

A METHOD OF STANDARDIZING BACTERIAL SUSPENSIONS.

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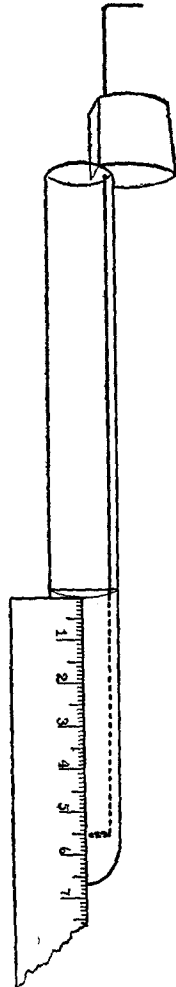
(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, October 23, 1919.)

When bacterial suspensions are used as vaccines or in serum reactions it is necessary or desirable to determine their concentration, at least approximately. Many methods have been advocated. The bacterial count per cc., the weight of the moist or dried bacterial substance, the capacity of a platinum loop, the opacity of various bacterial or chemical suspensions, the volume of the sedimented organisms, all have served as standards in various methods of determining concentration already described. The more accurate methods involve much time and labor; the simpler ones give only a rough approximation of bacterial content.

If a wire loop is gradually pushed down into a suspension of organisms in a test-tube (Text-fig. 1) and viewed by looking down into the tube through its mouth, the depth at which the loop disappears will be determined by the opacity of the supervening column of suspension. If two suspensions of the same organism are compared in this manner, a longer column of the thinner suspension will be required to effect the disappearance of the loop. The lengths of the columns may be measured and compared, and the measurements might be interpreted in terms of bacterial concentration if a suitable standard were determined. Tests with such an instrument show that with a little practice the length of the column of a bacterial suspension that will just hide the loop (the depth of disappearance) can be measured with considerable accuracy. For example, with suspensions of such an opacity that the loop disappears between 1 and 4 cm. below the surface, the zone of most accurate measurement, the depth of disappearance can be read within 1 mm. repeatedly, an error of less than 10 per cent. Within certain limits, neither the diameter of the test-tube nor the size of the wire loop affects

the readings appreciably. Even in the laborious method of counting the organisms the accepted error is often twice as great. If the relation of the depth of disappearance to the concentration could be



TEXT-FIG. 1. The simplest form of the apparatus used to measure the depth of disappearance of a wire loop in a suspension of bacteria.

determined, this would seem to be a quick and simple method of standardizing bacterial suspensions with a minimum of apparatus and manipulation.

It might be supposed that if a suspension containing 1,000 million bacteria per cc. caused the loop to disappear at 2 cm. below the meniscus and this suspension was diluted so as to contain 500 million bacteria per cc., the loop would disappear at 4 cm., or, in other words, that the opacity of a solution would vary directly with its concentration or inversely with the depth of disappearance of the wire loop. This is found not to be the case. In the second instance the loop will disappear at some distance less than 4 cm.; for example, at 2.8 or 3.3 or 3.6 cm. This discrepancy is due to the presence in each reading of a constant which must be eliminated by subtraction in order to bring the readings into ratio with the bacterial concentrations.

The constant appears to be a function of the size and opacity of the individual bacteria in the suspension. Two portions of a suspension of starch grains in cold water, one of which has been heated to boiling, give two parallel series of readings upon successive dilutions, but the constant with the heated specimen is larger than that with the unheated one, corresponding to an increase in size and translucency of the starch grains in the heated suspension. Subtraction of its own constant from each series brings the corresponding readings together and thus indicates that the heated and unheated specimens contain the same amount of starch, which is, of course, the case.

While this constant is the same for any series of readings on the same suspension, it varies with each suspension examined. The problem, therefore, is to eliminate the constant and so to bring opacity and concentration into accord. In practice this is easily done.

A series of readings taken on a suspension at successive dilutions and plotted in graphic form with the readings as ordinates and the corresponding volumes as abscissæ will be found to lie approximately in a straight line. In reality it lies along a flat curve that approaches a straight line as the suspension is further diluted. If the original concentration of the suspension is such that the loop is visible at a distance greater than 1 cm., the error introduced by assuming that successive readings fall along a straight line is not appreciable. For purposes of illustration, therefore, the straight line may be employed, as in Table I, *A*. The line is plotted in Text-fig. 2, *A—A*.

