A MICROSCOPIC METHOD OF DISTINGUISHING DEAD FROM LIVING BACTERIAL CELLS

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For a long time the biologist has been attempting to discover a criterion by which a living cell can be distinguished from a dead cell by simple microscopic examination. The most popular method has been the application of dyes, either in the form of the so-called vital staining, or in the usual way on dried and fixed smears.

1. METHODS OF “VITAL” STAINING

Dilute solutions of dyes were applied to animal or plant cells nearly as far back as a century ago. By this means it was sought to gain an insight into various life processes of the cells, to demonstrate certain cellular structures, or to find out whether the cell were dead or alive. The work started by Ehrlich on nerve cells and blood cells, and the stimulus it gave other workers, have given us a respectable literature on the subject, to which contributions are still being made from time to time.

The first application of “vital” stains to bacteria was made by Metchnikoff (1887) in his immortal study of phagocytosis. He says:

To convince myself of the bactericidal action of the microphages, I made use of an old solution of vesuvin which was not in condition to alter living bacteria but stained dead ones bright brown. By adding a few drops of this solution to preparations of leucocytic exudates, I was able to observe that the majority of the rods (of attenuated anthrax organism) inclosed in the protoplasm of the microphages immediately
took up the brown color, while the cells (microphages) remained colorless and continued to live, manifesting their amoeboid movements.

Among the basic anilin dyes, methylene blue and neutral red have been the most popular, especially neutral red, introduced and warmly recommended by Ehrlich (1894). Plato (1900) used that dye in his extensive investigations, and Metchnikoff (1901) himself adopted it in his later work.

Among "vital" staining methods may be mentioned that of Růžička (1905) who recommends the use of a staining solution made by mixing equal volumes of 0.05 per cent neutral red and 0.05 per cent methylene blue solutions in distilled water. A few drops of this mixture are spread over the surface of a clean slide and allowed to evaporate at 35°C. This leaves a thin, dry film of dye on which a drop of the bacterial suspension to be investigated is placed and examined under a cover-glass. Within a short time, living cells take on a violet color in which the red tone predominates. Dead cells show a predominance of blue tone.

Here belong also those "vital" staining methods with acid dyes, like that of Seiffert (1922) who used congo red. Seiffert's stain was a dilution of 1:10 of a saturated congo red solution in physiological salt solution. Others have used this dye in negative preparations. Dead bacteria are supposed to take up the dye, living bacteria to remain colorless.

2. METHODS OF APPLYING STAINS TO DRIED AND FIXED SMEARS

There is a variety of these methods described in the literature. The best known among these are Bordet's (1895) eosin-methylene blue method, and that of Proca (1909), especially in the form of Kayser's modification.

Bordet observed in the course of his studies of phagocytosis that phagocytosed bacteria gradually undergo a change in their staining reaction. At first they are cyanophilic and are colored blue, when the smear is stained with eosin and then with methylene blue. Gradually, under the action of the phagocyte they become eosinophilic and take up a pink color when stained in the same way. A continuous transition in color is seen between the two stages.
Proca's method consists in staining the dried and fixed smear for one minute with a solution made up by mixing 8 cc. of Ziehl's carbol fuchsin with 100 cc. of Loeffler's methylene blue and 100 cc. of distilled water. Dead bacteria stain red and living bacteria blue. Applied to spores (Proca and Danila, 1909), it was found that viable spores remain colorless and dead spores are stained blue. Proca also observed that bacteria killed by heat or disinfectants and stained with Loeffler's methylene blue, lose their blue color and turn red if subsequently stained with dilute Ziehl's carbol fuchsin (1:10). This observation was applied by Kayser (1912) in his modification. Kayser recommends staining of the smear three to five minutes with Loeffler's methylene blue, rinsing, and staining from five to ten seconds with Ziehl's carbol fuchsin, diluted 1:10. Recently this modification has been warmly recommended by Gay and Clark (1934).

The gram reaction has been suggested as a means of differentiating dead from living cells in gram positive species, and an improved method based on that reaction has been recently described by Frazier and Boyer (1934).

