THE PROPORTION OF VIABLE BACILLI IN AGAR CULTURES OF *B. AERTRYCKE* (MUTTON), WITH SPECIAL REFERENCE TO THE CHANGE IN SIZE OF THE ORGANISMS DURING GROWTH, AND IN THE OPACITY TO WHICH THEY GIVE RISE.

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(With one Chart.)

In studying the relation existing between the age and the virulence of cultures of *B. aertrycke* (mutton) (Wilson, 1926), it was necessary to work out a technique that would enable us to estimate the number of living and dead organisms in suspensions prepared from agar slope cultures.

Our first experiments were made by washing off the growth completely with sterile water, standardising an aliquot portion of it against an opacity tube containing 500 million *B. coli* per c.c., and counting the viable organisms by the roll-tube method (Wilson, 1922). Using a 5-hour agar slope culture, we obtained evidence that only about 15 per cent. of the bacilli were viable, and as we had reason to suspect the accuracy of this result, we determined to repeat the experiment, checking the opacity count by means of a direct count of the total number of organisms on the Helber slide (Wilson, 1922). To our surprise we found that the Helber count gave a total of only 160 million organisms per c.c., instead of the expected 500 million. Repeating the experiment on a culture 24 hours old, we obtained a figure of 510 million per c.c., and on a culture 1 week old of 679 million. The complete results are given in Table I.

Table I.

Age of culture	Opacity count	Helber count	Viable count
5 hours	500 million	160 million	63 million
24 ,,	500 ,,	510 ,,	251 ,,
168 ,,	500 ,,	679 ,,	26 ,,

The opacity standard with which we worked was a phenolised suspension of *B. coli* prepared from a 24-hour agar slope culture which had been counted on the Helber slide. From Table I it is seen that it is only the 24-hour culture which agrees in numbers with those in the opacity tube. In the 5-hour culture the total number of organisms is only about one-third of the expected; in the one week culture the number is considerably higher than the expected.

Before coming to any conclusions regarding the cause of this discrepancy, we decided to make a series of careful estimations of the number of total and

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viable organisms in suspensions prepared from agar slope cultures at different stages of growth corresponding to our opacity standard.

Technique.

A 5 c.c. tube of broth that had been seeded from a stock agar stab culture of *B. aertrycke* (mutton) was incubated for 14 to 18 hours at 20–22° C. From this six or more agar slopes of the same batch of medium in tubes of the same diameter were inoculated, one loopful of culture being spread evenly over each. These were incubated at 37° C. After times varying from 4 hours to a fortnight a tube was withdrawn, the growth washed off with sterile tap water, and a portion of the suspension matched with an opacity tube of *B. coli* standardised to 500 million per c.c. The suspensions were contained in tubes of hard glass of an inside diameter of 15 mm. and the comparison was made by a concealed light in a dark chamber, the tubes being placed against small print.

As soon as the standardisation was complete, a count of living organisms was made on the suspension by the roll-tube method. For a full description of this method see Wilson (1922). Briefly it consists in diluting the suspension to be counted in sterile water by means of accurately calibrated dropping pipettes, delivering a varying number of drops of the final dilution into test-tubes containing 2 c.c. of melted agar at 45–50° C., and rolling these rapidly in an almost horizontal position, so that the agar sets in a thin layer extending half way up the tube. On incubation the bacilli develop into colonies scattered evenly throughout the medium; after 3 days at 37° C. these are counted with a hand lens against a strongly illuminated dark background. In all experiments four tubes have been put up for each count, and the mean number of colonies taken. The results are multiplied by a factor to compensate for the degree of overcrowding.

