method introduced by Phelps (1908) to estimate the *B. coli* content of water from presumptive test data. In his method it is assumed that the reciprocal of the highest dilution which shows growth represents the most probable number of organisms present. His method was adopted by a Committee on Standard Methods of Water Analysis of the American Public Health Association (1920). In case "skips" occurred, that is, a positive presumptive test from a dilution higher than one which was negative, the result taken was the reciprocal of the dilution next higher than the smallest one giving a positive test.

A more accurate method of interpreting dilution data has been supplied by McCrady (1915). In developing his equations he begins with the proposition that there is only one organism for each 100 cc. in the sample. He stated that this one organism must obviously be contained in one of the 1 cc. volumes and that the probability of not getting the organism when a single cubic centimeter is removed from the 100 cc. volume is 0.99. The following quotation from his article (p. 185) shows how he has developed his equation.

Now suppose 2 *B. coli* are in the sample. The probability of each organism's not being contained in the 1 cc. withdrawn for the fermentation test has been shown to be (0.99). Then, by the principle just il-

illustrated, the probability of neither organism's appearing in this 1 cc. is equal to the product of the separate probabilities, or $(0.99)(0.99) = 0.9801$. And if a great number of such samples were examined, about 98.01 per cent of the results would be "0/1 in 1 cc."

In general if V represents the number of volumes in the sample, and x the number of *B. coli* in the sample, and one volume is withdrawn, the probability that this volume will contain no *B. coli* is given by

$$\left[\frac{V-1}{V} \right]^x$$

Thus when 1 cc. of the sample is withdrawn for the test, V becomes 100 and the formula becomes $\left[\frac{99}{100} \right]^x$. When a 10 cc. quantity is withdrawn, V becomes 10 (there are ten 10 cc. volumes in the sample), and the formula becomes 0.9^x .

With this reasoning as a foundation for his later work, McCrady developed the equations from which it is possible to calculate the most probable number of organisms per cubic centimeter from data obtained by inoculating a series of tubes with the same dilution or from several series of tubes inoculated with several different dilutions. For the special case where a series of tubes are inoculated with 10 cc., a second with 1 cc., and a third with 0.1 cc., the following formula is given:

$$(p+q)(\log 0.9) + (r+s)(\log 0.99) + (t+u)(\log 0.999) = \frac{p(\log 0.9)}{1-0.9^x} + \frac{r(\log 0.99)}{1-0.99^x} + \frac{t(\log 0.999)}{1-0.999^x}$$

In this equation, p , r , and t represent the number of tubes showing growth in the different series and q , s , and u represent the number of tubes showing no growth in the corresponding series of dilutions. To simplify the use of the method, McCrady (1918) has solved the equation for all possible combinations for several special cases, including those when 5 and 10 tubes are used in each dilution. These solutions have been put into tables so that the method may be used without tedious calculation.

While McCrady's tables and equations may be regarded as solving the problem of calculating, from dilution data, the most probable number of bacteria present, they do not offer a solution

to the more general problem of variation in accuracy with the number of tubes used. In attempting to solve this problem with McCrady's equations, we find that the mathematics become very cumbersome. McCrady's equations are each limited to a special case, so that to solve them one must resort to rather tedious calculation. A more general solution whereby all cases could be evaluated from a single equation and a single table is very desirable. We have accordingly approached the problem from a different angle and have worked out tables somewhat analogous to those of McCrady.

On the basis of minor assumptions, Wolman and Weaver (1917) have simplified McCrady's formula. Their equations are convenient for the calculation of the most probable number of bacteria, but they do not enable one to evaluate the accuracy of the data.

Various methods of interpreting dilution data have been considered in a series of articles by Wells (1918; 1919; 1921), and in an article by Wells and Wells (1922). Their treatment does not lead to results that can be used in the general solution of the problem.

The methods advocated by Wells have been objected to by several investigators. Notable among these is Cairns (1918).

More general considerations of the dilution method have been contributed by Stein (1922), Greenwood and Yule (1917), Fisher (1925), and Reed (1925). All of these men have shown that the number of tubes showing no growth when inoculated with a fixed quantity of a single dilution is equal to e^{-ax} , where x represents the number of organisms per cubic centimeter in that dilution, and a represents the volume of the dilution used for the inoculation.