SIGNIFICANCE AND LIMITATIONS OF THE PRESENT METHODS OF DISTINGUISHING DEAD FROM LIVING BACTERIA

The above brief review gives the general lines along which methods, based on staining reactions, have been developed in an attempt to distinguish microscopically a dead from a living cell. In spite of some very optimistic reports, we may safely say that none yet has proved to be dependable. The following discussion shows why this is to be expected.

"Vital" staining methods

The basis of the use of vital staining has been so far indefinite. The general assumption is that living cells do not stain with "vital" dyes, while dead ones do. This assumption has never been satisfactorily justified, and investigators hold conflicting views on the subject. There is first the school led by Ehrlich which believed that the living protoplasm never stains, and that the appearance of stain is an evidence of death. Intracellular
structures which take up the stain are, according to this school, metabolic products taking no part in cell functions. In this connection we may mention the interesting “theory of micellar immunity” formulated by Lumière (1925), in which he explains the non-stainability of living protoplasm by its colloidal structure. The opposite camp is led by Przesmycki (1897), who believed that both the cytoplasm and the nucleus of the living cell may stain, and that, upon death, decoloration takes place. Similar views were held in recent years by Roskin and Semenoff (1933) and by Gavaudan (1933, 1934) and others. Between these two schools we find all kinds of transitional views expressed by investigators like Prowazek (1897), Arnold (1899) and Plato (1900). This latter investigator, although denying stainability of the living protoplasm in blood cells and in protozoa, yet believes that stained bacteria inside of the phagocytes may still be alive.

The above views regarding blood cells and protozoa were gained from microscopic observations of motility, or of the behavior of the stained intracellular granules. In one or two cases, claims of correlation with the division of a protozoan were made.

A different line of attack was followed by Fraser (1920) who compared the number of viable yeast cells as obtained by “vital” staining, with the number of colonies developing on the petri plate. After trying out a number of basic and acid dyes, Fraser adopted methylene blue. He found that the plate count was consistently less than the number of unstained cells and concluded that the cell loses the power of reproduction before it stains. Methylene blue was also used by Fulmer and Buchanan (1923). Rahn and Barnes (1933) used Congo red in addition to methylene blue and compared their results with the fermenting power and the plate count of yeast cells. Their conclusions are, likewise, that yeast cells lose the power of reproduction first, followed, in order, by the loss of fermenting power and by the acquisition of the ability to stain with the dyes used.

On going over the literature, one can but feel that much of what is assumed in using “vital” dyes to distinguish dead from living cells is arbitrary and indefinite. This feeling becomes even
stronger when one undertakes such a study experimentally. It seems natural to consider dead any cell that takes up a dye and living any one that does not; for, if we suspend growing cells in dilute dye solutions, they remain colorless; and, if we kill the cells by heat or chemicals, they immediately stain. But is every stained cell a dead one or every non-stained one alive? We have seen that the authorities are not in agreement on that point. And then we have the more complex question of degree of staining. How about cells that barely show color? And cells that contain stained vacuoles of various kinds? Much has to be determined before "vital" staining methods can become dependable.

Methods using dried and fixed smears

Even less is known of the principles behind the methods involving the staining of dried and fixed smears. In general, such methods are evolved empirically as follows: A smear made from a living culture and stained in a certain way shows certain color or predomiance of a certain shade of color, while a culture, killed in certain ways and stained by the same method, shows a different color or predominance of a different shade. In attempting to explain these, at best, rough reactions, some wild assumptions have been made; for instance, the assumptions that in a dried film fixed by heat, the bacteria are still alive, if the film came from a living culture.

In order to get an idea of the value of these methods, the reader is referred to an article by Bickert (1930) who investigated a variety of them and arrived at the conclusion that none of them is of any practical value.

I, myself, have investigated the most recommended of all these methods, the Proca-Kayser method, and my conclusion is that it does not show the difference between dead and living cells, but merely the difference in the degree of destruction to which the cells have been subjected. In the case of very young cells of *Escherichia coli* killed by quick cooling from 45° to 5°C. (Sherman and Cameron, 1934) the method fails completely. There are two more objections to this method. First, the shade of
color is rarely pure blue or pure red. It is usually violet with a predominance of blue or red shades, and all kinds of transitional shades in between. Second, it is not suitable for quantitative work because many of the cells are washed off on rinsing.