At the same time as the count on dilutions of viable organisms was made, a suitable dilution in 1 per cent. phenolised saline was made by dropping pipettes for the total count. This was performed in a counting chamber modified from Helber's original by Topley and Barnard. The chamber consists of a glass slide from the upper surface of which a circular ring has been removed. Within this ring is a circle of glass whose surface is 0·02 mm. below the level of the rest of the slide. A portion in the centre is ruled into a large square, which is itself subdivided into 400 small squares. The area of each of these small squares is $\frac{1}{400}$th square millimetre, and the cubic capacity $\frac{1}{20000}$th cubic millimetre. A small drop of the suspension to be counted is placed on the circle, and an optically plane coverslip is lowered over it, and adjusted until coloured rings are seen spreading in every direction. The important modification of the chamber introduced by Topley and Barnard was in limiting the distance from the upper surface of the central circle to the lower surface of the slide to between 0·9 and 1·1 mm. This is essential for use with the dark background. When the coverslip is suitably adjusted, the slide is placed on the stage of a microscope which is fitted with a dark-ground con-

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denser, and illuminated by a strong light—preferably a pointolite or a mercury vapour lamp—focussed by a bull's eye condenser and examined with a $\frac{2}{3}$ objective and a low eye-piece. Once the illumination is satisfactory, the low ocular is replaced by an 18 compensating one, and the bacilli counted. If examined before they have time to settle, they are in active Brownian motion. With a little experience they can be counted with considerable accuracy. From a large number of experiments we have found the error to be not more than 5 per cent. The advantages of this method are that no staining fluids are required; that the apparatus is simple and such as is to be found in every well-equipped laboratory; that little time is consumed; and chiefly that, provided the chamber is properly calibrated—this is absolutely essential and must never be neglected—the method is the most accurate one that we possess.

In the work described in this paper we have made a practice of putting up two preparations and counting 60 small squares on each. In our hands this has given more consistent results than counting a greater number of squares on one preparation. The time required for the two counts was about 20–25 minutes.

A second culture tube was removed after 7 hours' incubation and treated in the same way; similarly with others later.

We had hoped to be able to perform the whole series of counts on one and the same agar culture, but this proved impossible, owing to the small amount of growth during the early stages of incubation. It is not to be expected therefore that the counts will show the same accuracy as those made on a broth culture, but, as will be seen from the curve given later, the agreement has been sufficiently close to enable us to obtain a definite answer to the question we had in mind.

Total and "Viable Counts[1]" of Agar Cultures.

In all we have carried out eight separate experiments, in each of which total and viable counts were made at intervals in the way already described. The results of these are set out in Tables II to IX. The third column labelled "Total count" refers to the total number of organisms alive and dead as estimated by the Helber chamber.

Table II. *Growth Exp.* 1.

Hours	Opacity count	Total count	Viable count	V./T. × 100 %
4	500×10^6	139×10^6	68×10^6	48·9
6	,,	219 × ,,	122 × ,,	55·7
9	,,	549 × ,,	482 × ,,	88·8
25	,,	754 × ,,	425 × ,,	56·4
31	,,	784 × ,,	400 × ,,	51·0
50	,,	813 × ,,	305 × ,,	37·5
74	,,	837 × ,,	97 × ,,	11·6
147	,,	*1066 × ,,	64 × ,,	6·0
315	,,	823 × ,,	14 × ,,	1·7

* Probably an error in dilution.

[1] Term used for brevity's sake as denoting enumerations of living organisms in a suspension.

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Table III. *Growth Exp.* 2.

Hours	Opacity count	Total count	Viable count	V./T. × 100 %
17	500×10^6	565×10^6	380×10^6	67·2
19·5	,,	574 × ,,	324 × ,,	56·4
24	,,	598 × ,,	285 × ,,	47·6
84	,,	844 × ,,	68 × ,,	8·1
186	,,	810 × ,,	19 × ,,	2·3

Table IV. *Growth Exp.* 3.

Hours	Opacity count	Total count	Viable count	V./T. × 100 %
11	500×10^6	502×10^6	393×10^6	78·3
15	,,	605 × ,,	396 × ,,	65·5
18	,,	625 × ,,	356 × ,,	56·9
36	,,	695 × ,,	233 × ,,	33·5
186	,,	841 × ,,	21 × ,,	2·5
371	,,	855 × ,,	3·8 × ,,	0·44

Table V. *Growth Exp.* 4.

