ON THE TITRATION OF BACTERIOPHAGE AND THE PARTICULATE HYPOTHESIS.

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INTRODUCTION.

One of the methods most frequently used to estimate the concentration of bacteriophage in a fluid medium is that of serial dilution. If several parallel titrations of the same solution are made by this method, it will be found usually that the results are not entirely consistent; that, although in most cases the number of tubes in which the bacteria dissolve will be the same, let us say n, a few cases will yield $n \pm 1$.

Dr. Bronfenbrenner,[1] of The Rockefeller Institute, in whose laboratory many thousands of such titrations have been made on solutions of various degrees of concentration, estimates that, if the dilution factor be .1, about 85 per cent of such parallel runs yield the same value of n. This degree of consistency is about 40 per cent higher than one would expect if it is true, as is quite generally believed, that bacteriophage exists in the state of particles, a single one of which is sufficient when added to a culture of susceptible bacteria to start the destructive processes.

Dr. Bronfenbrenner's estimate is based largely on the general impressions gained by himself and his coworkers in the course of much experimental work rather than on definite records. The discrepancy between this estimate and the results of analysis is so great, however, that it deserves consideration. It should be checked by experiment. If the predictions of theory are upheld, it would constitute an interesting verification of the simple particulate hypothesis. If not, it would require a further consideration of the hypotheses on which the analysis

[1] I am indebted to Dr. Bronfenbrenner for his kindness in furnishing the material on which this paper is based.

is based, which in itself might prove to be of interest. Inasmuch as the labor involved in making the experiments is very great, such a check can best be made as a by-product of titrations made for other purposes. A brief presentation of the analysis together with a discussion of the hypotheses on which it is based, may, therefore, be of interest.

The Serial Dilution Method.

The method will be explained briefly by an example. We put 10 cc. of the solution to be titrated, which contains broth as well as bacteriophage, into the first of a series of test-tubes; into each of the other tubes, we put 9 cc. of sterile broth. We now remove 1 cc. of the fluid from the first tube and introduce it into the second. After very thorough stirring, we remove 1 cc. from the second tube, using a clean pipette, and put it into the third tube. We continue this process indefinitely, in so far as the theory is concerned. The expectation of bacteriophage in any tube is, therefore, exactly one-tenth as great as that for the preceding tube. The quantity, .1, is called the dilution factor. Susceptible bacteria are now put into each of the tubes. In the first n tubes, they dissolve; in all of the others, they live and multiply. 10^{n-1} is taken as a measure of the concentration of the original solution.

Statistical Treatment of the Problem.

It will be assumed for the present that the presence of one or more particles of bacteriophage in any tube always results in the dissolution of all of the bacteria, that particles neither dissociate nor coalesce during the process of dilution, and that none of the particles are lost by adsorption or otherwise. The effects of changing these hypotheses in various ways will be discussed later.

Let x = the exact number of particles of bacteriophage placed in the first tube,

p_n = the probability that the last (most dilute) tube in which the bacteria dissolve will be the nth tube of the series, and

a = the dilution factor.

In what follows, it will be assumed that $a = .1$ unless otherwise stated.

The probability that the $(n+1)$th tube receives *a particular one* of the particles originally in the first tube is a^n; the probability that it does *not* receive it is $1 - a^n$; and the probability that it receives *none*

of the original x particles is, therefore, $(1-a^n)^x$. Likewise, the probability that the nth tube receives none is $(1-a^{n-1})^x$. These probabilities are not independent, however; whenever the nth tube receives none the $(n+1)$th tube *must* also receive none. In every other case in which the $(n+1)$th received none, the nth must have received *some*, and it must have *retained* them. Therefore, the probability, which is in effect p_n, that the nth retains at least one and the $(n+1)$th receives none is given by

$$p_n = (1-a^n)^x - (1-a^{n-1})^x \tag{1}$$

If x and n are infinite, xa^n being finite, this equation may be written

$$p_n = e^{-xa^n} - e^{-xa^{n-1}} \tag{2}$$

These are the fundamental equations with which we shall have to deal in what follows.

The Maximum Value of p_n for Small Values of n.

Let P_n = this maximum value of p_n, and
X_n = the value of x which corresponds to P_n.

If $n = 1$, it is obvious that $X_1 = 1$. Tube 1 must retain at least one particle, and the smaller the number of particles it receives, the less the probability that it will lose one of them to Tube 2. Equation (1) shows, then, that $P_1 = 1 - a$ which is .9.

