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Modifications of Micromanipulative Practice Suitable for Single Cell Isolation and Cultivation of (a) Aerobic and Transiently Chain-forming, (b) Lipophilic and (c) Micro-aerophilic Bacteria

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SUMMARY: The methods described allowed successful cultivation of 60 % of single cell isolations in the case of an aerobic, transiently chain-forming and polymorphous organism; 40 % in the case of an organism of related morphology and similar ready viability, but presenting technical difficulties because of the lipophilic nature of the surface membranes; and approximately 2 % in the case of a micro-aerophilic or anaerobic organism of restricted viability.

The commonest test objects for bacterial single cell isolations have been such cells as staphylococci or coliform bacteria which are not only monomorphous in aspect, but which grow and withstand micromanipulative treatment with equal ease. In the course of some years' experience with cells that are constantly polymorphous, and frequently difficult if not impossible to cultivate singly in accordance with the standard technique of de Fonbrune (1949), certain modifications of practice were adopted which form the substance of this communication. Throughout the work a de Fonbrune microforge was used for making the instruments, while for the manipulations the de Fonbrune micromanipulator was employed in conjunction with a Watson Bactil binocular microscope having a 3 mm. apochromatic objective. Illumination was given by a Pointolite lamp. The detailed procedures will be described, under the appropriate group heading, for aerobic and transiently chain-forming organisms, lipophilic organisms and anaerobic and micro-aerophilic organisms.

AEROBIC AND TRANSIENTLY CHAIN-FORMING BACTERIA

Under this heading we have placed those organisms which present no difficulty as regards viability of the individual cell elements, but which produce a coherent complex of cells liable to subdivision in varying ways, while the cells themselves are outstandingly polymorphous. Here the problem was to secure a number of cultures of known provenance (from rod, coccoid cell, filament, etc.), while establishing the mode of growth in each case. *Nocardia turbata* (see Erikson, 1954*a*) was selected as an example of this class.

Methods

Several rows of sterile droplets of medium were deposited in the normal way on the underside of the coverslip of a de Fonbrune oil chamber. In each experiment the position of these droplets was carefully charted on squared

paper. The medium employed was nutrient broth. The pool of cells used as inoculum generally consisted of young cells, diluted in sterile medium, taken from a 1- to 2-day old nutrient agar slope. When the desired number of cells had been transferred into the several droplets, their position and shape (long or short filament, with one or more branches, rod or coccoid cell) were noted on the chart. A proportion of separated cells were left to grow in the droplets in the oil chamber under visual inspection. The remaining cells were then transferred one by one to small Durham tubes containing 0.1 ml. medium. Incubation was at 25°. The instruments used for the transference of cells were micro-loops and micropipettes of varying diameters. Frequently it was found desirable to remove small aggregates of cells from the pool by means of a coarse micropipette to a number of medium-sized droplets, and allow them to grow for 1-2 days before picking out the single cells into small droplets with a small microloop. This ensured vigour of the minute stock of cells dealt with in any one experiment.

In the second stage of the operations—growth from micro-colony to normal culture—those single cells that had multiplied within their droplets in the oil chamber were removed by means of hand-drawn mouth pipettes to Durham tubes containing 0.5 ml. medium; and then, as soon as visible growth appeared, they were transferred by Pasteur pipette to 4 in. culture tubes containing 2 ml. medium. Where the development of separate cells had taken place on agar blocks on slides maintained in a moist chamber, the ensuing growth was taken up (after microscopical observation) in a drop of broth in a mouth pipette and sown into small tubes of liquid medium, thence to nutrient agar slopes. The progeny of all single cells carried the chart labels of their parent on the micromanipulating slide. Single cell isolations were made twice weekly until the development of each type of cell was established. Varying with the ease with which a particular batch of cells could be manipulated, the number of cells isolated was generally between fifteen and thirty in any one experiment. The usual precautions of bacteriological asepsis were observed throughout.

