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The Isolation of Single Bacterial Cells.

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THE writer has three reasons for introducing what, to many workers, is a somewhat threadbare subject: (1) In the minds of not a few people with whom the author has discussed the matter, the impression still persists that the technique of the isolation of single bacterial cells is extremely difficult, involving months, if not years, of practice for its successful acquisition. Heller (1921) stated that she found the Barber (1914) method " wasteful of time, material, eyesight and nervous energy " in the isolation of anaerobic bacteria. There is some justification for saying that a wastage of time and energy occurs when, after the successful isolation of ten to twelve bacteria or spores, not one germinates. This point, the germination of the single germ, especially when it concerns the anaerobes has not received the same amount of attention that the actual isolation has. The latter is a simple mechanical process, the former one over which the operator has not the same degree of control. (2) A method of isolation, to be of real practical value and to enjoy a constant and wide application, must be simple and devoid of tedium and time wastage. Preferably should it be such that the worker would prefer to employ it rather than use plate or shake methods of isolation. Again, the learning of the technique should not require months of practice nor should it be of such a nature that only those people with " hands " can easily acquire it. Reyniers (1933) described a method by which beautiful micro-pipettes may be made mechanically. The writer gained the impression, wrong, he hopes, that the making of the apparatus would be one requiring considerable skill and time. Doubtless, however, to see the designer himself at work would dispel this idea. The method, used by the writer for over four years, has proved to be so simple that three colleagues, after half-an-hour's practice in pipette-making have settled down and isolated (with successful germination) single germs of *Cl. welchii* and *B. anthracis.* (3) The third reason advanced for the presentation of this note is in the nature of a plea for the more extended use of the single cell isolation method as a routine measure. The saving of labour and of time involved in obtaining definitely pure cultures and the feeling of assurance in having these pure cultures greatly outweighs the only disadvantage (in the writer's opinion) of the expenditure of the money for the micromanipulator. But even a machine costs no more than a good microscope and where the outlay is impossible a locally-made apparatus or the modification used by Malone (1918) may be employed. Finally, there is the possibility that less would be heard of bacterial mutation if cultures were first purified by the single-cell method.

The Making of Micro-pipettes.—On the making of satisfactory pipettes depends the success of isolation. The writer was quite unable to make an efficient article by the " tiny flame method " as described by Barber (1914) and Chambers (1922)). That this method gives highly satisfactory results there is no doubt; never having had the procedure demonstrated to him and the use at these laboratories of " paraffin gas " (Mansfield system) are doubtless the reasons for the failure. The electrical heater described (Mason 1933) solved the difficulty. The two elements, arranged in the form of a V, may be made broad or narrow. If narrow, the type of pipette most desirable for isolation work is easily prepared. It is advantageous that the micro-portion should not be more than 1·5 to 2 cm. long and that it should leave the thick portion of the hand drawn capillary sharply. A long tapering micro-pipette trembles easily and when raised against the cover slip in the process of isolation " gives " considerably, necessitating much adjustment of the control screws of the machine. Such a tapering pipette is liable to be pulled when the breadth of the elements is 3·0 cm. or more. Elements of 2·0 cm. give satisfactory results.

Details.

1. Choose soft glass tubing with an external diameter of about 7·5 mm. and an internal diameter of about 6 mm. Cut portions about 30 cm. long. Soak for an hour or two in 5 per cent. hydrochloric acid, rinse thoroughly in tap water and then in distilled water. Allow to drain and dry. Plug both ends with wool and sterilize in the hot-air oven. Over a Bunsen flame, prepare two ordinary Pasteur pipettes from each portion of glass tubing. Store in a dust-free receptable until required.

2. Make a match-head flame by connecting, by means of rubber tubing, a fairly large bore hypodermic needle to a Bunsen burner. Carefully pull a much smaller capillary from the capillary end of the Pasteur pipette. Care should be taken to pull steadily and *out* of the flame whenever the heated portion of glass is nearly molten. The length of fine capillary pulled will vary in length from 3 to 6 cm.