Stein not only made use of this exponential relationship to interpret dilution data, but he attempted to simplify its application by graphical means. He also showed by graphical means how the accuracy of the method varied with the number of tubes used. These considerations were, however, limited to a few special cases.

Greenwood and Yule considered not only the special case of a

single dilution inoculated into a series of tubes but also the general case of several dilutions inoculated into a series of tubes. Although they give the equation from which the most probable number of organisms can be calculated from experimental data involving several dilutions and several tubes in each, they have made no attempt to simplify the solution of this general case. Since the general equation is rather involved, it is necessary to simplify the solution by means of tables in order to make it applicable for practical use. Greenwood and Yule show how the accuracy of dilution data can be evaluated. Here again, simplification is needed for general application.

Their equation for the general case is as follows:

$$(a_1n_1 + a_2n_2 + \dots + a_n n_n) = \frac{a_1 m_1}{1 - e^{-a_1 x}} e^{-a_1 x} + \frac{a_2 m_2}{1 - e^{-a_2 x}} e^{-a_2 x} \dots \dots \frac{a_n m_n}{1 - e^{-a_n x}} e^{-a_n x}$$

In this equation x is the most probable number of bacteria per cubic centimeter that will give n_1 negative and m_1 positive results when N_1 tubes are inoculated with a_1 cc. each, and will give n_2 negative and m_2 positive results when N_2 tubes are inoculated with a_2 cc. each, etc.

Reed's contribution is very helpful in the special cases where one tube in each of several dilutions or where several tubes of the same dilution are used, but his solution is not extended to the more general problem of several tubes in each of several dilutions.

The dilution method has been used by Cunningham (1915), by Cutler (1919a), and by Cutler, Crump, and Sandon (1922) to evaluate the number of protozoa in soil. In the first two of these publications the dilution data were interpreted by the method of Phelps (1908), while Cutler, Crump, and Sandon used a table calculated by Fisher (1925). This table by Fisher is useful only for a very special case, so that it is not of any material value in general application. Fisher's table appears to be calculated from the exponential function e^{-x} .

A theoretical consideration of the dilution method has also been contributed by Clark (1927), who applied the method to determine the numbers of bacteriophage in a suspension.

After a careful review of the literature on the subject we still

feel it is necessary to reconsider the entire problem. General as well as special equations should be developed that are based on the reasoning used by Stein (1922), Greenwood and Yule (1917), Fisher (1925), and Reed (1925). These equations should be solved for all the special cases that are in common use, and the solutions should be arranged in tabular form, as McCrady did for his equations. Furthermore, tables should be made available which will aid in the solution of any special case not in common use.

A more detailed consideration of the accuracy of the method is needed. The calculation of the accuracy should be simplified so that anyone using the dilution method will be able to determine, to a reasonable degree, the limits of accuracy of his data. This must be made simple enough so that it can be applied generally.

The mathematics involved in the dilution method should also be applied to other problems in bacteriology, such as the determination of the percentage of insects that may be infected with certain viruses, or the interpretation of data obtained when a series of animals are injected with a single dilution or several dilutions of a given pathogenic bacterium or virus. It is also desirable to investigate the effect produced on dilution data if we accept the theory that single cells cannot develop.

A consideration of these and other probability problems will be published in a series of articles on the subject. It has been deemed necessary to verify by experimental data some of the mathematical considerations, and data thus obtained will also be presented in this series.

This first paper is confined to the development of equations to be used for the evaluation of bacterial populations by the dilution method. Tables are included which aid in the solution of these equations, as well as special solutions which simplify their general application.

THEORY

In order to determine the number of bacteria in a sample of liquid material by the dilution method it is necessary to dilute

the material to such an extent that when a sample is removed, bacteria may or may not be present. The problem at hand, then, is to determine the probability of getting bacteria in a certain sample. This probability will depend upon the number of organisms present.

A number of earlier investigators have shown how this probability is related to the number of organisms present. It is believed desirable, however, to develop these relationships from fundamental reasoning so that it will be possible for those not familiar with Poisson's Series to understand the derivation of the equations without having to make a special study of the mathematics on which the Poisson's Series is based. An understanding of the derivation of the equations is necessary for their proper application.

There are several ways in which the bacterial population can be determined by the dilution method. One may determine the number present by inoculating a large number of tubes of media with an equal volume of the sample to be tested, and determine the number present by the percentage of tubes that show growth. Or one may inoculate a series of tubes of media with a given volume, another set with a smaller volume, and a third set with a still smaller volume, and then determine the number of organisms present by the number of tubes showing growth in the different series.

