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A NEW METHOD OF OBTAINING CULTURES FROM SINGLE BACTERIAL CELLS.

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(With Plate I.)

WHILE investigating variations in agglutinability, occurring among certain strains of paratyphoid bacilli, it became desirable to obtain cultures derived from single bacterial cells. The technique at first adopted was that devised by Mutch (1919), but the results obtained were not satisfactory. Certain modifications of this method were attempted, but were no more successful.

The main difficulty, and one which appears to us to have been far too little emphasised in all the methods hitherto described, has been encountered in obtaining satisfactory conditions for the microscopical observation of the bacillary suspensions employed. The difficulty of accurately observing hanging-drop preparations, and still more of controlling manipulation of them by microscopical observation, would seem to us to have been insufficiently realised; and the ease with which faulty interpretation of microscopical appearances may introduce fatal errors into work of this kind has not, perhaps, been fully taken into account.

For this reason we first turned our attention to the problem of securing really satisfactory conditions for microscopical observation, and adopted the dark-ground method of illumination for this purpose. Using this technique, with a relatively granule-free medium for suspension, and examining thin films between a slide and a cover-slip, it is possible to pick out a single bacterial cell, lying apart from any other organism, with a certainty quite unobtainable in the observation of hanging-drop preparations by directly transmitted light, or of organisms situated on the surface of any solid nutrient medium. The conditions of this method of observation, on the other hand, entirely preclude subsequent isolation of an individual bacterial cell by mechanical means, while the experience met with in attempts to examine large series of such preparations, until one was found which contained a single bacterium only, did not offer any inducement to continue work along these lines.

For these reasons we definitely abandoned all attempts to obtain the desired results by methods such as those described by Barber (1908, 1911, 1914, 1920), Mutch (1919), Hort (1920), Hewlett (1918), Malone (1918), and others; and tried to discover a solution of the difficulty along entirely new

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lines. The vital factor of good optical conditions for the examination of the preparations appeared to us to be satisfactorily dealt with by the use of thin film-preparations of young bacterial growths in granule-free nutrient gelatine, using dark-ground illumination. Such preparations overcome the difficulty introduced by the motility of certain organisms.

Previous investigations by one of us (Barnard and Morgan, 1903 *a* and 1903 *b*) suggested the possibility of utilising the sterilising action of ultra-violet light to kill all bacteria in the preparation, with the exception of the single cell from which growth was to be obtained. The problem remained of devising a method by which this cell alone might be protected. The solution was found in covering the organism selected with a small mercury globule, between 50 μ and 120 μ in diameter. Such small globules cling to the surface of the cover-slip so tenaciously that there is no fear of displacing them during the subsequent manipulations, if reasonable care be exercised.

THE TECHNIQUE EMPLOYED.

The only apparatus required, in addition to that which will be found in any bacteriological laboratory, is a mercury arc as the source of ultra-violet radiation, and a supply of circular quartz cover-slips of about 12 mm. diameter. The actual procedure is as follows.

A culture of the bacterial strain, from which the isolation is to be made, is put up in ordinary nutrient broth and allowed to grow for about 18 hours at 23° C., or about 6 hours at 37° C. A tube of 10 per cent. gelatine in 1 per cent. peptone-water is then melted, cooled to 37° C. and inoculated with a loopful of the actively growing broth-culture. This is then replaced in the incubator at 37° C. for another two hours. These times are of course only approximate, but if the most satisfactory results are to be obtained they must not be widely departed from. It is of great importance to work with young, actively growing cultures, containing relatively few bacteria, the great majority of which are viable.

Several (6–12) preparations are now put up by placing a small drop of the liquid gelatine culture on a quartz cover-slip, using a platinum loop with an external diameter of 3 mm., and inverting this on a glass slide, both slide and cover-slip having been previously sterilised by passing them through the flame of a Bunsen burner and allowing them to cool under a glass cover. The slide must be of a suitable thickness for use with a dark-ground condenser (0·95–1·1 mm.). The drop of culture should be of such a size that a very thin film is formed, completely filling the space between the slide and the cover-slip without any escape beyond the edge of the latter. This film should be entirely free from air-bubbles.