PRESENT INVESTIGATIONS

A satisfactory solution of the present problem involves the solution of several preliminary problems. In the first place a suitable dye must be selected. After trying out several basic and acid dyes, neutral red was chosen on account of its low toxicity and its low reduction potential \( E^\circ = -0.330 \) volts at pH 7). The concentration used was such that it had no retarding effect on the organism studied when added to the culture medium. In the case of *Escherichia coli* with which most of the present work was done, a concentration of 0.005 per cent was adopted, while some of the yeasts used required as little as 0.001 per cent of neutral red. It was also ascertained that, when the organisms were most actively growing in a neutral-red free medium, the addition of neutral red in the proper concentration caused no injury.

On the other hand neutral red has two disadvantages which, however, do not interfere with the method as it is here described. The first disadvantage is the fact that neutral red acts as an acid-base indicator in the pH range of 6.8 to 8.0. The alkaline color is yellow and hard to see under the microscope. The method automatically takes care of this in the process of diluting with neutral-red gelatin of pH about 6. The addition of a fermentable sugar to the original medium will also eliminate this trouble.

The second disadvantage is encountered when a strongly reducing organism like *Escherichia coli* is grown in a medium in which acid is produced. It has been known for a long time that *Escherichia coli* produces discoloration in neutral-red sugar media in the form of a yellowish-green fluorescence. Clark and Perkins (1932) have shown that this is due to an irreversible reduction of neutral red in the pH range of 4 to 6. Although the criteria and results given in this paper have been obtained by direct
experimentation and are independent of aberrations in the behavior of neutral red in the conditions described, yet numerous experiments were performed to elucidate the circumstances of the appearance of fluorescence in coli cultures. The conclusion from these experiments is that, when coli cultures in the neutral red medium used attain their most negative potential, the pH is about 6 and only about 25 per cent of the dye is reduced, which is not noticeable to the unaided eye. Fluorescence appears much later, usually in cultures twenty-four hours old or more, at 30°C. The pH of the culture is then about 5.2 and the dye should be reduced, only, to the extent of about 1 per cent. This apparently paradoxical fact has probably its explanation in the continuous accumulation of small amounts of the irreversible fluorescent material. As long as the bacterial population in fluorescent cultures is such that they must be diluted with neutral-red gelatin, the objection suggested by this phenomenon disappears.

The next step was to find out whether a cell can stain (in whole or in part) and still be alive. The test organisms were Escherichia coli, Schizosaccharomyces pombe and another yeast isolated from fermenting ale. Use was made of the technique of microcultures described by the author in a previous paper (Knaysi, 1933). The investigated organism was grown in glucose broth containing neutral red far below its inhibiting concentration. Micro-cultures were made from such cultures or from neutral-red glucose broth inoculated from these cultures. The microcultures were incubated and observed at room temperature. Each preparation contained about 30 droplets deposited in rows, and these were mapped immediately after the preparation was completed, and descriptions of the cells contained in some of them were recorded, and this correlated with viability. And, again, after growth in certain droplets had proceeded far enough or was completed, micro-transfers were made to fresh droplets of the neutral-red glucose medium or, in certain cases, the medium was sucked out and fresh medium added, thus insuring the observation of some particular cell or cells.

The conclusions drawn from numerous such experiments may be stated as follows:
1. Normal, healthy cells do not show the slightest evidence of color either of the cytoplasm or the vacuoles and other inclusions.

2. Any cell showing evidence of staining of its cytoplasm, no matter how faint it is, is a dead cell.

3. Cells in which the vacuoles alone are stained, may or may not be able to grow. In the first case, the vacuole loses its color completely before growth takes place. In the second, the stain diffuses out into the cytoplasm, giving it a faint tinge of color.