We are interested, therefore, in several cases: (1) the probability of getting growth in a single tube, when it is inoculated with a definite volume of the sample; (2) the probability of getting a certain number showing growth out of a series of tubes, all of which are inoculated with the same volume of a given sample; (3) the probability of getting a certain combination of tubes showing growth out of several series of tubes when the tubes in each series are inoculated with different sized samples.

Case I we can designate as a single tube of a single dilution; case II, as several tubes of a single dilution; and case III, as several tubes of each of several dilutions.

CASE I

To calculate the probability that an organism will or will not be contained in a certain sample, let us assume that a large volume (N cc.) of the material to be sampled be at hand, and that in this material there are x organisms per cubic centimeter. Let us assume further, that the volume of an organism is v cc. (unit volume), that all organisms have the same volume, and that the water present be divided up into particles of the same unit volume as a bacterium. If we imagine now that we remove one of these particles, and let P equal the probability that our selection will not be an organism, then

$$P = \frac{\frac{N}{v} - Nx}{\frac{N}{v}}$$

This will be true because $\frac{N}{v}$ represents the total number of particles in a volume N . Nx is the number which are bacteria, and $\frac{N}{v} - Nx$, the number which are water. The probability Q

that the selection will be an organism is $\frac{Nx}{\frac{N}{v}}$. These expressions

can be simplified to $P = 1 - vx$; $Q = vx$.

Let us assume that enough of these small particles are removed so that the aggregate is 1 cc. The total probability will be expressed by the binomial $[(1-vx) + vx]^{\frac{1}{v}}$. The first term of the binomial will be $(1 - vx)^{\frac{1}{v}}$, which represents the probability that no organisms will be contained in a 1 cc. sample.

$$P = (1 - vx)^{\frac{1}{v}}$$

This can be simplified as follows:

$$\ln P = -x - \frac{vx^2}{2} - \frac{v^2x^3}{3} - \frac{v^3x^4}{4} \dots$$

Since v is very small, the second and following terms will be very small in comparison with the first term as long as relatively small values of x are considered.

Therefore

$$\ln P = -x$$

or

$$P = e^{-x} \quad (1)$$

If instead of taking out 1 cc., we remove a cc., then

$$\begin{aligned} P &= (1 - vx)^{\frac{a}{v}} \\ \ln P &= a \left[-x - \frac{vx^2}{2} - \frac{v^2x^3}{3} \dots \right] \\ &= -ax \end{aligned}$$

or

$$P = e^{-ax} \quad (2)$$

and

$$Q = 1 - e^{-ax} \quad (3)$$

CASE II

If several tubes are to be inoculated with the same dilution the number of tubes which show growth will depend upon the number of organisms present and upon the element of chance.

If n tubes are inoculated, the total probability will be expressed by the binomial $[e^{-ax} + (1 - e^{-ax})]^n$. By expanding this binomial, the following series is obtained:

$$(e^{-ax})^n + n(e^{-ax})^{n-1}(1 - e^{-ax}) + \frac{n(n-1)}{2!}(e^{-ax})^{n-2}(1 - e^{-ax})^2 \dots$$

The first term of this series gives an expression for the probability that none of the tubes will show growth, the second term for the probability that only one of the tubes will show growth, etc.

If we let p equal the number of tubes that show growth and q the number that show no growth, in which case $p + q = n$, the series becomes:

$$(e^{-ax})^q + n(e^{-ax})^q (1 - e^{-ax})^{p'} + \frac{n(n-1)}{2!} (e^{-ax})^{q''} (1 - e^{-ax})^{p''} \dots$$

If we let P be the probability, then the following equation will hold for any combination of p and q :

$$P = \frac{(p+q)!}{p!q!} (e^{-ax})^q (1 - e^{-ax})^p \tag{4}$$

If $p, q,$ and a are kept constant in this equation, P will vary only with x . From the equation and the nature of the problem, it is evident that there must be a maximum value of P corresponding to some particular value of x . This optimum value of x can be found by differentiating equation (4), which gives the following:

$$\frac{dP}{dx} \frac{1}{P} = -qa + \frac{pa e^{-ax}}{1 - e^{-ax}}$$

By putting the derivative equal to 0, it is possible to find the value of x that corresponds to the maximum value of P . If we let \bar{x} be the optimum or most probable value of x , its value may be found by substituting \bar{x} for x in the above equation, when the derivative is placed equal to 0. The resulting equation simplifies to