3. Lower the fine capillary portion into the glowing heater, and so arrange its position that the micro-capillary comes off sharply. Whenever the glass " gives ", pull gently and evenly, raising the pipette slightly in the heater. Very little practice is necessary to learn the degree of traction necessary for the making of a satisfactory article. As a rule separation of the micro-capillary portion from the distal end of the fine capillary occurs, but if not, the distal end is severed with sharp scissors.

4. The end of the micro-pipette must be turned up for a distance of about 2 mm. This is accomplished by holding it above the match-head flame. As the capillary turns up quickly, care should be exercised to ensure that too much is not bent or that too acute an angle does not occur. As the distal end of the turned-up portion is almost certain to be fused, enough is cut off with sterile scissors to leave approximately 2 mm. To ensure the patency of the opening, air is pumped, by means of hand bellows, through the pipette into alcohol. One can judge of the diameter of the hole by the size of

the bubbles produced. The type of bellows used is important. One of the writer's colleagues nearly discarded what were excellent pipettes because he was unable to blow air through. He was using the common type of barbers' bellows, made of thin rubber with intermediary bulb surrounded by string netting. Sufficient pressure was not obtainable. The use of thick walled bellows with only a small intermediary bulb (also thick walled and without the netting) showed that the opening were patent.

The completed pipette is clamped to the manipulator.

The Manipulator.—The writer uses the machine manufactured by the Zeiss company and has found it eminently satisfactory in every way. Whatever apparatus is used, no matter how simple of construction, a *sine qua non* is that the movements must be precise and steady. A jerkiness will produce poor results and loss of patience.

The moist chamber, cover slips and hollow-ground slides.—The moist chamber was prepared from an ordinary microscopic slide by luting to it (by means of sealing wax) strips of slides so as to form a chamber enclosed on three sides, its depth being 6 mm. This depth may without disadvantage be increased to 1 cm. One end is left open for the introduction and withdrawal of the pipette.

The cover slips and hollow-ground slides present no special features. The concavity of the hollowed slide is 25 mm. in diameter and the cover slips are of such a size that they cover the area with plenty of room to spare. Just prior to use, both are cleaned with a clean cloth and sterilized by flaming.

The Medium.—This will depend on the germ to be isolated but it must be capable of being transformed into a solid or semi-solid transparent condition.

The writer has used as routine horse-muscle infusion peptone agar plus 10 per cent. of a mixture of equal parts of sheep haemolysed cells and serum and a saline extract of guinea-pig liver. The preparation of the haemolysed cell and serum mixture has already been described (Mason 1934). The guinea-pig liver extract was added because it was found to have, as Tarozzi (1905) originally discovered, a stimulating effect upon the growth of anaerobes, the group of germs in which the writer was chiefly interested. Portions of the liver of a healthy guinea-pig were removed with sterile precautions and placed in thick walled test tubes. By means of a glass rod inserted through the cotton wool plug of the tube, the tissue was ground up. Enough ether was added to cover the liver and the tubes placed in an incubator at 37° C. for three to five hours. Then sufficient sterile saline solution (0·85 per cent.) was added to make, approximately, a 10 per cent. suspension of tissue. After stirring, the suspension was incubated for twenty-four hours. For use, equal parts of the serum, haemolysed cells and the clear supernatant of the liver extract were mixed, and added to the melted agar. The sterility of the mixture was ensured prior to its addition.

Care should be taken to have a very clear nutrient agar, as the presence of granules makes isolation very difficult.

The writer does not insist that the above described medium is the best that could be desired; it is mentioned because it has given excellent results with many different germs, and in particular with anaerobes. However, experience has shown that the admixture of some additional nutrient, such as raw serum, is advantageous, if not essential, in the obtaining of positive growth results even of such easily-grown microbes as *Bact. coli* and *B. anthracis*.

The Culture.—For the isolation of aerobes, young (twelve to twenty-four hours) surface cultures have been used, and suspended in broth just prior to use. The anaerobes will be discussed under the appropiate heading.