These preparations are then examined, using a 1/6 objective and an 18 compensating ocular. A suitable preparation will show 5 to 20 bacteria in the whole film, any one cell being widely separated from its nearest neighbour. In choosing a cell for isolation certain facts should be kept in mind.

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It is important to select an organism which is surrounded by a wide zone of medium, in which careful examination fails to reveal any other bacteria. It is best to choose a cell which already shows signs of incipient division, and further manipulations are facilitated by selecting an organism which lies near the centre of the preparation, rather than one near the periphery.

The choice having been made the cell selected is moved to the centre of the field, and its position is read off on the verniers of the mechanical stage. (Fig. 1 shows such a cell in position.) The 1/6 objective is now replaced by a 2/3, and the cell is again identified.

A large drop of carefully cleaned mercury is now placed in a glass dish and broken up into minute droplets by a smart tap with the end of a finger. One of these minute droplets is picked up on the point of a needle and transferred to the surface of the cover-slip, at a point as close as possible to the centre of the field of illumination. For this procedure it is best to employ a needle with a roughened point which has been allowed to rust. The mercury droplet will often refuse to cling to a clean and polished needle. The exact position of the mercury droplet is then ascertained by examination with the 2/3 objective and 18 ocular. The appearance presented when the edge of the droplet is focussed is shown in Pl. I, Fig. 2. It has now to be moved into position, so as to lie with its centre over the cell to be protected. It is simply pushed into its proper place with the point of an ordinary steel needle, under observation through the microscope. This procedure is singularly easy after a little practice. The needle is held at a slight angle with the horizontal, and the point only should be used. The manipulation is greatly facilitated by the fact that the reflection of the point of the needle is clearly observable on the surface of the mercury droplet, long before the point itself appears in the field. To make use of this fact the tube of the microscope is racked up slowly, so that successive bright rings, which are reflections from the upper convex surface of the mercury droplet, come into view. The appearance of such a drop is shown in Fig. 3. At the upper part, between the inner and outer rings is seen a point of light, and below this there is a small break in the inner illuminated ring. This appearance is the reflection of the needle point. The needle employed at this stage should be scrupulously clean and polished, since a dirty or roughened point may cause the droplet to cling to it and be dragged back when the needle is withdrawn. The droplet having been successfully manœuvred into the centre of the field, the 2/3 is again replaced by the 1/6 objective. The drop should now be found to fill at least half the field, but must not fill it completely. On racking down the tube of the microscope the bacterial cell may now be observed in the centre of the field, below the centre of the mercury droplet. The possibility of thus controlling the position of the droplet in relation to the bacillus, by direct observation, depends upon the fact that the size of the former is very small, relatively to the aperture of the objective. On lowering the tube of the microscope the mercury droplet passes out of focus and disappears from view, while the

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rays entering the objective peripherally form an image of the bacterium, which is readily observable, though less bright than that obtained without the interposition of the droplet. If the droplet employed be so large as almost to fill the microscopic field, a central dark area will be left when the tube is lowered; so that, if the bacillus be lying beneath the centre of the droplet, it will not come into view. This very fact may, however, be taken as evidence that it is satisfactorily covered. The slide is now removed from the stage of the microscope, and the process is repeated with two or three similar preparations. At least two further preparations, without mercury droplets, are reserved as controls.

All these preparations are now exposed to ultra-violet radiation. Using the medium referred to above, we have found that an exposure of one minute, at a distance of three inches from the source of light, gives entirely satisfactory results with such non-sporing organisms as we have studied. The exposure is made through a tube of about 24 mm. diameter, to prevent the incidence of very oblique rays, which might pass beneath the edge of the droplet and reach the underlying cell.