$$\bar{x} = \frac{1}{a} \ln \frac{n}{q} \tag{5}$$

In the case of several tubes in a single dilution, the most probable number of organisms per cubic centimeter is given by the natural logarithm of the ratio of the total number of tubes to those that show no growth. Changing from base e to base 10, the expression becomes

$$\bar{x} = \frac{2.3026}{a} \log \frac{n}{q} \tag{6}$$

In deriving this equation, it has been assumed that in bacterial suspensions, all values of x are equally likely to occur between

0 and a number determined by the maximum bacterial population that can occur in a sample. By means of this formula it is easy to calculate the most probable number of organisms present in a sample from the number of tubes that show no growth. This can also be obtained by means of equation (5) and table 1 (appendix). From equation (5), we have

$$\frac{q}{n} = e^{-ax}$$

The function e^{-ax} then gives the fraction of tubes which show no growth. By means of a table showing values of e^{-x} for different values of x , it is possible to determine the most probable number of organisms present corresponding to any value of $\frac{q}{n}$. Such tables have been calculated by other investigators (L. von Bortkewitsch (1898) and H. E. Soper (1915)). The table of Bortkewitsch is carried out to only four places and that of Soper to six places. Whereas these tables may be adequate for the calculation of the most probable number from dilution data, we found that they did not suffice for the calculation of the frequency distribution of experimental values that might be obtained on a single suspension. Such calculations are essential in order to show a convenient relationship between the accuracy of the data and the number of tubes used in each dilution. For this reason we found it necessary to calculate these tables to nine decimal places. They are included in the appendix. Values of the logarithm of $(1 - e^{-x})$ are included in this table to simplify the solution of other problems which will be discussed later.

Examples: Suppose that in an experiment 0.100 of the tubes inoculated with a cc. each showed no growth, then from the table we find that the value of x corresponding to $e^{-x} = 0.100$ is 2.30. This number is therefore the most probable number of organisms present in the size sample used for the inoculation.

By means of this table it is also possible to determine the percentage of tubes showing growth which are inoculated with different sized samples. Let us assume that a liquid contained 0.650 organism per cubic centimeter, or 65 organisms in a 100 cc.

sample. In this case there will be 0.0065 organism in 0.01 cc., 0.065 organism in 0.1 cc., and 0.65 organism in 1 cc. Referring to the table, we see that for

$x = 0.0065$	$e^{-x} = 0.9935$
$x = 0.065$	$e^{-x} = 0.9371$
$x = 0.650$	$e^{-x} = 0.5222$
$x = 6.500$	$e^{-x} = 0.0015$

This means that for every 10,000 tubes inoculated with each of these dilutions, on the average, 15 of those inoculated with 10 cc., 5222 of those inoculated with 1.0 cc., 9371 of those inoculated with 0.1 cc., and 9935 of those inoculated with 0.01 cc. would show no growth; or if 100 tubes were inoculated with each dilution, one would expect that all the tubes receiving 10 cc., 48 receiving 1 cc., 6 receiving 0.1 cc., and 1 receiving 0.01 cc. would show growth. If a person wished to determine the most probable number of organisms by the result of inoculating a series of tubes with a single dilution, the best size of inoculum to use would in that case be 1 cc.

When a person inoculates a series of tubes with a single dilution and finds the percentage which show growth, he will also be able, by means of this table, to determine what size inoculum would have produced growth in 50 per cent of the tubes.

CASE III

In this case several dilutions are used, and several tubes are inoculated with each dilution.

For this development the following terms will be used:

- $w_1, (1 - w_1)$ = the probability of a success and failure respectively, in a sample of size a_1 ,
- $w_2, (1 - w_2)$ = the probability of a success and failure respectively, in a sample of size a_2 ,
- $w_3, (1 - w_3)$ = the probability of a success and failure respectively, in a sample of size a_3 ,
- n_1 = the number of samples of size a_1 that are taken,
- n_2 = the number of samples of size a_2 that are taken,
- n_3 = the number of samples of size a_3 that are taken,
- p_1, q_1 = the number of failures and successes obtained out of n_1 trials.
- p_2, q_2 = the number of failures and successes obtained out of n_2 trials,
- p_3, q_3 = the number of failures and successes obtained out of n_3 trials.