The Isolation.—Most workers isolate the single germs in droplets of broth on a cover slip, mark these drops, remove the contained germ with a new sterile pipette and break off the pipette end in a tube of broth. If ten such isolations are made, this involves the use of the same number of pipettes and the changes involved in fixing and adjusting them. What is, to the writer, a much simpler process, viz. the expelling, with one pipette, single germs on to the surface of a solid medium does not seem to have found the application it deserves. The methods described by Hewlett (1918), Hort (1920), Fortner (1930), and Koblmüller and Vierthaler (1933) allow of single cells being isolated on the surface of agar. In the case of the first three, one must inoculate a thin film of medium with the germs and then search for and mark a microbe lying free. This involves considerable time and eye strain and its consistent success would depend upon always inoculating with a bacterial suspension of a suitable density. With the last-named authors' technique, a number of single germs are mechanically removed from the edge of a young growing colony on an agar plate and the colonies resulting from them are observed and subcultured. With this method, the writer has had no practical experience.

Details of Isolation (use of a Zeiss manipulator, microscope and lenses).—The microscope is clamped to the manipulator and the prepared micro-pipette with attached hand bellows fastened in its holder. The bottom of the moist chamber is moistened with distilled water (a few drops should be placed at one end, the chamber tilted to allow excess to run off and the bottom rubbed with the clean finger to produce a thin even layer of water). A thin even layer of vaseline is deposited on the tops of the walls of the chamber. It is then fixed on the mechanical stage. The tip of the micro-pipette is centred, first under the low power lens (No. 8) and then under the higher power lens (No. 40). A No. 5 ocular is used. The open end of the turned-up tip should be the only part of the pipette in focus. The micro-portion of the pipette should now be lying approximately in the middle of the chamber. A cover slip and a hollowed slide, held in clamp forceps are sterilized in the flame and left " face " downwards in their holders. Meanwhile nutrient agar has been boiled and cooled to about 50° C. With a pipette, about 1·5 c.c. is deposited in a sterile tube and immediately enough of the serum-cells-liver mixture to make a 10 per cent. concentration is added and thoroughly mixed. With another pipette which has been opened by filing with a sharp file so as to produce a clean cut, the serum-agar mixture is sucked up and a thin micro-plate prepared. The cover

slip in the forceps is tilted to an angle of about 45°-50° and a drop of the melted agar allowed to run down it. A little practice is necessary to learn how much agar has to be deposited so that it does not run right down the slip and over the end. The agar solidifies almost as it runs, and a thin transparent micro-plate is thus formed. With a very small platinum loop, a droplet of a previously prepared suspension of the bacteria to be isolated is deposited on a portion of the cover slip. It is best to place this drop on the cover slip, some distance from the agar. The slip is then placed, agar side downwards, on the moist chamber, pressing the sides lightly so that the vaseline seal is firm. The droplet of culture is focussed first with the low and then with the high power. The pipette point, having been previously centred will be just below the drop. Carefully raise the point into the culture, when the bacteria will be seen to flow in. When some dozens or hundreds have entered, lower the point and, with the mechanical stage, bring one end of the agar strip into view. Raise the point until it touches the agar; the moment of contact is easily seen. Lightly press the bellows and note the exit of bacteria. If, for example, only three emerge, it is worth while to pick up two leaving only a single germ. This is accomplished by bringing the point just under the germ, and raising it until it touches the agar. Often, the bacterium floats into the hole. If not, or if say ten germs emerge it is best not to waste time but rather to move to a new portion of agar (by means of the mechanical stage) and again try to expel only one germ. This may sound rather difficult, but actually by varying the pressure on the bellows and by allowing the pipette point just to touch the agar or to be slightly buried in it, ten to twelve isolations may be made in five to ten minutes. When one is certain that only one germ is lying free the area is ringed with a nose piece diamond marker. The writer has been accustomed to make from eight to ten isolations on one plate, arranged in two rows. Having finished the isolations, the pipette is lowered and withdrawn from the chamber, and the cover slip is carefully detached and placed over the concavity of the hollow slide. Vaseline is used to lute it in position. The slide is then incubated at 37° C. If one wishes to observe the progress of multiplication, the slide may be examined at frequent intervals, the individual germs being easily found in the ringed areas. Otherwise the slide is removed and examined after twenty-four hours and those areas containing colonies noted. The colonies may be removed in two ways, (1) by the use of the naked eye and a fine platinum loop or needle or (2) the cover slip is again placed on the moist chamber and the desired colony emulsified in a drop of broth expelled on to it from a coarse micro-pipette and the emulsified bacteria allowed to run back into the capillary and finally expelled into a tube of nutrient. With the first method one must work quickly as the agar film dries out in a matter of five minutes; with the second procedure, a fresh pipette is needed for each colony. If desired, one may suck up a portion of the emulsified colony in a micro-pipette and then, on a fresh micro-plate, carry out another series of isolations.