4. There is a certain number of healthy, normal-appearing cells that fail to grow, and yet they do not take up the dye for a long time. These were probably alive but somehow lost the power to reproduce. They are numerically few.

5. Before autolysis sets in, a dead cell stains deep red, but as autolysis proceeds, staining becomes gradually fainter, and one finds a certain number of cells that look disorganized and have a low refractive index without showing evidences of staining. Such cells are not numerous and are easy to recognize. They probably contain no more stainable material.

ADDITIONAL TESTS OF RELIABILITY AND SENSITIVITY OF THE METHOD

Aside from the direct viability test described above, other means have been used to gain an idea about how reliable and sensitive the method is.

Cultures of Escherichia coli killed by heating to 65°C., in flowing steam, or in the autoclave, take up the dye immediately, as do cultures killed by phenol and mercuric chloride. The dye may not penetrate the cell instantaneously on account of coagulation of the protoplasm or the like, but the membrane of the cell stains deeply and there will be no mistake about the cell being stained.

A most crucial test for the sensitivity of the method is to apply it to young cultures cooled quickly from 45° to 5°C. It may be remembered that Sherman and Cameron (1934) have recently noted that when very young, rapidly growing cultures of Escherichia coli are quickly cooled from 45° to 10°C., over 90
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Per cent of the cells may be killed. This discovery is surprising in view of the fact that both temperatures are included within the temperature range of growth of Escherichia coli. The criterion of death used by Sherman and Cameron having been the failure to grow on petri plates, it is reasonable to consider the possibility that cooling merely interfered with the growth mechanism of the cell instead of causing instantaneous killing. When the present method was applied to such cultures, it was found that almost all the cells took up the dye, indicating an immediate death.

The present method also shows that in a smear of Escherichia coli, dried and fixed by heat, the cells are dead, contrary to a statement of Ficker (1929) adopted by various investigators, including Bickert (1930) and Gay and Clark (1934).

In addition to the above tests it was also thought desirable to compare the numbers of viable bacteria as obtained by the present method with those obtained by the petri plate method.

NUMBERS OF VIABLE CELLS IN CULTURES OF ESCHERICHIA COLI, AS DETERMINED BY THE NEUTRAL RED AND BY THE PETRI PLATE METHODS

Technique

A certain volume of the culture of Escherichia coli in which it was intended to enumerate viable cells was diluted with 15 or 20 per cent of nutrient gelatin at 35°C., containing 0.005 per cent neutral red. The percentage of gelatin used depended on the degree of dilution and on the temperature of the room. The mixture was shaken thoroughly for a few minutes and replaced in the water bath until most of the foam disappeared. Then 1 cc. was measured into a 99 cc. water blank previously warmed to 30°C. and, immediately following this, a loopful of the bacterial suspension was used to fill a Petroff-Hauser counting chamber. The chamber was allowed to remain one minute at 37°C. while filling, in order to keep the viscosity of the gelatin low and prevent it from raising the cover-glass, which would introduce a large error. During the time the chamber was at 37°C., the water
blank which received 1 cc. of suspension was being shaken. The chamber was then placed in a 10°C. incubator to allow hardening of the gelatin and to slow down possible growth of the organism, and it was left there until plating was completed. This procedure, it was ascertained, causes no injury even to the most sensitive cells. The value of the gelatin is to check motility of the organism, and to supply rigidity, as counting was made with the oil-immersion objective.

The Petroff-Hauser counting chamber has similar ruling to ordinary haemacytometers and possesses the advantages of being adapted for use with the oil-immersion objective and with the dark-field condenser. Its depth is 0.02 mm.; that is, it is one-fifth as deep as the chambers of ordinary haemacytometers. An average of one cell per small square is equivalent to 20 million cells per cubic centimeter. With the possible large error involved, the degree of accuracy attained is remarkable, even when the suspension contained less than 10 million cells per cubic centimeter. Usually about 50 small squares were counted.