The total probability will therefore be the product of several binomials, and by expanding each of them and multiplying the expanded forms together, the different terms in the product will be the probability of getting any set of combinations of failures and successes, as

$$p_1q_1; p_2q_2; p_3q_3; \dots$$

The general formula for the probability of any one of these terms will then be:

$$P = \frac{(p_1 + q_1)!}{p_1! q_1!} (w_1)^{q_1} (1 - w_1)^{p_1} \frac{(p_2 + q_2)!}{p_2! q_2!} (w_2)^{q_2} (1 - w_2)^{p_2} \frac{(p_3 + q_3)!}{p_3! q_3!} (w_3)^{q_3} (1 - w_3)^{p_3} \dots \dots \dots (7)$$

This equation is obtained by multiplying together several equations, as (4), each being derived from a different binomial.

Now if the probabilities w_1, w_2, w_3 , etc., are functions of x , it is possible to find the most probable values of x by differentiating the above equation with respect to x and putting the derivative equal to zero. This equation can be differentiated most readily by taking the logarithms of both sides, thus:

$$\ln P = \ln \frac{(p_1 + q_1)!}{p_1! q_1!} + \ln \frac{(p_2 + q_2)!}{p_2! q_2!} + \ln \frac{(p_3 + q_3)!}{p_3! q_3!} \dots \dots \dots + q_1 \ln w_1 + q_2 \ln w_2 + q_3 \ln w_3 \dots \dots \dots + p_1 \ln(1 - w_1) + p_2 \ln(1 - w_2) + p_3 \ln(1 - w_3) \dots \dots \dots$$

Differentiating and substituting $n_1 - p_1$ for q_1 , etc., we get

$$\frac{dP}{dx} \frac{1}{P} = d \frac{\ln w_1}{dx} \left[n_1 - \frac{p_1}{1 - w_1} \right] + d \frac{\ln w_2}{dx} \left[n_2 - \frac{p_2}{1 - w_2} \right] + d \frac{\ln w_3}{dx} \left[n_3 - \frac{p_3}{1 - w_3} \right] \dots \dots \dots$$

Now if x represents the percentage of a certain kind that are present in a unit, and a_1, a_2, a_3 , etc., represent the number of these units that are selected, then

$$w_1 = (1 - x)^{a_1}; w_2 = (1 - x)^{a_2}; w_3 = (1 - x)^{a_3}; \text{etc.}$$

and

$$\frac{d \ln w_1}{dx} = \frac{-a_1}{1 - x}; \frac{d \ln w_2}{dx} = \frac{-a_2}{1 - x}; \frac{d \ln w_3}{dx} = \frac{-a_3}{1 - x}, \text{etc.}$$

Substituting these values in the above differential equation, putting it equal to zero, and factoring out all common multipliers, we get:

$$a_1 \left(n_1 - \frac{p_1}{1 - w_1} \right) + a_2 \left(n_2 - \frac{p_2}{1 - w_2} \right) + a_3 \left(n_3 - \frac{p_3}{1 - w_3} \right) = 0$$

$$\frac{p_1 a_1}{1 - w_1} + \frac{p_2 a_2}{1 - w_2} + \frac{p_3 a_3}{1 - w_3} \dots \dots = a_1 n_1 + a_2 n_2 + a_3 n_3 \dots \dots \quad (8)$$

This is the same type of equation as that developed by Greenwood and Yule (1917), but is a little more general. With this formula a_1, a_2, a_3 , etc., n_1, n_2, n_3 , etc., are constants for any given experiment. p_1, p_2, p_3 , etc., can be determined by experimental data. Since w_1, w_2, w_3 , etc., are functions of x , it is then possible to solve for x the most probable value of that variable. The solution of the equation can be simplified if tables are worked out that give the relationship between x and w_1, w_2, w_3 , etc.

To apply this to the problem of determining the most probable number of bacteria per cubic centimeter, let us assume that a set of tubes are inoculated with various amounts of the sample, one set inoculated with 10 cc. each, another set with 1 cc. each, and a third set with 0.1 cc. in each tube. In this case,

$$\begin{array}{lll} w_1 = e^{-10x} & w_2 = e^{-x} & w_3 = e^{-\frac{x}{10}} \\ a_1 = 10 & a_2 = 1 & a_3 = 0.1 \end{array}$$

Let us assume that ten tubes are used in each set, Then

$$n_1 = n_2 = n_3 = 10$$

The general formula then becomes

$$\frac{10 p_1}{1 - e^{-10x}} + \frac{p_2}{1 - e^{-x}} + \frac{p_3}{10(1 - e^{-\frac{x}{10}})} = 111$$

in which p_1 = the number of tubes receiving 10 cc. that show growth,

p_2 = the number of tubes receiving 1 cc. that show growth,

p_3 = the number of tubes receiving 0.1 cc. that show growth.