If $n > 1$, we can find between what two consecutive integral values of x the desired value lies by treating x as a continuous variable. Accordingly, we set $D_x P_n$ equal to zero. From equation (1), we find that

$$D_x P_n = (1-a^n)^x \log_e (1-a^n) - (1-a^{n-1})^x \log_e (1-a^{n-1}).$$

Setting this expression equal to zero, simplifying, and writing X_n in place of x, we have

$$X_n = \frac{\log [-\log (1-a^{n-1})] - \log [-\log (1-a^n)]}{\log (1-a^n) - \log (1-a^{n-1})} \tag{3}$$

in which the base of logarithms is arbitrary.

Column 2 of Table I contains the values of X_n found by setting a equal to .1, and n equal to 2, 3, and 4 in equation (3), and Column 3 contains the corresponding values of P_n found by substituting X_n in equation (1). These quantities cannot be less than the true values corresponding to the best integral values of X_n. Inspection of Column 3 shows that as n increases from 1 to 4, P_n apparently approaches a limiting value very rapidly. To make sure of this, we must find the value of P_n when n is infinite.

TABLE I.

1	2	3	4	5	6	7	8
		$a = .1$				$a = .09$	
n	X_n	P_n	X'_n	p'_n	$\bar{p}_n$	P_n	p'
1	1.000	.900	7.27	.466		.910	.469
2	24.60	.706	76.6	.463	.604	.720	.467
3	255.0	.698	770.	.463	.602		
4	2558.	.697					
∞		.697		.463	.602	.717	.467

X_n is the value of x corresponding to P_n, the maximum value of p_n which in turn is the probability that the last (most dilute) tube in which bacteria dissolve is the nth tube of the series.

X'_n is the value of x for which $p_n = p_{n+1}$. At this point, p'_n, the degree of consistency of parallel runs, has a minimum value.

$\bar{p}_n$ is the mean value of p_n over the range of values within which p_n is greater than p with any other subscript.

The Value of P_n When n Is Infinite.

From equation (2), we find that

$$D_x P_n = a^{n-1} e^{-xa^{n-1}} - a^n e^{-xa^n}$$

setting this expression equal to zero, simplifying, and writing X_n for x, we find

$$X_n = \frac{-\log_e a}{a^{n-1}(1-a)}$$

After substituting this expression for x in equation (2), and simplifying, it appears that

$$P_\infty = a^{\frac{a}{1-a}} - a^{\frac{1}{1-a}} \tag{4}$$

The value of P_∞ given in Column 3 was found by setting a equal to .1 in equation (4).

The Value of p'_n for Small Values of n.

Between X_n and X_{n+1}, there must be a value of x for which $p_n = p_{n+1}$. We denote this value by X'_n. When $x = X'_n$, the degree of consistency of titrations of samples containing the same number of particles will have a minimum value inasmuch as it is equally probable that a run will yield either n or $n+1$. The value of p_n corresponding to X'_n will be denoted by p'_n. We proceed to find the values of X'_n. Setting the expression given by equation (1) for p_n equal to a similar expression for p_{n+1}, rearranging terms, and writing X'_n for x, we have

$$\left[\frac{1-a^n}{1-a^{n-1}}\right]^{X'_n}\left\{2 - \left(\frac{1-a^{n+1}}{1-a^n}\right)^{X'_n}\right\} = 1 \tag{5}$$

Equation (5) shows that $X'_1 = 7.27$. For higher values of n, the equation cannot be solved for X'_n explicitly, but the values of X'_n can be found to any desired degree of approximation as follows: We set the quantity inside the brackets equal to zero, thus—

$$X'_n = \frac{\log 2}{\log (1-a^{n+1}) - \log (1-a^n)} \tag{6}$$

Having found a value of X'_n for any small value of n from equation (6), we use this value as the exponent of the first parenthesis of equation (5). This gives a new value of the quantity inside the brackets slightly different from zero, and consequently a new equation like equation (6) except that the figure 2 is replaced by a quantity slightly less than 2. This process could be carried on indefinitely but inspection shows that the true final value of X'_n cannot differ from the value first found from equation (6) by as much as .1 of 1 per cent for any value of n. We, therefore, use equation (6) and ignore the error in-

volved. Column 4 of Table I contains the values of X'_n thus found and Column 5 contains the corresponding values of p'_n found by substituting the values in Column 4 together with the corresponding values of n in equation (1). Inasmuch as X'_n must, in fact, be an integer, these values are slightly too small. The error is certainly negligible if n is 2 or more.

The Value of p'_n When n Is Infinite.