Table I, *A*, represents a series of readings on a bacterial suspension taken at dilutions obtained by adding one, two, three, and four volumes of the diluent to the original volume of the suspension. Inspection of the readings shows that they are not in the same ratios to each other as the corresponding volumes are. But if a constant quantity, in this instance 0.5, is subtracted from each reading, the remainders fall into direct ratios with the volumes, as is seen in Table I, *B*. Similar results are obtained from the graph. If the straight line is projected across the zero abscissa it crosses it at an ordinate distance of 0.5 cm. A line *B—B* parallel to *A—A* and passing through the zero point cuts each volume abscissa 0.5 cm. below the corresponding observed reading for that volume. The

TABLE I.

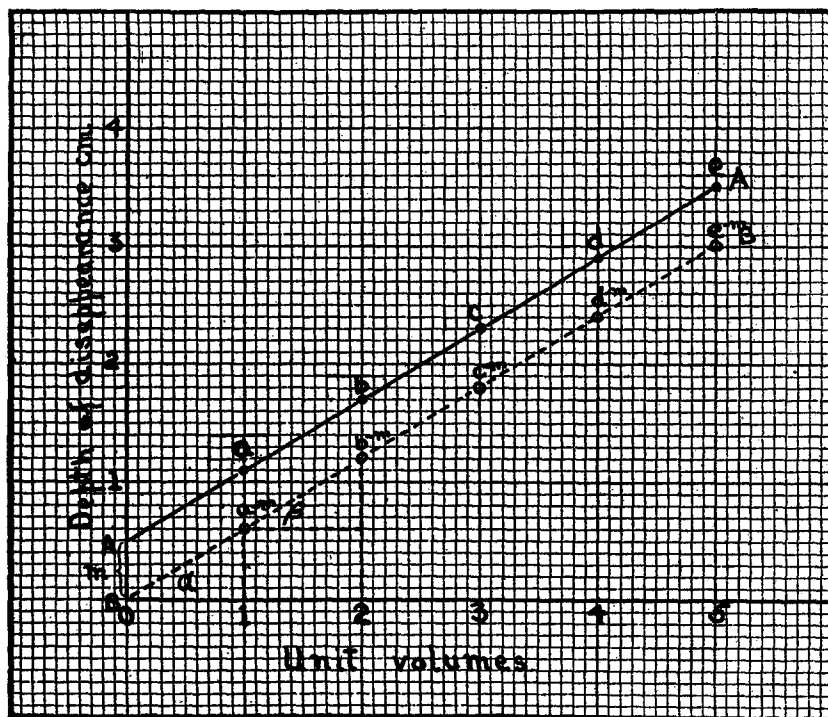
A		B	
Unit volumes.	Depth of disappearance.	Constant.	Corrected readings.
	<i>cm.</i>		
1	1.1	0.5	= { 0.6 1.2 1.8 2.4 3.0
2	1.7		
3	2.3		
4	2.9		
5	3.5		

corrected reading, divided by its volume, is in each case the tangent of the angle alpha, and the corrected readings are therefore in the same ratios to each other as their corresponding volumes.

It is not necessary to plot the readings or even to find the constant in order to subtract and so to eliminate it, even though it is found to differ with each suspension examined. Each of the observed readings on a single suspension is the sum of the corrected reading and the constant. Let the successive observed readings be *a*, *b*, *c*, *d*, etc., and the constant *m*. Then the corrected readings are *a* - *m*, *b* - *m*, *c* - *m*, etc. If any corrected reading is subtracted from a subsequent one the constant is cancelled from the equation,

$$(b - m) - (a - m) = b - a \quad (d - m) - (b - m) = d - b$$

and the remainder is the difference between the observed readings. Obviously the converse is also true and the difference between any two observed readings equals the difference between the corresponding corrected readings. It is convenient to choose such dilutions of the suspension that the difference between successive readings is in



TEXT-FIG. 2. The ordinates represent the depth of disappearance in cm. of a wire loop in a suspension of bacteria. The abscissæ represent the corresponding successive dilutions of the suspension. *A—A*, observed readings, *a*, *b*, *c*, *d*, *e*. *B—B*, corrected readings, from which the constant *m* has been eliminated by subtraction. The corrected readings stand in equal proportion to the corresponding volumes,

$$\frac{a - m}{\text{vol } a} = \frac{b - m}{\text{vol } b} = \frac{c - m}{\text{vol } c} = \frac{d - m}{\text{vol } d}, \text{ etc.}$$

and may therefore be used to determine the concentration of the bacteria per cc.