Each preparation is now ringed round with melted paraffin, delivered from a capillary pipette. It is important that this manipulation should be carried out rapidly, and that the temperature of the paraffin should not be far above its melting point. If these precautions be observed there is no danger of melting the gelatine, which is the accident to be avoided.

All preparations are then incubated over night at 25° C. Next morning they are examined with the 1/6 objective and 18 ocular. Such examination should show that the single bacterial cell has now multiplied and formed a colony, containing few or many bacteria (Fig. 4), while any other bacteria in the preparation have failed to divide. These latter will usually present a definitely granular and degenerate appearance. Each of those preparations in which such a colony has formed, and the two or more controls which were exposed without the protection of the mercury droplets, are now subcultured by removing the cover-slip, the edge of the cover-slip and the surrounding surface of the slide being previously sterilised with a hot metal rod. The gelatine is then rubbed up in a loopful of broth, and this is transferred to a tube containing a suitable culture medium. Subsequent examination of these tubes should show growth in some or all of those inoculated from the microscopic colonies, while the controls should remain sterile. These controls are added to demonstrate that the action of the ultra-violet light has not been merely to prevent the multiplication of the unprotected bacteria in the gelatine preparation, but has actually killed them. In our experiments large numbers of such controls have been employed to test the adequacy of the technique. In routine isolation they are not, perhaps, strictly necessary, once the length of exposure needed in the case of the organism and medium to be worked with has been determined; but it is always more satisfactory to include at least two such preparations, and to discard any strains which have been

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isolated in an experiment, in which growth has occurred from the unprotected controls. The risk of air-borne contamination of the preparations during the necessary manipulations appears to be negligible, for in our experience it has never occurred.

A few further technical points may be mentioned. The constitution of the medium used in the actual isolation has a profound effect on the length of exposure needed to kill the unprotected bacteria. In many of our earlier experiments the gelatine medium employed was prepared with a tryptic digest of casein, containing large amounts of tyrosin and other amino-acids. Using this medium, the lethal dose of radiation at a distance of three inches lay between four and six minutes, as compared with the one minute required with peptone-gelatine. The mercury arc generates an appreciable amount of heat; and it is, of course, essential that no liquefaction of the gelatine should occur during the exposure. With 10 per cent. gelatine there is little risk of this, except in very hot weather. Under these conditions the risk can readily be obviated by laying the slide on a block of ice while exposing it to the radiations.

With organisms which themselves produce liquefaction of the gelatine, the formation of circumscribed colonies cannot be observed. Since, however, no liquefaction occurs during the time of manipulation and exposure, the technique is readily applicable to such bacteria, and the sterility on subculture of the unprotected controls affords satisfactory evidence of the lethal action of the radiations.

The slides and cover-slips, after subcultures have been made from the gelatine preparations, are most readily sterilised and cleaned by immediately transferring them to 25 per cent. sulphuric acid containing 10 per cent. of potassium bichromate, and boiling them for a few minutes.

As regards the proportion of successful isolations, it is only in connection with the members of the paratyphoid group that our figures are sufficiently large to give representative results. After the main lines of the technique had been arrived at, but while we were still making small variations in the composition of the medium and the length of exposure, we attempted 148 isolations of organisms of this type. In 58 cases (39 per cent.) microscopic colonies were obtained from the single cells selected. In 39 instances (26 per cent.) these microscopic colonies were successfully subcultured. In the last 46 isolations, when the technique described above was strictly adhered to, the percentage of colony-formation was 41 and of successful subculture 33. During the 148 isolations referred to 137 unprotected controls were exposed. Of these 128 remained sterile. The 9 which grew were included in three experiments, in two of which a considerable interval had been allowed to elapse between putting up the preparations used for isolation and those used as controls. During this interval multiplication had been going steadily forward, so that the control preparations probably contained far too large a number of bacteria. Actually, therefore, the number of isolations which have to be discarded through non-sterility of the unprotected controls is very small indeed.