Hours	Opacity count	Total count	Viable count	V./T. × 100 %
4	500×10^6	172×10^6	100×10^6	58·2
7	,,	447 × ,,	309 × ,,	69·1
9	,,	432 × ,,	410 × ,,	94·9
26	,,	688 × ,,	279 × ,,	40·6
170	,,	1092 × ,,	19 × ,,	1·7

Table VI. *Growth Exp.* 5.

Hours	Opacity count	Total count	Viable count	V./T. × 100 %
4	500×10^6	145×10^6	98×10^6	67·6
7	,,	423 × ,,	—	—
9	,,	552 × ,,	418 × ,,	75·7
27	,,	735 × ,,	279 × ,,	38·0
195	,,	951 × ,,	17 × ,,	1·8

Table VII. *Growth Exp.* 6.

Hours	Opacity count	Total count	Viable count	V./T. × 100 %
9·25	500×10^6	481×10^6	316×10^6	65·7
10·5	,,	573 × ,,	447 × ,,	78·0
12	,,	615 × ,,	532 × ,,	86·5
15	,,	644 × ,,	464 × ,,	72·0

Table VIII. *Growth Exp.* 7.

Hours	Opacity count	Total count	Viable count	V./T. × 100 %
4	500×10^6	137×10^6	56×10^6	40·9
7	,,	423 × ,,	322 × ,,	76·1
9	,,	539 × ,,	441 × ,,	81·8
26	,,	722 × ,,	372 × ,,	51·5

Table IX. *Growth Exp.* 8.

Hours	Opacity count	Total count	Viable count	V./T. × 100 %
4	500×10^6	145×10^6	81×10^6	55·9
7	,,	514 × ,,	309 × ,,	60·1
9	,,	560 × ,,	481 × ,,	85·9
26	,,	772 × ,,	395 × ,,	51·2

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In Chart I the figures obtained in all the experiments have been plotted and a free-hand curve drawn through them representing the total and viable counts. Along the ordinates are plotted the logarithms of the numbers of bacilli, and along the abscissae the time in hours elapsing since the inoculation of the culture. Considering that separate culture tubes were used for each