To make sure that p'_n approaches a limiting value as n increases, we find the value of p'_n when n is infinite. As in the preceding section, we first set p_n equal to p_{n+1} to find X'_n. Using equation (2) for the purpose, writing X'_n for x, and introducing a new variable, y, such that

$$X'_n = \frac{\log_e y}{a^{n-1}(1-a)}, \tag{7}$$

we find that

$$y(2 - y^a) = 1 \tag{8}$$

Equation (8) is the analogue of equation (5), and the value of y can be found by the same method of approximation. Inspection shows, as before, that

$$y = 2^{\frac{1}{a}} \tag{9}$$

gives log y with a maximum error of .1 of 1 per cent. Substituting this value of y in equation (7), we have

$$X'_n = \frac{\log_e 2}{a^n(1-a)} \tag{10}$$

and this expression when substituted in equation (2) gives

$$p'_\infty = 2^{\frac{1}{a-1}} - 2^{\frac{1}{a(a-1)}} \tag{11}$$

p'_∞ is, therefore, equal to .463 with an error of less than .1 of 1 per cent.

The Value of $\bar{p}_n$ for Small Values of n.

If we are working with solutions of a great variety of degrees of concentration, we are justified in considering the mean value of p_n for the values of x which lie between X'_{n-1} and X'_n. This quantity is denoted by $\bar{p}_n$. If n is greater than 1, we may treat x as a continuous variable without introducing an appreciable error. We simply integrate $p_n dx$ (using equation (1) for the purpose) between the limits X'_{n-1} and X'_n, and divide by the difference of the limits. We find, thus, that

$$\bar{p}_n = \frac{1}{X'_n - X'_{n-1}} \left[\frac{(1 - a^n)^{X'_n} - (1 - a^n)^{X'_n - 1}}{\log_e (1 - a^n)} - \frac{(1 - a^{n-1})^{X'_n} - (1 - a^{n-1})^{X'_n - 1}}{\log_e (1 - a^{n-1})} \right] \tag{12}$$

The values of $\bar{p}_n$ for $n = 2$ and $n = 3$ shown in Column 6 were found by substituting the figures of Column 4 together with the appropriate values of n in equation (12).

The Value of $\bar{p}_n$ When n Is Infinite.

As before, we integrate $p_n dx$ from X'_{n-1} to X'_n (using equation (2)) and divide by the difference of the limits. X'_n is given by equation (10) and X'_{n-1} is a similar expression with the value of n reduced by one unit. After integrating, substituting these expressions for the limits, and simplifying, it comes out that

$$\bar{p}_\infty = \frac{1}{\log_e 2} \left[2^{\frac{a}{a-1}} + a \cdot 2^{\frac{1}{a(a-1)}} - (1 + a)\, 2^{\frac{1}{a-1}} \right] \tag{13}$$

which proves to be .602.

The Effect of Altering the Dilution Factor.

In the preceding pages, a has been taken as .1. Increasing the value of a would result in a lowering of the values of the various p's; and conversely a decrease in the value of a would have the opposite

effect. To make sure that a slight change in the dilution factor could not produce a great change in the results, I have recalculated P_n and p'_n taking .09 for a. The results are shown in Columns 7 and 8. If we set a equal to zero in equation (13), we find that the limiting value of $\bar{p}_\infty$ is .722.

DISCUSSION.

In the foregoing, it has been necessary to deal with x as a continuous variable and to consider the case in which x is infinite. One must be careful not to confuse these analytical devices with the idea that the active substance is itself infinitely divisible; they were used simply for the purpose of studying equations (1) and (2) which are based on the particulate hypothesis. The low values of the p's in Table I are brought about by the fact that, however nicely the active substance may be divided by serial dilution in the first stages where the number of particles per cc. is great, a time comes as the dilution continues when the number of particles per cc. is so small that the probability variations are considerable. It is by the indications at this point that the state of affairs in the first tube is judged.

In practice, n is much greater usually than 1 or 2. We may, therefore, ignore these two cases. We take a as .1. Table I shows that a value 10 per cent lower makes little change in the results; we may, therefore, ignore the effects of slight errors of dilution.

The table shows that if $n>2$ all of the p's are practically independent of n. It makes little difference, then, whether a particular tube, (the first tube as we have taken it) receives exactly x particles, whether it is made from a parent tube the concentration in which is ten times as great, or whether it is merely a sample of stock solution.

It appears from Column 3 that, with a fortunate choice of the solution to be titrated, about 70 per cent of parallel runs might yield the same value of n. On the other hand, if the choice were unfortunate, less than half of them would yield the same value of n. In the long run, working with a great variety of solutions, we should expect 60 per cent to yield the same value. The discrepancy between this figure and Dr. Bronfenbrenner's estimate, 85 per cent, based on the actual yield of the method in practice is, in Dr. Bronfenbrenner's opinion, too great to be ignored.