In plating, water blanks warmed to 30°C. were used. Warming the water blanks was for the purpose of keeping the gelatin in the liquid form. The medium used for plating was nutrient agar containing 0.5 per cent glucose. The agar was poured at 45°C. Before this procedure was decided upon, experiments were performed in substituting broth blanks for water blanks, and in using different percentages of agar to make pouring possible at 35°C. No advantage was found for any of these modifications, even with most sensitive cells. The glucose was incorporated, however, because it made the organisms grow much faster and form larger colonies. Counting was usually made after 24-hour incubation at 37°C.

Results

The results of comparative counts are recorded in tables 1 and 2. Table 1 includes counts of young cultures, while table 2 includes the results with mature and old cultures.

In comparing any microscopic count with the plate count, it is necessary to compare the number of groups observed under
the microscope with the number of colonies developing on the plate. By group, is meant one cell or two or more cells that are still united by their cell wall. This point seems too evident to need mention, and yet, strangely enough, in the few instances where a direct count of viable cells was compared with the plate count, no mention was made of groups, except in Frazier and Boyer's report. This, alone, is sufficient to magnify the discrepancy found between the two methods to the proportions recorded. The argument that, upon shaking, groups (in the sense here used) are broken up to individual cells does not hold in the

<table>
<thead>
<tr>
<th>EXPERIMENT</th>
<th>AGE OF CULTURE</th>
<th>MICROSCOPIC COUNT PER CUBIC CENTIMETER OF CULTURE</th>
<th>PLATE COUNT PER CUBIC CENTIMETER OF CULTURE</th>
<th>PLATE COUNT GROUP COUNT X100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cells x 10^n</td>
<td>Groups x 10^p</td>
<td>Groups x 100</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>185.0</td>
<td>111.0</td>
<td>60.0</td>
</tr>
<tr>
<td>2</td>
<td>4 1/2</td>
<td>107.0</td>
<td>60.0</td>
<td>56.0</td>
</tr>
<tr>
<td>3</td>
<td>3 1/2</td>
<td>72.0</td>
<td>48.0</td>
<td>66.6</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>703.0</td>
<td>356.0</td>
<td>50.6</td>
</tr>
<tr>
<td>5</td>
<td>1 1/2</td>
<td>2.2</td>
<td>1.1</td>
<td>50.0</td>
</tr>
<tr>
<td>6</td>
<td>3 1/2</td>
<td>11.2</td>
<td>5.8</td>
<td>51.8</td>
</tr>
</tbody>
</table>

light of the figures reported in tables 1 and 2, even for as "individualistic" an organism as Escherichia coli. The gelatin suspension was usually vigorously shaken for nearly five minutes, and yet the ratio of groups to individuals never reached unity, although in a few experiments with old cells it approached that value. In young cultures of coli, where the cells are actively growing, the number of groups is nearly half of that of individuals, because most of the population consists then of pairs of sister cells. In cultures where growth is very slow, this circumstance is non-existent, and the cells tend to be single with few pairs and an occasional short chain.
When the number of viable groups is compared with the number of colonies, it is evident from the tables that these numbers check very closely. In the many experiments performed, there were some showing discrepancy. But the number of such experiments is small and almost reached the vanishing point as the technique became more and more refined. On the other hand, in the great majority of the experiments, the agreement was surprisingly close, often closer than counts given by duplicate plates. The tables give a representative account of the results. Out of 22 experiments reported, 13 give an average number of 90 to 110 colonies, the group count being considered as 100. One

### TABLE 2

**Number of viable cells in mature and old cultures of Escherichia coli as determined microscopically and by plating**

The organism was grown in glucose broth containing 0.005 per cent of neutral red at 30°C.

