EXPERIMENTAL AND STATISTICAL EVIDENCE OF THE PARTICULATE NATURE OF THE BACTERIOPHAGE*

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In spite of the fact that the bacteriophage is invisible under the microscope and passes readily through porous filters, most observers seem to be agreed that it is distributed throughout a medium as some kind of a particulate entity. Whether it is of an organized corpuscular nature as claimed by d'Herelle (1) or whether it is a chemical of a colloidal nature adsorbed upon the particles of broth constituents or upon the particles left after the disintegration of bacteria during lysis as claimed by Bronfenbrenner (2) and others has not as yet been settled.

If the bacteriophage is a particulate entity in the sense that a single unit is capable of initiating complete lysis, the absence of a single "particle" in a portion of liquid can be experimentally determined. Its similarity in behavior in this respect to a bacterium, known to be particulate, makes it possible to apply the same dilution method of determination now familiar in the sanitary testing for *B. coli* in drinking water. It was first shown by McCrady (3) that the probability of a single bacterium being absent in any given portion of a larger volume containing a given number of bacteria can be tested experimentally and expressed mathematically. Eminent mathematicians have abundantly confirmed and extended the McCrady concept, discovering finally that it is but a special case of Poisson's law of small chances. It states simply that the concentration of particles in a volume is equal to the negative natural logarithm of the proportion of volumes in which particles are absent. Wells and Wells (4, 5) have discussed the application of this law to the bacteriological system of geometric dilution

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series, and have offered a simplified index which is consistent with the theory and adapted to practical use. In the application of Poisson's law to the bacteriophage, the logic is reversed to secure a statistical approach to the question as to whether or not the bacteriophage satisfies the conditions required if it were particulate. This paper records the results of experiments made to test the hypothesis.

Bronfenbrenner (6) set up an experiment in which there were 20 tubes in each of two series. In one series the dilution was 1×10^{-10} and four tubes (20 per cent) failed to show lysis. In the other series the dilution was 1×10^{-11} and 19 tubes (95 per cent) failed to show lysis. The variation he accounted for on the law of chance that some of the tubes in the first series would receive more than their share of the particles while others would receive less, which in four tubes happened to be none. In the same way in the second series, which usually contains no bacteriophage, an occasional tube will receive a phage particle and show lysis.

Clark (7) discussed the theory of serial dilutions and stated that Bronfenbrenner's estimate, that 85 per cent of parallel titrations of the same bacteriophage would show lysis in the same tube of the series, did not check closely with what he calculated to be the most probable per cent of agreement that might be expected, which he claimed should be 60 per cent. He noted that Bronfenbrenner's estimates were more in the category of impressions and not computations from actual records. Likewise he did not feel justified in offering this lack of agreement as evidence against the particulate nature of the bacteriophage.

If the bacteriophage is actually particulate, observations must approximate statistical requirements more closely than would be indicated in Clark's paper. To determine whether or not the required conditions are fulfilled, experiments were set up in which the number of observations would be great enough to be statistically significant.

In Experiment I five dilutions (1×10^{-8} , 1×10^{-9} , 1×10^{-10} , 1×10^{-11} and 1×10^{-12}) of a bacteriophage active against the Hiss Y dysentery bacillus were made and each flask was seeded with the Hiss Y dysentery bacillus. Each dilution was then distributed in 5 cc. quantities into 160 sterile tubes and 18 hours later the number of tubes in which lysis failed to occur was recorded. All the tubes in the 1×10^{-8} series showed lysis. The per cent failing to show lysis in the other four dilutions are shown in Table I. The labor of distributing the dilutions in long series was greatly lessened by using special racks holding 10 tubes each which eliminated the necessity of handling cotton plugs. These racks were generously loaned to us by the Department of Sanitary Engineering of Harvard University.

In each of the three succeeding experiments the dilutions were made closer together and were chosen nearer and nearer the center of the curve, where observations tend to be more uniform than at the ends of the curve. The results of these experiments are likewise recorded in Table I.

TABLE I
Comparison of Theoretical and Observed Values

Dilution of bacteriophage	Tubes failing to show lysis							
	Experiment I (160 tubes in series)		Experiment II (160 tubes in series)		Experiment III (100 tubes in series)		Experiment IV (150 tubes in series)	
	Calculated*	Observed	Calculated*	Observed	Calculated*	Observed	Calculated*	Observed
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
10 $\times 10^{-10}$	0.0	1.8						
5 $\times 10^{-10}$			0.05	2.2				
3 $\times 10^{-10}$					1.8	2.0		
2 $\times 10^{-10}$					6.9	14.0		
1.58 $\times 10^{-10}$			17.4	11.4				
1 $\times 10^{-10}$	28.9	33.1			26.3	31.0		
0.8 $\times 10^{-10}$							31.7	31.9
0.7 $\times 10^{-10}$							36.8	37.8
0.6 $\times 10^{-10}$							42.2	41.8
0.5 $\times 10^{-10}$			57.5	58.7	51.3	47.0	48.6	47.9
0.4 $\times 10^{-10}$					58.6	54.0	56.3	58.6
0.3 $\times 10^{-10}$					67.1	64.0	65.3	65.3
0.2 $\times 10^{-10}$					76.9	75.0	75.0	76.0
0.158 $\times 10^{-10}$			83.9	85.0				
0.1 $\times 10^{-10}$	88.3	85.6			87.5	88.0	86.7	84.7
0.05 $\times 10^{-10}$			94.6	93.7				
0.01 $\times 10^{-10}$	98.7	98.1						

* Estimated on the assumption that dilution 1×10^{-10} contained the following number of phage particles per distributed volume: Experiment I, 1.244; Experiment II, 1.105; Experiment III, 1.337; Experiment IV, 1.439.