Some Practical Considerations.

1. *The Size of the Hole in the Micro-pipette.*—Contrary to what would be expected, a hole just large enough to allow of the passage

of a bacterium has not proved suitable for isolation purposes. The germs do not flow into the pipette easily or at all and further, great difficulty is experienced in expelling them. An aperture two to five times the size of the microbe has given the best results.

2. *The Density of the Inoculum.*—This should be such that when the pipette point is raised into the inoculation droplet, each individual germ should be observed to enter. When they enter *en masse*, the suspension is too dense.

3. *The Thickness of the Agar Film and the Spacing of the Isolations.*—The film should be as thin and even as possible and its area should be such that when the cover slip is luted to the hollowed slide, no part of the agar touches the slide. The distance between each isolation should be so arranged that the resulting colony can be picked without danger of touching another colony.

4. *The Bellows.*—As previously stated, this should be made of thick rubber with no or only a small intermediate bulb. If the type with the net-covered intermediary bulb is used, the pressure can be released only by disconnecting the bellows from the pipette. One must be able to apply or release the pressure at will. Further, considerable pressure is often required to expel the germs.

5. *The Magnification Required.*—The writer has found it of advantage to use as low power an eye piece as possible, otherwise loss of definition with resultant eye strain occurs. For large germs such as the anthrax bacillus, and also for bacteria as *Cl. welchii* and *Cl. septicum* a Zeiss No. 5 eye-piece with a No. 40 objective have proved suitable (according to the Zeiss company this gives a magnification of X 200). When higher magnifications are required the oil immersion lens should be brought into use. With a suitably prepared agar film, sufficient working space is still available for this lens. A higher power eye piece (the Zeiss No. 7 or 10) should be used only to make certain that a single germ is lying in the field; it is not advised to carry out the actual isolation under such a lens. The condensor must be adjusted so that the bacteria stand out sharply. With ordinary daylight as illuminant, the writer has found that the lowering of it half to three quarters of its full distance gives the sharpest definition.

6. *Actively Motile Bacteria.*—The method detailed is not suitable for the isolation of germs such as *B. proteus;* a confluent film will be produced, rendering the work valueless. One may still employ the agar film method, but one bacterium only may be implanted on the whole surface. Rather should one isolate the microbes in droplets of liquid medium, in which case the cover slip should be carefully prepared as described in detail by Gee and Hunt (1928). Or one may employ the standard technique of isolating the germs in droplets and transferring these to tubes of medium.

7. *Percentage of Positive Results.*—With germs such as *B. anthracis, Bact. coli, B. typhosus, B. pullorum* and *staphylococci* one may expect six to ten of ten isolated cells to multiply. As one approaches the more " fastidious " microbes, as streptococci, the percentage of positive results drops, but the writer has had no difficulty in obtaining three to six positive results out of ten isolations of a delicately growing streptococcus.

8. *Application to Anaerobes.*—It was with this aspect that the writer was chiefly interested.

The culture to be used for isolation was, in the case of *Cl. welchii* and *tertium*, an eighteen hours' surface culture of the germ. The same could also be used for *Cl. septicum, bifermentans, sporogenes and oedematiens* but experience showed that a young (twelve to eighteen hours) serum-cells-liver broth culture gave better results. Ordinary infusion broth was boiled for fifteen minutes, then cooled, the mixture added, and after heavy inoculation (to ensure rapid growth) was incubated in a gas-filled jar. A young meat broth culture could not be used owing to the presence of tiny meat particles.