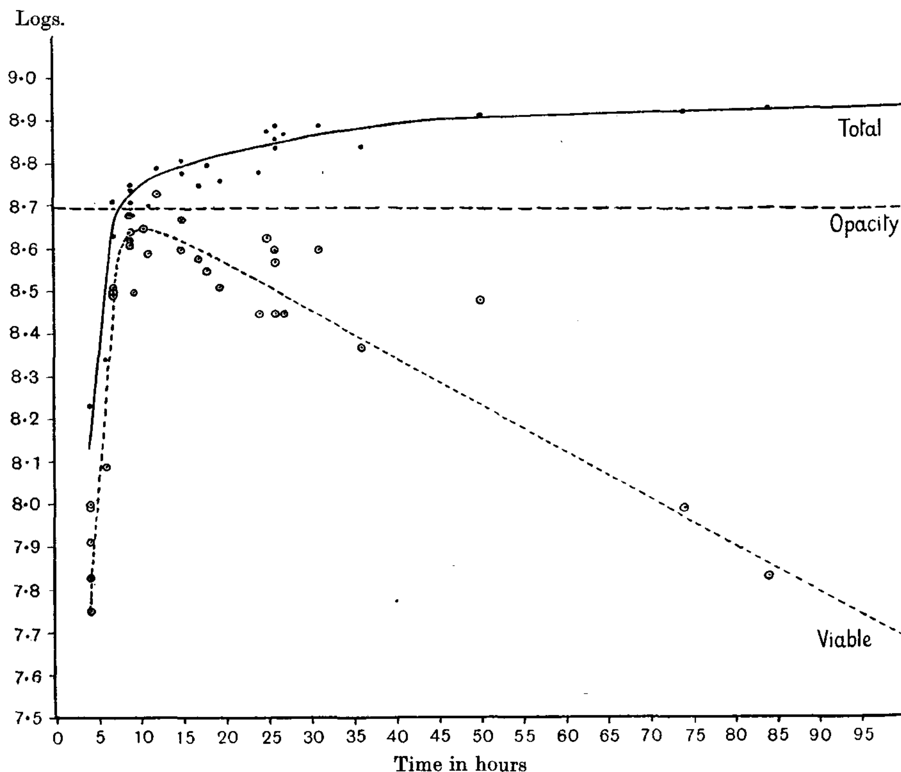

Chart I. Curves showing the relationship of Viable to Total Bacilli in Growth Experiments 1–8.

count, and that the eight experiments were made at different times—some on different batches of agar—it is surprising that the figures obtained are in such close agreement. This gives us confidence in asserting that the relation between the viable and total counts, expressed in the curves, may be accepted as approximately correct.

The Fallacies of the Opacity Method of Counting.

The figures given in Tables II to IX show conclusively that the opacity caused by a given number of bacilli depends upon the age of the culture from which they are taken. This will be more clearly recognised when the data collected in the eight growth experiments are summarised (Table X).

At 4 hours 148 million bacilli give rise to the same opacity as 526 million bacilli at 9 hours, 705 million at 25 hours, and 914 million at 178 hours.

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Table X.

Age of culture	No. of estimations	Opacity count	Helber count
4 hours	5	500 million	148 million
7 „	4	500 „	452 „
9 „	5	500 „	526 „
24–26 „	5	500 „	705 „
170–186 „	3	500 „	914 „

Unless, therefore, the age of the culture is taken into account, the opacity method is not a reliable index of the total number of organisms present.

To what can this be ascribed? It would seem that it is dependent either on a difference in the translucency or in the size of the organisms.

Brown (1914) drew attention to the variation in opacity between young and old cultures; he found that a given weight of dried organisms from a 48-hour growth on agar caused greater opacity than the same weight of organisms from a 6-hour growth. This observation is clearly open to more than one interpretation.

We have not repeated Brown's experiments, but have approached the problem in a different way. In one experiment, besides making the usual counts, we measured 20 consecutive organisms from a 4- and from a 26-hour culture so as to ascertain their relative size. The results are shown in Table XI.

Table XI.

Age of culture in hours	Opacity count	Helber count	Average size of organisms
4	500 million	137 million	$2{\cdot}35 \times 0{\cdot}79\,\mu$
26	500 „	722 „	$1{\cdot}13 \times 0{\cdot}49\,\mu$

These measurements show that the organisms from the 4-hour culture are about twice the size of those from the 26-hour culture. With regard to the relationship existing between the size of the organism and the opacity it produces, Mr Barnard has very kindly given us his opinion as follows: he says that the greater part of the light entering a bacterial suspension passes through the organisms and is refracted; some of the rays are reflected from the surface, and a very small proportion is diffracted. He states, however, that our present knowledge is insufficient to permit of an answer to the main question.

For the sake of interest we have calculated the volume of a single bacillus and found that the cubic capacity of an organism from a 4-hour culture is 1·152 cu. μ and from a 26-hour culture 0·213 cu. μ. That is to say, the volume of the bacillus from the young culture is 5·4 times that from the older one. This compares closely with the quotient obtained by dividing the number of organisms in the 26-hour by that in the 4-hour culture, or $\frac{722}{137} = 5{\cdot}3$. In another experiment we found the average size of an organism from a 4-hour culture to be $2{\cdot}39 \times 0{\cdot}805\,\mu$, and from a 26-hour culture to be $1{\cdot}075 \times 0{\cdot}495\,\mu$. The volume of the former is 1·216 cu. μ, of the latter 0·2068 cu. μ. Dividing as before, we have a quotient $\dfrac{1{\cdot}216}{0{\cdot}2068} = 5{\cdot}88$, as compared with a quotient of $\frac{772}{145} = 5{\cdot}3$ for the numbers of organisms present. Whether this agreement is

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merely fortuitous or whether there is a direct relationship between the volume of the organism and the opacity it produces must be regarded for the present as uncertain. But it does show that the variation in opacity with age is due to a corresponding variation in size of the organisms. And it suggests further that the opacity method of standardisation is a gauge not of the numbers of bacteria present, but of the total volume of bacterial protoplasm.