Various workers have discussed methods of determining the most probable number of particles in a suspension when the number of tubes of various dilutions which fail to receive a particle is known. Given the percentage of negative tests in various dilutions, the probable number of particles in each dilution can be selected from tables, such as those recently published by Halvorson and Ziegler (8).

The most probable numbers of phage particles were read from this table by looking up the values of e^{-x} , corresponding to the percentage of tubes negative for each dilution. This was done for each of the experiments. The values for the various dilutions in Experiment IV are recorded in Table II. The values were then reduced to a common base (1×10^{-10}) by dividing by the factor necessary to make the dilution equivalent to that of the base. It will be seen that the figures in this column fall quite closely together, the lowest being 1.300 and the

TABLE II
*Calculation of the Most Probable Number of Phage Particles in a Suspension
(Experiment IV)*

Dilution of bacteriophage	Tubes negative	Most probable number of phage particles per distributed volume*	Values in previous column reduced to common base (dilution 1×10^{-10})†
	<i>per cent</i>		
0.8×10^{-10}	31.9	1.150	1.440
0.7×10^{-10}	37.8	0.975	1.300
0.6×10^{-10}	41.8	0.870	1.430
0.5×10^{-10}	47.9	0.745	1.470
0.4×10^{-10}	58.6	0.535	1.340
0.3×10^{-10}	65.3	0.425	1.417
0.2×10^{-10}	76.0	0.275	1.375
0.1×10^{-10}	84.7	0.165	1.650
			8)11.512
			1.439

* Values taken from table published by Halvorson and Ziegler, mentioned in the text.

† Obtained by dividing 1.150 by 0.8, 0.975 by 0.7, etc.

highest 1.650 phage particles per distributed volume. If a mean of these values is calculated, it gives the most probable number of phage particles in dilution 1×10^{-10} when all the dilutions are given equal weight. This mean in Experiment IV is 1.439. (In the other experiments the values are: Experiment I, 1.244; Experiment II, 1.105; Experiment III, 1.337.

Using these means we can now go back to the table of Halvorson and Ziegler and read off the number of tubes which would be expected to be negative if various dilutions of the phage were set up against the homol-

ogous organism. A number of such values are recorded in Table I. In reading across this table, it will be seen that in most instances the observed values are fairly close to the calculated values. Such discrepancies as are noted are due to experimental errors and to chance variation. The variations due to chance could be still further reduced by increasing the number of tubes in each series to 500 or 1000,

GRAPH 1. This figure shows how closely the observed per cent (represented by circles) of tubes failing to show lysis in various dilutions of bacteriophage come to the theoretical curve (represented by the heavy line).

but the values obtained seem to be accurate enough for the present discussion.

In order to be able to see at a glance how close the agreement is, a curve represented by the heavy line was plotted in Graph 1 from the calculated values for Experiment IV. The actual observations were shown as circles with figures to indicate to which experiment they belonged. The circles follow the curve closely. For the sake of sim-

plicity the curves for the other three experiments were omitted and all of the observed values are compared to the one curve. The other three have the same shape but fall slightly higher than that of Experiment IV, which explains why so few of the observations of the first three experiments are below the line. Plotted against their own curve some observations would be above and some below the line.

GRAPH 2. This figure demonstrates clearly that by decreasing the influence of chance through increasing the number of tubes in a series the agreement between theoretical and observed values becomes closer and closer (graph made from figures of Experiment IV).

In order to show that it is necessary to make a large number of observations before conclusions can be drawn, Graph 2 was made. In this graph the theoretical curve and the actual observations in Experiment IV were plotted. Each of the large circles represents 150 tubes, as in Graph 1. By breaking up each series into three groups of 50 tubes, we can observe how much farther away from the curve the observa-

tions fall because of the decrease in the number in each series. The smaller circles represent observations of 50 tubes each. Breaking up each series still farther, we have 15 groups of 10 tubes each and the dispersion is still greater, as shown by the black dots. It is easy to see that if we had used only 10 to 20 tubes to a series there might have been some chance of the figures being so irregular as to cause doubt concerning whether there was any agreement between theoretical and observed values. Conversely, if we had used 1000 tubes in a series, the parallelism would have been more striking, because of the decrease in the influence of chance.

These observations point strongly to the particulate nature of the bacteriophage. It is difficult otherwise to explain the closeness of fit, over so wide a range, of observed values with values computed from laws characteristic of particles in suspension. The bacteriophage fulfills this important requirement of particulate entities in that it does not appear to exist in a medium except in association with something which is particulate. Speculation on how such an association would be possible without the bacteriophage being in itself particulate is not the purpose of this paper.

SUMMARY

Experimental evidence is brought forward to show that the bacteriophage obeys very closely the laws of chance distribution of particles in suspension.

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