Results

Little difficulty was experienced in working with the cells of *Nocardia turbata* provided they were young, filamentous and non-motile. The very small coccoid cells that have been described in the earlier paper (Erikson, 1954*a*) as resulting from fragmentation of aged growths on nutrient agar or other substrates were subject to Brownian motion and too difficult to see and apprehend under the 3 mm. dry objective used throughout the micromanipulating operations. The short motile rods and filaments could be picked up from time to time, but their pursuit was necessarily slow. It was found easier and quicker to remove non-motile filaments and rods for experimental work, while noting the position and observing the subsequent growth of any coccoid or motile cells left behind in the droplets. All cells developed in the same way—by elongation, branching, subdivision into small mycelia—but the rate of progress varied with different cells, and subdivision might frequently precede branching.

The following is an example of vigorous unchecked growth. The medium employed for the droplets was nutrient broth, the temperature at which the manipulating slide was incubated (in a moistened metal slide box with tight fitting lid) between operations was 25°. In one droplet (A) four short filaments were deposited from a pool of 1-day cells taken from a nutrient agar slope.

1st day: each filament increased in length;

2nd day: filaments had grown together into a small mycelium already showing fragmentation, with a few motile cells at large; nine long filamentous cells were transferred to separate droplets;

3rd day: five of the nine filamentous cells had grown with 6-8 branches; many of these cells were already fragmented, or separated apart with the intrusion of a microloop; in some instances small coccoid cells were beginning to develop. The progeny of the five single cells were now removed by microloop to separate Durham tubes containing 0.5 ml. medium as follows:

A1 5 filaments transferred: 1 long filament and 1 short coccoid cell remain in droplet;

A2 6 long cells transferred and 1 very short cell remains;

A3 5-6 long filaments transferred; no cells remain;

A4 2 filaments removed and 2 short cells remain;

A5 (1) } 3 out of 7 filaments in drop removed;

A5 (2) } another 3 filaments removed; 1 left in drop.

Meanwhile those portions of the original filaments left in droplet A, which had been replenished with fresh medium by means of a micro-pipette, have grown again into a ramifying network showing fragmentation with the production of some actively motile cells; of these 13 were removed to separate sterile droplets;

4th day: 9 of the 13 cells had multiplied, and the microcolonies were removed to little tubes—series B; when replenished with medium the coccoid cells remaining in A1 and A2 had elongated; 6 other single filaments from another sub-pool transferred directly by means of a micro-pipette carrying minute agar columns (de Fonbrune, p. 165) to Durham tubes—series G;

5th day: A2, A3, A5 (1), in the little tube series all showed visible minute granular growth with clarity of medium; the short cells that had elongated in droplets A1 and A2 were now showing fragmentation of their branched filaments;

6th day: 7 of series B and 3 of series G now showed tiny granules of growth with clarity of medium in the small tubes; in the 3 tubes of the A series the granular growth had now disintegrated and multiplied with the result that there was a slight turbidity of the medium;

7th day: the granular growths in series B and G were disrupted with a diffusion of cells through the slightly turbid medium; normal mass cultures were obtained from all these tubes.

Sixty per cent of all attempted single cell isolations of *Nocardia turbata* were successful, and it required 7-10 days for the preparation of a macroculture

from a single cell. The repeated separation into fresh droplets of the different types of cells as they appeared allowed visual inspection of every stage of growth, from an individual cell point of view. On the other hand, the sowing of such single cells of varying shapes into small tubes of media made evident a stage which could not be demonstrated by other means. Because of surface tension factors and the varying depths of the droplets, single cells are frequently apt to divide into a number of rods or coccoid cells (see history of droplets A1 to A5), before branching or chain-formation take place. In the very much larger volume of liquid contained in a small tube (0.5 ml.), this early subdivision apparently did not take place, and the organism was able to produce the initial coherent growth characteristic of its family group. In this way only was it possible to demonstrate a phylogenetic relationship, since the use of even very dilute suspensions as inocula for mass cultures constantly resulted in turbidity of growth from the beginning.

LIPOPHILIC BACTERIA

This class comprises organisms which grow readily on suitable substrates, but which offer considerable resistance to the manipulator because of the nature of their surface membranes. The example taken was a paraffin-utilizing organism, *Nocardia sebivorans* (see Erikson, 1954*b*). This species is further distinguished from *N. turbata* by the relative stability of the cell-mass formed (liquid cultures being consistently clear), and by the development of a secondary aerial growth the component cells of which are hydrophobe in nature.