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As a further proof of the adequacy of the method it may be mentioned that we have, on many occasions, worked with a mixed culture of *B. coli* and paratyphoid bacilli, or with a mixture of different serological types of the latter group, and have in every case demonstrated the purity of the cultures finally obtained.

Although 33 per cent. of successful isolations may not appear an entirely satisfactory result, it is in practice quite adequate for the purpose. With a little practice, it is easily possible to carry out the entire manipulations concerned with putting up and exposing a series of 6–10 preparations within an hour; while the unprotected controls are dealt with in a few minutes. As the result of an hour's work, excluding the preliminary and final subcultures, it is thus possible to make practically certain of obtaining a culture derived from a single bacterial cell, the whole process being controlled at each step under satisfactory optical conditions. We would again emphasise that it is this last requirement that appears to us to be the essential factor.

As mentioned above, the majority of our work has been carried out on paratyphoid bacilli, but we have tested the applicability of the method to certain other organisms. *B. coli*, *Staphylococcus albus*, *Staphylococcus aureus* and *Streptococcus haemolyticus* are all killed by exposure for one minute at a distance of three inches from the source of radiation, and successful isolations have been made from these organisms. Our experiments with spore-bearing bacilli are as yet too few to warrant any definite statement, but, as would be expected, the time of exposure must be very greatly increased. With regard to anaerobic organisms, we have so far attempted the isolation of single-cell cultures of *B. welchii* only. This has been successfully carried out, but the best method of ensuring the requisite conditions for growth, and the applicability of the technique to other anaerobes, have not yet been established.

We are indebted to the Medical Research Council for supplying a mercury arc with which some of these experiments have been carried out.

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EXPLANATION OF PLATE I.

Fig. 1. Bacterial cell, showing incipient division, chosen for isolation.

Fig. 2. Appearance presented by mercury droplet when the edge is focussed.

Fig. 3. Appearance presented by mercury droplet when the tube of the microscope is slightly raised. The reflection of the needle-point is shown in the upper part of the droplet.

Fig. 4. Multiplication of the protected bacterial cell after incubation.

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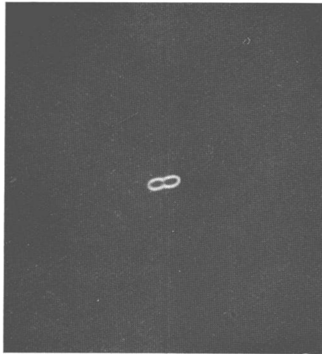

Fig. 1

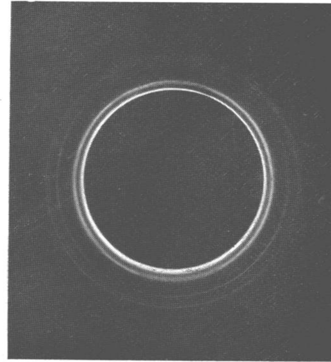

Fig. 2

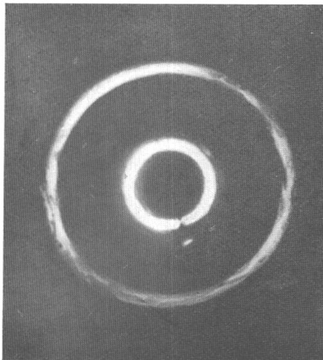

Fig. 3

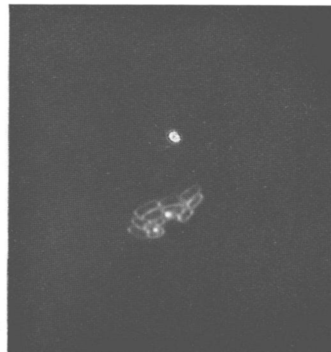

Fig. 4

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