ON A MODIFICATION OF WRIGHT'S METHOD FOR COUNTING THE BACTERIA CONTAINED IN A CULTURE.

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In all work in connection with bacterial vaccines the necessity for an accurate method of standardising the cultures used is paramount. In a recent investigation, in which the writer took a part, the inadequacy of the methods which are at present in vogue was brought forcibly to mind and he was, in consequence, induced to attempt to devise a method which would give more uniform and accurate results than the present processes do.

Wright has described a method which depends on the proportion existing between blood-cells and bacteria in a mixture of definite volumes of blood and culture; he aims at obtaining by this means a precise numerical expression of the bacteria, both living and dead, present in a volume of the culture. Unfortunately, in actual practice, the process gives very variable results, and, even in the hands of its author, it gives figures with a variation of as much as 22 per cent. above and below the average of two counts. These unsatisfactory results are due, I believe, in part to the difficulty of thoroughly mixing the blood and culture; to the influence of the blood fluids in producing agglutination of the bacteria, and possibly bacteriolysis during the process of mixing; to uneven distribution of the blood-cells and bacteria during the spreading of the film; and lastly, to the fact that, during the process of fixing and staining, a proportion of the bacteria are washed away while the more bulky red cells remain. Very probably the last two factors are the chief ones which conduce to fallacy, and especially the last; it happens not infrequently that one gets a lower reading by this method than one gets from enumeration of the colonies on an agar culture, and in the results quoted below, it will be seen that three counts made by Wright's method gave much lower results than those given by the method about to be described.

It seemed, however, that it was possible to overcome these causes of error, and the process described below is the result of attempts in this direction. Briefly, the method consists in:

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(1) Preparing a suspension of the cells of a definite volume of blood in a similar fluid to that in which the bacteria to be counted are suspended; (2) mixing the suspension of blood-cells with a convenient number of volumes of the culture, the bacteria of which have been previously stained in bulk; (3) placing a sample of the mixture under a cover-glass and luting the specimen with vaseline; (4) counting the red cells and bacteria in the specimen, field by field, and calculating the bacterial content of the culture from the figures thus obtained. By these means one avoids all fallacies due to the action of the blood fluids, which have been removed during the process of preparing the suspension of blood-cells; one gets an even mixture, the red cells and bacteria are evenly distributed in the specimen, and are not further interfered with, so that there is no risk of losing bacteria during processes of fixing and staining.

The details of the technique employed are as follows, the whole of the processes being done in duplicate:

The Preparation of the Suspension of Blood Cells.—The red cells of the blood to be used having been counted in the usual way, a stout capillary pipette, such as is used in sedimentation tests, is taken and bent, at a point about half an inch below the commencement of the capillary portion, into the form of a long narrow U, as shown in the accompanying rough sketch; the terminal quarter of an inch of the capillary portion of the pipette is bent over to one side so as to form an angle of 120° or thereabouts with the
remainder of the tube; a mark is made on the tube at the point (c). The tube is filled by dipping the point (b) into the drop of blood resulting from a prick in the observer's finger, the blood is allowed to run along the pipette to the mark (c), the end of the pipette is rapidly wiped and then inserted into a 0.75 per cent. solution of citrate of soda in normal saline solution, the fluid is allowed to run along the pipette, washing the blood before it, until the chamber (a) of the pipette is about half filled; the apparatus is then placed in a centrifuge and the blood-cells driven down into the bend of the U, the supernatent clear fluid is removed and the pipette refilled in the same manner as before, this time with broth or normal saline solution, according to the fluid in which the bacteria to be estimated are suspended; the mixture is again centrifuged and the supernatent fluid again removed; the operation is repeated; thus making a total of three washings of the blood-cells, and leaving them suspended in a fluid of the same kind as the fluid in which the bacteria is suspended. After the last washing the bulk of the fluid is brought to the same volume as the original quantity of blood taken as indicated by the mark on the pipette, by removal of the supernatent clear fluid. No blood-cells are lost in the process of washing and the pipette retains its original form and size, and remains available for measuring the volumes of culture which are to be added to the blood-cell suspension at a later stage of the proceedings. There are one or two minor details in the manipulation of the little tube which it may be useful to mention. When one removes the supernatent fluid from the chamber of the pipette the fluid will be found to recede from the capillary end and the resulting cushion of air effectually prevents the refilling of the pipette; the difficulty is easily overcome by pressure with the tip of a finger over the end of the chamber. Another point is that the larger end of the tube should be placed towards the outer side of the cup of the centrifuge, otherwise, when the centrifuge is slowing down and the cups are approaching the vertical position, the heavy end tends to bend towards the periphery of the circle described by the centrifuge and the tube snaps; the point is a trifling one, but well worth attention if one wishes to save one's self annoyance.

The Staining of the Bacterial Culture.—The culture having been thoroughly mixed by vigorous shaking, nine volumes of it are mixed with one volume of a 1 per cent. solution of methylene blue (in the case of a thick emulsion it is better to dilute it to 1 in 10 and then treat it in the same way as a broth culture). The culture and dye are thoroughly mixed, sealed off in a pipette and placed in a water-
bath at 45° C., the temperature of the bath is raised to 60° C., and kept at that level for fifteen minutes; the temperature must be kept constant and must not be exceeded, else there is a tendency for the dye to form large blocks of deposit which spoil the specimen.