Methods

When attempts were made to grow *Nocardia sebivorans* in the same manner and in the same complex nutrient broth and agar as had been used for *N. turbata*, which it was thought would afford the maximum chances of survival for isolated cells, various difficulties of a practical nature were encountered at the outset. They were concerned with the following points.

(1) *Nature of the inoculum.* A 1-day growth on nutrient agar required mechanical disruption by shaking, which was readily accomplished but with a consequent diminution of viability. A 7-day growth in similar circumstances yielded a non-homogeneous suspension of fragments from the surface network of filaments together with short aerial cells. These very short cells proved difficult to manipulate in numbers, although several individuals were isolated successfully. Similar objections applied to the material taken from the surface growth on liquids, while the bottom growth in nutrient broth appeared to have a surface coating that resisted seizure by the micro-instruments. The most suitable material was obtained by growing the organism in a simple Czapek glucose nitrate liquid medium, subculturing daily 3 or 4 times, finally taking a small portion of the bottom growth for the initial pool. The longer filaments so obtained separated readily in the droplets, and, since they appeared more refractile than the majority of the cells tried previously, could be seen and picked up more quickly.

(2) *Effect of prolonged growth at 37° upon droplets.* Even with the precaution of placing a large drop of water in the well of the oil chamber, a number of small droplets of medium dried up during the necessary 2–3 days' incubation at 37°, and frequent replenishment by micropipette was needed. A more serious matter, when nutrient broth was used for the droplets, was the occasional formation of a thin skin at the oil/medium interface after incubation. The skin was quite tough, resisting the passage of micro-instruments, and no growth at all took place of any cells within such droplets. It appeared, therefore, that the film prevented the diffusion of air into the droplets. The same broth supported good growth of the organism in ordinary tube cultures, and it may be that the batches of broth which yielded such results were slightly richer in fatty substances than usual, and with incubation this concentration was enhanced. It is known that solutions of sterols and other fatty substances as well as of proteins have the property of forming monolayers, not only at air/water interfaces, but also at oil/water interfaces (see Sexton, 1953, p. 70). When the broth was diluted, the phenomenon did not occur. In general, it was found best to employ Czapek glucose nitrate medium for the droplets.

(3) *Attraction of oil phase for the growing cells.* The lipophilic nature of the organism derived from non-fatty media was very evident when small aggregates of cells were placed in sub-pools, and within a matter of seconds had swung over to the boundary of the drops, generally with the longer branching filaments projecting into the paraffin oil of the chamber. When single cells were placed in droplets, they tended to lie close and with their long axes parallel to the boundary. After incubation, it seemed merely a matter of chance whether or not the resultant growth was still partially contained within the droplet. In fact, it was frequently found that when a small complex of cells had developed at the margins of a droplet, it migrated overnight into the oil phase and was lost. While, on the other hand, droplets which had been sterile, and to which nothing had been consciously added, might become the hosts of one or more cells or aggregates of cells that were at large in the oil phase. There was, of course, no question of motility on the part of any of these cells, merely one of physical attraction (cf. Webley, 1953).

Different oils were then tried as substitutes for paraffin oil. The vegetable oils such as olive oil, castor oil, etc., were unsatisfactory, either because of poorer visibility or because they did not permit good growth. Polyrice oil (Edward Gurr Ltd.), which is used as an immersion oil, allowed very good growth and visibility, but it was found that the aqueous droplets did not adhere very well to the coverslip, and the growing cells were readily attracted into the oil phase. Non-oily substances that are non-miscible with water such as diethyl phthalate were not sufficiently viscous for incubation in the chamber. The use of the standard paraffin oil chamber was therefore continued, but as a rule only for the minimum period of time required for separating and charting the cells. Many pools of cells might be grown successfully and their course of development observed in the manipulating slide, but because of the lack of effective boundary at the oil/medium interface, individual cells were removed by micropipette to small tubes or slide cultures.