In the appendix, table 2, will be found values of $\frac{1}{1 - e^{-x}}$ for

the different values of x . With the aid of these tables it is an easy matter to solve the equation by trial and error by selecting the value of $\frac{1}{1 - e^{-10x}}$, $\frac{1}{1 - e^{-x}}$, $\frac{1}{1 - e^{-\frac{x}{10}}}$, that will make the left hand side of the equation equal to 111. The values of x corresponding to these values of $\frac{1}{1 - e^{-x}}$, etc., will be the most probable number of organisms per cubic centimeter.

To demonstrate the method of calculation, a specific example is included.

Suppose in an actual experiment 160 tubes were inoculated with each of a series of dilutions. Suppose that the following results were obtained:

DILUTION	NUMBER SHOWING GROWTH	NUMBER SHOWING NO GROWTH
10^4	160	0
10^5	160	0
10^6	158	2
10^7	61	99
10^8	8	152
10^9	0	160

The critical dilutions are therefore 10^6 , 10^7 , 10^8 . The most probable number of organisms present in the 10^7 dilution can be obtained from the general equation:

$$\frac{a_1 p_1}{1 - e^{-a_1 x}} + \frac{a_2 p_2}{1 - e^{-a_2 x}} + \frac{a_3 p_3}{1 - e^{-a_3 x}} = a_1 n_1 + a_2 n_2 + a_3 n_3$$

In this experiment, a_1 is 10, a_2 is 1, and a_3 is 0.1; $n_1 = n_2 = n_3 = 160$; $p_1 = 158$, $p_2 = 61$, $p_3 = 8$. Substituting these values in the above we get

$$\frac{10 \cdot 158}{1 - e^{-10x}} + \frac{1 \cdot 61}{1 - e^{-x}} + \frac{8}{10(1 - e^{-\frac{x}{10}})} = 160(10 + 1 + 0.1)$$

$$\frac{1580}{1 - e^{-10x}} + \frac{61}{1 - e^{-x}} + \frac{8}{10(1 - e^{-\frac{x}{10}})} = 1776.0$$

An approximate idea of the value of x can be obtained by considering the middle dilution in which the proportion of tubes

showing no growth is $\frac{99}{160} = 0.6188$. From table 1 (appendix)

the value of $e^{-x} = 0.6188$ is found to correspond to a value of $x = 0.4800$. By obtaining the reciprocals of $1 - e^{-10x}$, $1 - e^{-x}$, and $1 - e^{-\frac{x}{10}}$ from table 2 (appendix) and substituting in the above equation, we can obtain a more accurate value of x . Substituting the proper reciprocals in the left hand side of the equation and solving, we get

$$\begin{aligned} 1776.898 & \text{ for } x = 0.4650 \\ 1774.598 & \text{ for } x = 0.4700 \\ 1770.194 & \text{ for } x = 0.4800 \end{aligned}$$

By interpolation, we find that the function becomes

$$1776.00 \text{ for } x = 0.4669$$

In the case of the ten tubes inoculated with each of the dilutions this equation has been solved for all the combinations that are likely to occur. Table 3 (appendix) shows the most probable number of organisms which correspond to each of the combinations.

When 10 tubes are used in each of these dilutions, and when $a_1 = 10$ cc., $a_2 = 1$ cc., and $a_3 = 0.1$ cc., the general equation (7) becomes

$$P = \frac{10!}{p_1! q_1!} \frac{10}{p_2! q_2!} \frac{10}{p_3! q_3!} (e^{-10x})^{q_1} (e^{-x})^{q_2} (e^{-\frac{x}{10}})^{q_3} (1 - e^{-10x})^{p_1} (1 - e^{-x})^{p_2} (1 - e^{-\frac{x}{10}})^{p_3}$$