It is clear that the current use of a standard opacity tube for estimating the numbers of bacteria in any given suspension is open to serious error unless both suspensions are made from cultures of the same age grown under the same conditions. Even then the results will be only approximate, for growth is rarely the same in different experiments. Thus in five determinations of 4-hour cultures put up under conditions resembling each other more closely than is usual in practice the numbers varied from 139 to 172 million per c.c., and in similar determinations of 25–27 hour cultures they varied from 688 to 772 million per c.c.

These experiments were all carried out with one strain of *B. aertrycke* (mutton). But in practice not only do the strains compared differ, but frequently other species are compared with the standard tube, and their numbers estimated by a reduction factor (Brown and Kirwan, 1915, and Cunningham and Timothy, 1924). And even though the reduction factor is calculated from a culture of the same age as that used for the standard, there must be ample opportunity for error to creep in.

We maintain, therefore, that the opacity method of counting bacteria cannot be expected to yield more than an approximate result. Provided cultures of the same age are compared, and provided that the comparison is made as accurately as possible, the method allows of a rough estimate of the number of organisms present. For vaccine work, where an error of 20–30 per cent. is immaterial, there is no objection to its use, but for accurate experimental work it is unsuitable.

Incidentally, if it is true that the opacity method measures the total volume of bacterial protoplasm rather than the number of organisms, it should find its main use in the preparation of vaccines, for here we are concerned not with the action of the organisms as entities, as we are in virulence work, but with the antigenic effect of the bacterial substance.

The Relation of the Viable to the Total Number of Organisms.

An examination of Tables II to IX shows that the number of viable organisms in an agar culture never equals that of the total number of organisms. At 4 hours the proportion of viable to total bacilli is 54 per cent., at 6–7 hours 65 per cent., at 9 hours 82 per cent., at 24–27 hours 47 per cent., and at 147–186 hours 2 per cent. That is to say, in the early stages of growth only about half the bacilli are viable, or, more strictly, capable of reproduction when transferred to a fresh medium. During the period of 4–9 hours, when the organisms are presumably growing at maximum rate, the proportion rises

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till over 80 per cent. are viable. Then a decline sets in, the number of viable organisms decreasing, till after a week or so only about 2 per cent. are alive. This relationship is seen more clearly in Chart I.

The most striking point about these curves is the close resemblance they bear to those representing the growth of *B. aertrycke* (mutton) in broth cultures (Wilson, 1922). Not only is the relationship of viable to total organisms similar in each case, but their general configuration is so alike as to lead one to imagine that the curves of the agar cultures represent not simply the number of organisms corresponding to a given opacity at different ages, but the rate of growth of the bacilli. We have, in fact, been much troubled by the interpretation that should be put upon them. It is difficult to believe that the resemblance is entirely fortuitous. On the other hand, as the technique we employed in counting our agar cultures was not designed to give a quantitative estimation of the rate of growth, it is puzzling to understand why the resultant curves should so closely resemble those which we should have expected to obtain had it been so. The solution that we are inclined to accept is that since the opacity method is apparently a measure of the total volume of bacterial protoplasm in the suspension, what we have actually been doing is to estimate the number of organisms in similar volumes of culture. And as the number of organisms in a given volume is necessarily determined by their size, it follows that our curves form a graphical representation of the change in size that occurs during growth. That this is the correct solution is rendered probable by reference to the results obtained by Clark and Ruehl (1919). These workers, using a micrometer, measured the size of 70 strains of organisms belonging to 37 species at different times during their growth. With the exception of members of the diphtheria group, they found that the young organisms in cultures from 4–9 hours old were much larger than those examined after 24 hours. And though no experiments correlating the size of the organisms with their rate of growth are recorded they consider that the largest forms are produced during the most rapid period of cell division.