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each instance equal to the corrected reading for the original concentration of the suspension. This is most easily explained from the graph. Reference to Text-fig. 2 shows that since angle alpha equals angle beta, tan alpha equals tan beta, or

$$\frac{a - m}{\text{vol } a} = \frac{(b - m) - (a - m)}{\text{vol } b - \text{vol } a}$$

Then

$$a - m = \frac{\text{vol } a (b - a)}{\text{vol } b - \text{vol } a}$$

and if

$$\text{vol } b - \text{vol } a = \text{vol } a$$

then

$$a - m = b - a$$

When two observed readings are considered, the difference of whose volumes equals the original volume of the suspension, then the first, subtracted from the second, will give the corrected reading for the original volume of the suspension (Table II).

TABLE II.

Volumes.	Volume <i>a</i> .	Observed readings.	Corrected reading (<i>a</i> - <i>m</i>).
2-1 =	1	1.7-1.1 =	0.6
3-2 =	1	2.3-1.7 =	0.6
4-3 =	1	2.9-2.3 =	0.6
5-4 =	1	3.5-2.9 =	0.6

It is thus seen that the corrected reading for any suspension, by which its concentration may be compared with that of a standard suspension of the same organism, may be found by making a reading on the suspension, adding an equal amount of the diluent, making a second reading, and subtracting the first reading from the second. Any error in observation is considerably reduced, however, if the suspension is diluted several times instead of once before the second reading is made, and consideration of the equation

$$a - m = \frac{\text{vol } a (b - a)}{\text{vol } b - \text{vol } a}$$

shows that any dilution of the suspension may be used to obtain the corrected reading $a - m$. A concrete example will illustrate the point.

Suppose that in a given suspension whose volume is 2 cc. (*vol a* = 2) the loop disappears at a depth of 1.2 cm. ($a = 1.2$). The suspension is then diluted by adding, for example, 6 cc. of the diluent, so that the total volume is now 8 cc. (*vol b* = 8). Let the second reading be 3.6 cm. ($b = 3.6$). Then, substituting in the equation,

$$a - m = \frac{2(3.6 - 1.2)}{8 - 2}, \text{ or } a - m = 0.8$$

The corrected reading for the concentration of the given suspension is 0.8 cm. Now this corrected reading may be directly compared with corrected readings on other suspensions of the same organism. A suspension whose corrected reading is 1.6 cm. contains half as many organisms per cc.; one whose corrected reading is 2.4 cm. contains one-third as many. In this connection it should be remembered that the value obtained for $a - m$ applies to the original suspension before the dilution required in the test. If the diluted test specimen is to be used for any purpose its corrected reading may be obtained from the equation,

$$b - m = \frac{\text{vol } b (b - a)}{\text{vol } b - \text{vol } a}$$

If several suspensions of the same organism are simply to be compared with one another no further calculations are necessary. In many instances, however, a permanent standard is desirable and it is convenient to translate the opacity into terms of an accepted standard, such as the weight of dried bacterial substance or the number of organisms per cc. The bacterial count is the basis most widely employed, even though it is recognized that similar counts do not always represent equal amounts of bacterial substance, on account of variations in the size of the organisms under varying conditions of cultivation.

Given a corrected reading on the depth of disappearance of a wire loop in a suspension of a microorganism and the corresponding bacterial count, or better, a series of such correlated observations, to reduce the error in counting as well as in estimating opacity, the depth of disappearance of a standard suspension containing, for example, 1,000 million bacteria per cc. is readily found by inverse proportion.

$$\frac{\text{Opacity of standard suspension (unknown)}}{\text{Opacity of given suspension (in cm.)}} = \frac{\text{Count on given suspension (millions per cc.)}}{\text{Count on standard suspension (1,000 millions)}}$$

The opacity (depth of disappearance) of a standard suspension of 1,000 million bacteria of any species having been thus determined, the number of organisms per cc. in any suspension of such bacteria may be readily calculated from the same equation. A complete example follows.

Suppose that the readings quoted on page 111 were made on a suspension of normal type meningococci. It has been found that 1,000 million normal meningococci correspond to a depth of disappearance of 4.2 cm. In 2 cc. of the given suspension the loop disappears at 1.2 cm. When this suspension is diluted to 8 cc. the loop disappears at 3.6 cm. The corrected reading for this suspension is then