Taking the log to the base 10 of both sides of the equation, we get

$$\begin{aligned} \log P = \log \frac{10!}{p_1! q_1!} + \log \frac{10!}{p_2! q_2!} + \log \frac{10!}{p_3! q_3!} - x \left(10q_1 + q_2 + \frac{q_3}{10} \right) \log e + \\ 10p_1 \log (1 - e^{-10x}) + p_2 \log (1 - e^{-x}) + p_3 \log (1 - e^{-\frac{x}{10}}) \end{aligned}$$

By means of this equation it is possible to solve for the value of P for any given value of x and p_1 , p_2 , and p_3 . This has been done for all the combinations where P has a value greater than 0.01 per cent. These values show how often a certain combination can be expected if the number of organisms in the solution are as indicated by the most probable values of x . These frequencies

are helpful in interpreting data. If certain combinations are found to occur more often than indicated by these values of P , one may become suspicious that something is wrong either with the technic or with the medium.

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TABLE OF EXPONENTIAL FUNCTIONS

x	e^x	e^{-x}	$\log(1+x)$	$\log(1-x)$	$\log(1+x^2)$	$\log(1-x^2)$	$\log(1+x^4)$	$\log(1-x^4)$	$\log(1+x^8)$	$\log(1-x^8)$	$\log(1+x^{16})$	$\log(1-x^{16})$	$\log(1+x^{32})$	$\log(1-x^{32})$	$\log(1+x^{64})$	$\log(1-x^{64})$	$\log(1+x^{128})$	$\log(1-x^{128})$	$\log(1+x^{256})$	$\log(1-x^{256})$
0.00	1.000000	1.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
0.01	1.010050	0.990049	0.004713	-0.004713	0.009426	-0.009426	0.018852	-0.018852	0.037704	-0.037704	0.075408	-0.075408	0.150816	-0.150816	0.301632	-0.301632	0.603264	-0.603264	1.206528	-1.206528
0.02	1.020201	0.980198	0.009426	-0.009426	0.018852	-0.018852	0.037704	-0.037704	0.075408	-0.075408	0.150816	-0.150816	0.301632	-0.301632	0.603264	-0.603264	1.206528	-1.206528	2.413056	-2.413056
0.03	1.030454	0.970348	0.014139	-0.014139	0.028278	-0.028278	0.056556	-0.056556	0.113112	-0.113112	0.226224	-0.226224	0.452448	-0.452448	0.904896	-0.904896	1.809792	-1.809792	3.619584	-3.619584
0.04	1.040709	0.960499	0.018852	-0.018852	0.037704	-0.037704	0.075408	-0.075408	0.150816	-0.150816	0.301632	-0.301632	0.603264	-0.603264	1.206528	-1.206528	2.413056	-2.413056	4.826112	-4.826112
0.05	1.051066	0.950651	0.023565	-0.023565	0.047130	-0.047130	0.094260	-0.094260	0.188520	-0.188520	0.377040	-0.377040	0.754080	-0.754080	1.508160	-1.508160	3.016320	-3.016320	6.032640	-6.032640
0.06	1.061525	0.940804	0.028278	-0.028278	0.056556	-0.056556	0.113112	-0.113112	0.226224	-0.226224	0.452448	-0.452448	0.904896	-0.904896	1.809792	-1.809792	3.619584	-3.619584	7.239168	-7.239168
0.07	1.072086	0.930958	0.032991	-0.032991	0.065982	-0.065982	0.131624	-0.131624	0.263248	-0.263248	0.526496	-0.526496	1.052992	-1.052992	2.105984	-2.105984	4.211968	-4.211968	8.423936	-8.423936
0.08	1.082759	0.921113	0.037704	-0.037704	0.075408	-0.075408	0.150816	-0.150816	0.301632	-0.301632	0.603264	-0.603264	1.206528	-1.206528	2.413056	-2.413056	4.826112	-4.826112	9.648176	-9.648176
0.09	1.093544	0.911269	0.042417	-0.042417	0.084834	-0.084834	0.170008	-0.170008	0.341216	-0.341216	0.682432	-0.682432	1.364864	-1.364864	2.729728	-2.729728	5.459456	-5.459456	10.916352	-10.916352
0.10	1.104441	0.901426	0.047130	-0.047130	0.094260	-0.094260	0.189200	-0.189200	0.381824	-0.381824	0.763648	-0.763648	1.527296	-1.527296	3.054592	-3.054592	6.109184	-6.109184	12.218368	-12.218368