The Preparation of the Specimens for Counting.—A number of clean watch-glasses, slides and cover-glasses having been laid ready, the stained culture is blown out into a watch-glass and thoroughly mixed, a preliminary inspection of the culture is made under a one-twelfth objective in order to get a general idea of the number of bacteria present and of the number of volumes of the culture which it will be convenient to mix with the suspension of blood-cells. The blood-cell suspension is then blown out into a clean watch-glass, the cell remaining in the pipette being washed out into the watch-glass also, by means of one or two volumes of broth, then a convenient number of volumes of the stained culture are taken up in the same pipette and mixed with the blood-cells, each volume of the culture being of the same volume as the original quantity of blood taken, as shown by the mark on the pipette; as a rule it will be found that ten volumes of culture, or of diluted agar emulsion, to one volume of blood-cell suspension will form the best mixture, both for counting and for the subsequent calculation. The blood-cells and bacteria must be very thoroughly mixed by drawing in and out of a pipette several times, as a matter of fact, three or four minutes is not too long a time to spend over the process. The mixture having been made, a small drop from the pipette is placed on a slide and immediately covered with a cover-glass, the size of the drop being that which will just suffice to cover the whole space under the cover-glass without putting any pressure on it. The specimen is immediately luted with vaseline and is then ready for counting; it is as well to make two or three such specimens in order to have a selection from which to take the best spread one to count. In the whole of this process rapidity of working is essential, otherwise the blood-cells will settle in the mixture and the count be spoiled, hence the necessity of having everything ready before beginning the mixing operation.

The Counting of the Specimen and Subsequent Calculation.—The preparation is examined under a one-twelfth objective, the diaphragm of the microscope being half closed, and it is a convenience to restrict the field by means of a counting disc, a cover-glass marked with a square of about a quarter of an inch, which is dropped into the eyepiece. Having satisfied himself that the specimen is properly made, one proceeds to count it, taking field by field, and noting in each the
number of red cells and of bacteria present; the latter will be found to lie in different planes and, especially if the specimen is a little thick, some of them will be seen to show Brownian movement, but by focussing and by taking advantage of the landmarks made by the distribution of the red cells, it will be found that the bacteria can be counted quite readily. About fifty fields should be counted, and they should be taken *seriatim* without any conscious or unconscious selection. The counting having been completed, the total numbers of red cells and of bacteria seen are added up and from the figures obtained the calculation of the bacterial content of the culture is made as follows:—

<table>
<thead>
<tr>
<th>Number of red cells counted</th>
<th>Number of red cells in 1 cc. of blood used</th>
<th>Number of bacteria counted</th>
<th>Number of volumes of culture in mixture</th>
<th>Number of bacteria in 1 cc. of culture</th>
</tr>
</thead>
</table>

The nine-tenths in the last figure of the proportion sum is, of course, due to the fact that the culture was diluted with one-tenth volume of dye before staining and a correction is necessary for this.

**Results.**—The following are the results of eight consecutive counts made by this method. The term "error" is used to denote the extent to which either of the figures obtained exceeds, or is less than, the average of the two counts.

1. A broth culture of *B. typhosus*.
   
   1st count = 320 millions per cc.  
   2nd count = 283·4 millions per cc.  
   Error = ± 6 per cent.

2. A broth culture of *B. typhosus*.
   
   1st count = 188·3 millions per cc.  
   2nd count = 237 millions per cc.  
   Error = ± 11·4 per cent.

3. A broth culture of *B. typhosus*.
   
   1st count = 246·3 millions per cc.  
   2nd count = 238·09 millions per cc.  
   Error = ± 1·09 per cent.

4. A broth culture of *B. typhosus*. Two samples were tested, one of undiluted culture and the other of culture diluted with one-third broth.

   Undiluted culture = 471·6 millions per cc.  
   \(\frac{1}{3}\) culture = 314·32 millions per cc.  
   (== 471·49 millions per cc. in the original.)  
   Error = negligible.

A living count made on agar plates gave 112 million bacteria per cc., the culture being four days' old.

A count of the same culture made by Wright's method gave:—

Undiluted culture = 243·2 millions per cc.  
\(\frac{1}{3}\) culture = 223 millions per cc.
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(5) A fairly thick emulsion in normal saline solution of a twenty-four hour agar growth of B. typhosus. In one count the emulsion was counted undiluted, in the second the emulsion had been diluted with 1 in 5 of normal saline solution.

Undiluted emulsion = 2,744 millions per cc. 1/5 emulsion = 1,994.8 millions per cc.
(= 2,493.5 millions per cc. in the original.)
Error = ± 4.7 per cent.

A count of the same emulsion made on agar plates gave 2,066 millions per cc.

(6) A twenty-four hour broth culture of B. typhosus. The control used was the culture diluted with 1 in 5 of broth.

Undiluted culture = 183.9 millions per cc. 1/5 culture = 166.6 millions per cc.
(= 208.25 millions per cc. in the original.)
Error = ± 6.2 per cent.

A count of the same culture made on agar plates gave 126 millions per cc. A count made by Wright's original method gave 63.4 millions per cc.

(7) An emulsion of a three days' agar culture of B. typhosus. One sample was diluted to 1 in 9 before staining.

1 in 10 diluted emulsion = 203.98 millions per cc.
(= 2,039.8 millions per cc. in the original.)
1 in 9 diluted emulsion = 218.52 millions per cc.
(= 1,966.68 millions per cc. in the original.)
Error = ± 1.8 per cent.

(8) A very thick emulsion of a twenty-four hour agar culture of B. typhosus in normal saline. The emulsion was diluted to 1 in 50 before staining.

1st count = 27,665 millions per cc. 2nd count = 33,075 millions per cc.
Error = ± 8.9 per cent.

Conclusion.—The process, like many bacteriological methods, appears far more complicated on paper than it is in actual practice. The specimens are easily counted after a little practice, and the whole operation can be completed in three or four hours, it being done in duplicate throughout so as to provide a check on possible errors. In the writer's hands it has given better results than any method he had tried, and it seemed quite possible to keep the error below ± 10 per cent. in every case.