It will be remembered that our analysis of the problem was based on the simple assumption that only one particle need be put into a tube in order to dissolve the bacteria in it. It has not been assumed that the particles are alike. The particles may be molecules—all alike—or they may consist of particles of foreign matter on the surfaces of which one or more of the ultimate units of bacteriophage have been adsorbed. We have required only that particles neither divide nor coalesce during the process of dilution (only the second of these processes would make $\bar{p}_n$ greater). It is, of course, conceivable that, in concentrated solution, a change of concentration might have some influence on such particles, but it is hard to imagine how any such change could take place during the process of serial dilution after a point has been reached where there are only from one to ten particles in 10 cc. of broth. Such changes in the first part of the series would have a profound effect on the accuracy of estimates made by the method, but none on the degree of consistency of the results.

It is conceivable that the interaction of a bacterium and a particle of bacteriophage is, in itself, a matter of probability. The particle may be inactive, or it may attach itself to a bacterium which is not susceptible. It is reasonable to assume that, of the whole number of bacteria added to each tube, a constant fraction are susceptible. We may say, then, that there is a certain constant probability, q, that any particular unit of bacteriophage will act effectively. This could have been taken into account very easily in deriving equations (1) and (2), thus—if, instead of considering the probabilities, a^{n-1} and a^n, that a particular unit of bacteriophage would be transferred to the nth and $(n+1)$th tubes respectively, we had considered the probabilities that the particular unit would act effectively in these two tubes, we should have found them to be $a^{n-1}q$ and a^nq respectively. q may now be replaced by some unknown positive power of a. It is evident, therefore, that the effect of introducing q is to increase the value of n. This means that the limiting values of the p's remain unchanged and that the values of the p's for small values of n, are, for the same value of n, more nearly in coincidence with the limiting values than they would be if q were not introduced; in other words, if n is greater than 2, the introduction of q is without appreciable effect.

We have next to consider adsorption losses. During the process of

stirring and transferring fluid, some of the particles must come in contact with the surfaces of the tube and the pipette and it may be that some or all of them adhere to the glass. Although this would not necessarily render the particles inactive, it would effectively prevent transferring them to the next tube. Such losses, if they exist, must be very small. Dr. Bronfenbrenner[1] has found that a very dilute solution (corresponding approximately to Tube $n - 2$) gives the same value of n whether it is titrated immediately after preparation or after having been kept in glass for 72 hours. This means that, during the 10 minutes required to make a transfer, only a very small fraction, certainly much less than 10 per cent, of the whole number of particles in the tube will be adsorbed. Since the fraction is so small, and since the transfers to successive tubes require about the same length of time, we may say that there is a definite probability, which is the same for all of the transfers, that any particular unit of bacteriophage, which has been transferred to any tube, will escape adsorption until the transfer of fluid to the next tube has been made. This probability may be combined with the dilution factor, a, to give a new and slightly smaller value of a. If 10 per cent of the particles were lost at each transfer, a would be reduced from .1 to .09. Table I shows that the corresponding increases in the values of the p's amount to only 2 or 3 per cent.

If, therefore, it is true that when one active particle of bacteriophage comes in contact with a susceptible bacterium, all of the bacteria in the tube dissolve, we are justified in expecting that, in the long run, about 60 per cent of parallel runs will yield the same value of n. This figure will remain unaltered whatever value we assign to the probability either that a particle is by nature inactive, or that it is taken up by a bacterium which is not susceptible; and it will change only slightly as a result of the greatest adsorption losses which we have reason to consider.

If experiment should show definitely that the serial dilution method yields results with a degree of consistency much greater than 60 per cent, the most obvious explanation of the discrepancy will be that one particle is not usually sufficient to cause the dissolution of all of the bacteria in the tube, even though it is active and comes in contact with a susceptible bacterium. This idea is not seriously in conflict

with the most important feature of the particulate hypothesis as usually understood, *i.e.* that one particle can start the process of dissolution. It is conceivable that a single infected bacterium may not be able to produce enough particles of bacteriophage to infect all of the others within the time during which the bacteria remain susceptible.

SUMMARY.

1. The theory of the serial dilution method of titration of bacteriophage has been worked out on the basis of the simple particulate hypothesis.

2. It has been shown that, if the dilution constant is .1, only about 60 per cent of parallel runs on the same solution should give the same end-point, the average being taken over a great number of titrations of each of a great variety of solutions.

3. The discrepancy between this figure, 60 per cent, and Dr. Bronfenbrenner's estimate, 85 per cent, is considerable.

4. Inasmuch as the particulate hypothesis is well founded, no explanation of the discrepancy is suggested.