The reason, therefore, why our curves are similar to those recording the multiplication of bacteria in a broth culture is probably explained by there being a correlation between the size of the organism and the rate of growth. Hence curves representing either of these variables must necessarily be of similar configuration.

DISCUSSION.

It is difficult to understand why during the logarithmic phase of growth all the bacilli are not viable. Both in agar and in broth cultures the proportion of viable to total bacilli reaches a figure of about 80 per cent. at the end of this phase. It seems only natural to expect that as growth is occurring at a maximum rate all the progeny of a given generation should be capable of reproduction. Under the conditions of our experiments this however is not true. In a previous paper (Wilson, 1922) we suggested that there was a mor-

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tality amongst the bacilli in the early stages of growth, carrying off the weaker members of each generation. Since then we have found evidence in the literature to support this view. Schultz and Ritz (1910), for example, showed that in young 3–6 hour cultures of *B. coli* most of the organisms were thermolabile, being destroyed by heat for 25 minutes at 53° C., whereas in older 8–24 hour cultures they were nearly all thermostable. Sherman and Albus (1923) likewise found that in young cultures of *B. coli* the bacilli were more susceptible to the action of inimical agents, such as heat, cold and disinfectants, than were the bacilli from older cultures.

That the greater susceptibility of organisms in the early stages of growth is not confined to bacteria is amply demonstrated by Child (1915), who, working with different species of infusoria, flat worms and marine algae, found that the young actively growing cells were more rapidly killed by cyanide than the older slow-growing cells.

These observations assist us in understanding why the number of viable bacilli in a young culture fails to correspond completely with the total number of bacilli. The organisms that are generated during the logarithmic phase of growth are highly susceptible to inimical agencies. When transferred to a fresh medium, only about 80 per cent. are found to be alive. We can assume therefore either that some of them perish in the original culture, or that during the process of dilution some of them are injured sufficiently to prevent their reproduction. Which of these explanations is true we have at present no means of telling.

The objection may be raised that so far from bacilli in young cultures being more susceptible than those in older ones, they are actually more resistant. It will be remembered that Miss Chick (1908) in her original investigation into the laws of disinfection concluded that the bacilli in young cultures of *B. paratyphosus* were more resistant to phenol than those in older ones. A careful examination of her protocols shows that the evidence for this conclusion is unsatisfactory. No comparison was made between the bacilli at different stages of growth in the same culture; instead, a culture that had been repeatedly passed through broth at 3-hour intervals was compared with one 24 hours old. Knowing, as we do, the variations in resistance that may occur in different cultures of the same organisms, we consider that her experiments were not strictly comparable. So far as we are aware, Miss Chick is the sole author who has claimed that it is the organisms from young cultures that possess the greater resistance; the evidence to be gained from the literature all points in the reverse direction.

Summary and Conclusions.

1. Working with agar cultures of a strain of *B. aertrycke* (mutton) it was found that the number of organisms requisite to cause a given opacity was five times as great in a 26-hour as in a 4-hour culture.

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2. This difference is probably explicable by the fact that bacilli from young cultures are larger than those from old ones.

3. Evidence is produced which suggests that the opacity method gives a measure not of the number of organisms, but of the total quantity of bacterial protoplasm in the suspension. It should therefore be of special value in the standardisation of vaccines.

4. It is pointed out that the opacity method when used for the enumeration of bacteria is subject to a considerable error, and is hence unsuitable for accurate work.

5. A technique is described for the estimation of the proportion of viable to total organisms in an agar culture.

6. The results obtained are closely similar to those already described for broth cultures.

7. The maximum proportion of living bacilli is reached at the end of the logarithmic phase of growth, when about 80 per cent. of the organisms are alive and capable of reproduction.

8. It seems probable that there is a mortality amongst the bacilli that are generated in the early stages of growth, the least resistant organisms failing to divide.

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