<table>
<thead>
<tr>
<th>EXPERIMENT</th>
<th>AGE OF CULTURE</th>
<th>MICROSCOPIC COUNTS PER CUBIC CENTIMETER OF CULTURE</th>
<th>PLATE COUNT PER CUBIC CENTIMETER OF CULTURE</th>
<th>PLATE COUNT GROUP COUNT x100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cells x10⁶</td>
<td>Groups x10⁶</td>
<td>Groups x10⁶</td>
</tr>
<tr>
<td>1</td>
<td>48 hours</td>
<td>528.0</td>
<td>374.4</td>
<td>70.9</td>
</tr>
<tr>
<td>2</td>
<td>48 hours</td>
<td>674.0</td>
<td>488.0</td>
<td>72.4</td>
</tr>
<tr>
<td>3</td>
<td>118 hours</td>
<td>200.0</td>
<td>140.0</td>
<td>70.0</td>
</tr>
<tr>
<td>4</td>
<td>119 hours</td>
<td>220.0</td>
<td>180.0</td>
<td>81.8</td>
</tr>
<tr>
<td>5</td>
<td>36 hours</td>
<td>530.5</td>
<td>285.8</td>
<td>53.9</td>
</tr>
<tr>
<td>6</td>
<td>63 hours</td>
<td>317.7</td>
<td>198.9</td>
<td>62.6</td>
</tr>
<tr>
<td>7</td>
<td>40 hours</td>
<td>340.0</td>
<td>238.0</td>
<td>70.0</td>
</tr>
<tr>
<td>8</td>
<td>37 hours</td>
<td>412.5</td>
<td>259.0</td>
<td>68.8</td>
</tr>
<tr>
<td>9</td>
<td>42 hours</td>
<td>417.0</td>
<td>306.0</td>
<td>73.4</td>
</tr>
<tr>
<td>10</td>
<td>36 hours</td>
<td>731.0</td>
<td>544.0</td>
<td>74.4</td>
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<tr>
<td>11</td>
<td>38 hours</td>
<td>751.0</td>
<td>578.0</td>
<td>77.0</td>
</tr>
<tr>
<td>12</td>
<td>40 hours</td>
<td>548.0</td>
<td>364.0</td>
<td>66.4</td>
</tr>
<tr>
<td>13</td>
<td>62 hours</td>
<td>724.2</td>
<td>493.0</td>
<td>68.1</td>
</tr>
<tr>
<td>14</td>
<td>64 hours</td>
<td>724.2</td>
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<td>15</td>
<td>36 hours</td>
<td>632.0</td>
<td>500.0</td>
<td>79.1</td>
</tr>
<tr>
<td>16</td>
<td>35 hours</td>
<td>540.2</td>
<td>418.3</td>
<td>77.5</td>
</tr>
</tbody>
</table>
would have no objection if his duplicate plates came consistently as close as this. It must be added that, in most of the cases reported, the gelatin suspension contained less than 25 million per cubic centimeter, and in a couple of experiments with young cells it contained less than 2 millions. A much closer agreement is obtained when the number of cells counted in the gelatin suspension is made larger.

**SUMMARY**

This paper is concerned with the use of neutral red in distinguishing, microscopically, between dead and living cells.

The technique of micro-cultures was used to study the relation between the viability of a cell and its ability to stain in whole or in part. It was found that cells of *Escherichia coli*, *Schizosaccharomyces pombe* and a yeast isolated from ale are to be considered dead whenever the cytoplasm proper is tinged, even slightly, with stain. Yeast cells with stained vacuoles and unstained cytoplasm are weakened, sick cells that may or may not recover. In a culture, there is a certain number of cells that are not stained and look healthy, but are unable to multiply. These are numerically few. Others do not stain because their autolysis is too far gone. These look disorganized, have a low refractive index and are easy to recognize. Their number is usually not important.

When comparisons are made between numbers of viable cells in cultures of *Escherichia coli* as obtained microscopically, using neutral red as an indicator of viability, and numbers of viable cells as given by plating, a close agreement between the two is found when groups, instead of single cells, are taken into consideration.

The concentration of neutral red varies with the organisms. In the case of *Escherichia coli*, a concentration of 0.005 per cent was adopted. This concentration is harmless even to the youngest and most sensitive cells, and the organism grows readily in broth containing neutral red in that concentration.
REFERENCES

FICKER, M. 1929 In Kolle and Wassermann’s Handbuch der Pathogenen Mikroorganismen, 3rd edition, 9, 711-802.
GAVAUDAN, P. 1934 Comptes Rend. à l’Acad. des Sciences, 198, 848-850.