$$\frac{2(3.6 - 1.2)}{8 - 2}, \text{ or } 0.8 \text{ cm.}$$

which, substituted in the equation above, gives

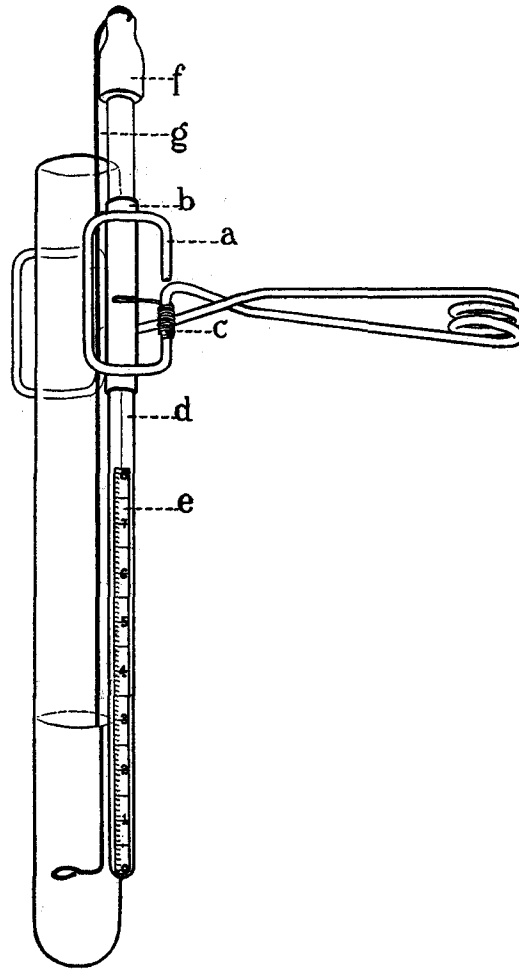
$$\frac{4.2}{0.8} = \frac{x}{1,000}$$

The suspension under examination contains 5,250 million meningococci per cc.

Several simple forms of apparatus have been used to measure the depth of disappearance. The first observations were made with a loop of No. 18 nichrome wire thrust through a cork, as in Text-fig. 1, and measured with a centimeter scale laid against the test-tube. A more convenient instrument, which has proved sufficiently accurate for ordinary use, is shown in Text-fig. 3.

To a wire test-tube clamp (*a*) is soldered a 4 cm. piece of 7 mm. (inside diameter) brass or copper tubing (*b*) slotted in the middle to receive the free end of a small coiled spring (*c*) which presses against an 18 cm. length of glass tubing (*d*), holding the tubing in place, but allowing it to be raised or lowered freely in the tube. A narrow paper centimeter scale (*e*), reading upward, is sealed within the glass tubing, which is surmounted by a stub of heavy walled rubber tube (*f*) to hold the end of the No. 18 gauge nichrome wire loop (*g*). Iron wire may be used, but it rusts and flakes off when repeatedly wet and heated. Nichrome or chromel wire retains its black color and is unaffected by repeated use. The free end of the wire is bent at right angles into a small circle, so that it lies horizontally in the center of the test-tube opposite the zero point on the centimeter scale when the instrument is held in the upright position.

In use a measured quantity of the specimen to be estimated is placed in a sterile test-tube, 1.6 by 16 cm., in the clamp. The wire loop, viewed by looking down into the mouth of the test-tube, is



TEXT-FIG. 3. A convenient modification of the instrument for measuring the opacity of bacterial suspensions.

lowered into the suspension and adjusted until it is just beyond the limit of vision through the fluid; *i.e.*, the opacity of the supervening column of suspension is just sufficient to hide the loop. This

end-point is more accurately observed than one with the loop faintly visible. The depth of disappearance is then read on the centimeter scale at the bottom of the meniscus, care being taken that the test-tube is held perpendicularly, with the meniscus at the level of the eye. A measured amount of the diluent is then added and mixed by agitation, and the second reading is made. The original volume of the suspension (*vol a*), the amount of diluent added (*vol b - vol a*), and the two observed readings (*a* and *b*) give the necessary data for obtaining the corrected reading (*a - m*) on the suspension. This corrected reading, by comparison with the standard for the given organism, figured by inverse proportion as already demonstrated, gives the concentration of the suspension in millions of organisms per cc. A separate sterile test-tube should be used for each suspension examined. The nichrome wire loop is dried and sterilized in a flame. The rubber cap (*f*) permits it to be held out at a right angle for this purpose.

The readings and the calculations, on a slide rule, can be made in 2 or 3 minutes when the standard for the given organism is known. Owing to differences in acuity of vision, a certain personal equation is involved in the reading of the end-point, and the standards should be worked out for each observer by comparison of corrected depth of disappearance readings and the corresponding bacterial count. Once the standards are established, suspensions of the same organism can be estimated rapidly. The method should be found useful in vaccine and serological laboratories in which many suspensions have to be standardized.

SUMMARY.

The opacity of a bacterial suspension is measured by the length of the column of the suspension required to cause the disappearance of a wire loop. By a simple formula the measured opacity is translated into terms of the concentration of bacteria per cc., and so made comparable with that of other suspensions of the same organism. An instrument for measuring the opacity of bacterial suspensions is described in detail.