Fortner's (1930) *B. prodigiosus* technique proved to be the best method of securing anaerobiosis. A slight modification was introduced. Instead of seeding a portion of the micro-plate with culture, the whole of two to four 18 hours' agar slope cultures was smeared on the concavity of the hollowed slide. By such technique, germination of *Cl. welchii* was repeatedly observed after three to six hours' incubation. Experience showed that if multiplication had not commenced after twenty-four to thirty hours, it did not take place at all (micro-plates observed for seven days). By this modified Fortner technique, positive results were easily obtained with *Cl. welchii* (Types A, B, C and D), *tertium, bifermentans and sporogenes*, and with somewhat more difficulty with *Cl. septicum, oedematiens, tetani* and *sordelii*. However, the greatest difficulty was experienced with *Cl. chauvœi* and out of about 150 isolations, multiplication was obtained in one instance only. A considerable number of variations was introduced in attempts to induce the single germs to multiply, but without success. Among them may be mentioned the following:—

(*a*) *B. prodigiosus* was replaced by *Bact. coli* and by *B. Proteus* OX 19 and by Dr. Fortner's own strain of *B. prodigiosus*.

(*b*) On the supposition that air leakage could occur through the vaseline seal, the set-up micro-culture (with *B. prodigiosus*) was incubated in a MacIntosh's and Fildes' jar.

(*c*) Cystein hydrochloride was incorporated in the medium.

(*d*) Cystein hydrochloride was incorporated in the medium and in addition, the micro-plate was dropped on to the surface of the same medium in a Petri plate and incubation carried out in a MacIntosh's and Fildes' jar (see Frei and Hall 1931).

(*e*) The micro-plate with the isolated germs was set up as one side of the apparatus (made on a small scale) described by the writer (1930) for obtaining shake cultures of anaerobes.

(*f*) Little chambers through which hydrogen could be passed or in which pyrogallic acid and NaOH could be placed were constructed and the micro-plate luted thereon.

(*g*) The method of isolating in droplets of fluid and removing each germ to a tube of suitable medium (Robertson's meat broth plus haemolysed cells, serum and liver extract) was carried out in about 50 instances with one positive result. The tubes were incubated for at least three weeks before being discarded.

(*h*) Obtaining sporulating cultures on Dorset's egg medium as recommended by Henderson (1932) and isolating only those germs with spores did not lead to positive results.

(*i*) The use of different media did not lead to positive results. Amongst those tried were—Viljoen's and Scheuber's medium (Viljoen and Scheuber, 1927) (a liver extract plus peptone), Colebrook's ox liver tryptic digest medium and horse-flesh infusion agar plus 0·5 per cent glucose and the serum-cells-liver mixture. No positive result was got with 0·25 per cent. sloppy agar as an inoculum.

(*j*) A small circular hole was cut in a glass slide and over this were placed sterile pieces of thin celluloid, on the under surface of which single germs were isolated. These pieces were then dropped into meat broth. Not one of the isolated germs grew.

(*k*) On one occasion, eight isolations were made on an agar film whilst hydrogen was slowly bubbling into the chamber. The results were negative.

In the foregoing attempts three strains of *Cl. chauvæi* were used.

As the solid and liquid media used for isolation gave excellent results when large quantities of culture were seeded, one may presume that only a very odd germ was viable or the exposure to air during the isolation killed them.

Percentage of Positive results with Anaerobes.—With *Cl. welchii* and *tertium* from five to eight of ten implanted cells should grow; with *Cl. septicum*, *bifermentans* and *sporogenes* two to five; with *Cl. oedematiens*, *tetani* and *sordellii* two to three and with *Cl. chauvæi* about 1 per cent.

Comment.

It is possible that, to one unfamiliar with micro-manipulation, the technique as described may appear more difficult than it really is. In actual practice, the writer has usually been able to carry out the whole process, from the making of the pipette to the placing of the micro-culture (ten to twelve isolations) in the incubator, within 45 to 60 minutes. With large germs and with a suitable pipette, ten isolations should not occupy more than 5 to 15 minutes' time. Small bacteria entail more time in their isolation, caused chiefly by the period spent in assuring ones' self that only one microbe has been expelled. As explained, this can best be ascertained by carrying out he isolation under an oil immersion lens with a low power eye piece.

The chief difficulty that may be encountered is in inducing the single bacteria to grow. When fastidious germs are being worked with, endeavours should be made to devise media and/or conditions most suitable for their growth requirements.

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