Cultivation of the small Durham tubes of liquid media was as previously described. Slide cultures employing solid media were made in order to obtain visual inspection of the development of the separate cells. The latter were delivered on to the moistened surface of a small block of nutrient agar on a slide in a sterile Petri dish, the block being immediately capped with a sterile coverslip and the cover of the dish replaced. When 18–20 slides were prepared, they were lifted with sterile forceps on to metal trays, each of which slid horizontally into its place in the internal scaffolding of a sterile metal container (see Pl. 1, Figs. 1, 2). The upper and lower sectors contained sterile wadding which was moistened with water, and so maintained a very efficient moist chamber during prolonged incubation (1–2 weeks). The container used was an especially stout cupro-nickel drum built to withstand evacuation for anaerobic cultivation, the only gas ingress being at the two leads which were fitted with pieces of cottonwool-plugged sterile glass tubing. It is probable that a less substantial tin or glass container would serve equally well for aerobic growth. The use of a single container for a number of slides was, however, found preferable to a large number of individual Petri dishes lined with moistened filter-paper. Where it was desired to follow the development of the secondary aerial cells, without the restriction imposed by coverslips, individual slide boxes were employed. These were made of the same cupro-nickel as the large vessel, had close-fitting lids, and were provided with two ledges on which the slide rested, while the space below was packed with sterile moistened absorbent cottonwool (see Fig. 1). Such slide boxes, made in two widths to fit ordinary slides or the broader micromanipulating slide, had the advantages of maintaining the slide cultures in position, preserving humidity over considerable periods, and economizing in incubator space.

Fig. 1. Slide box for moist incubation of droplets containing single cells. $\times 1$. *a*, *a*, supports extending whole width of box; *b*, moistened absorbent cottonwool; *c*, tight-fitting lid. *d*, standard manipulator slide with coverslip and oil chamber.

Results

Forty per cent of all attempted single cell isolations of *Nocardia sevivorens* were successful, and it required 3–4 weeks for the preparation of a mass culture from a single vegetative cell. This lengthy period was due in part to the necessity of the repeated transfers that have been described, and to the several lags so induced. Where growth could be kept under observation in the droplets on the slide, and the microcolony then removed to a small culture

tube in the normal way, cultivation was more rapid. Similarly, in those instances where the very minute aerial cells of a culture were transferred by microloop directly to small Durham tubes of nutrient broth, tiny granules of visible growth appeared within 2 days, and the mass culture could be obtained in 7–10 days. On the whole, more consistent results were secured with the slower methods.

ANAEROBIC AND MICRO-AEROPHILIC BACTERIA

In his account of the paraffin oil chamber as a menstruum for the cultivation of aerobic micro-organisms within aqueous nutrient droplets, de Fonbrune (1949) demonstrated that sufficient air would diffuse through the oil to enable the strictest aerobes to grow. He further stated (p. 136) that 'Par contre, nous n'avons pu obtenir, dans ce dispositif, des cultures de germes anaérobies, même en purgeant préalablement l'huile des gaz qu'elle tenait en dissolution.' We can confirm this, having tried very many times to cultivate the anaerobic *Actinomyces israelii*, which is sometimes regarded as micro-aerophilic, within the oil chamber, and having constantly failed in spite of the following precautions: flushing with nitrogen the tube containing the parent inoculum as well as the micromanipulating slide and the medium used for the sterile droplets just before operations, and removing the finished slide to the anaerobic jar immediately afterwards. No growth whatever occurred, no matter what additions were made to the medium, although minute just visible granules of cell material transferred to small Durham tubes containing suitable liquid substrate gave rise to good growth when incubated in an anaerobic jar. Accordingly, it seemed advisable to have recourse to the principle adopted with the lipophilic organisms (see previous section), and not to allow the cells to remain in the manipulating slide any longer than necessary.

Methods

The characteristic granular growth of *Actinomyces israelii*, which always leaves the liquid substrate clear, required mechanical disruption for the purposes of obtaining an initial pool of clearly visible cells; the cells which tended to fragment most easily were the shortest cells, which were correspondingly difficult to manipulate. In this group of organisms the cells which are compelled to grow against a plane surface, whether nutritive like agar or non-nutrient such as cellophan or coverslip, have frequently been found (Erikson, 1949) to be proportionately longer than those which have been allowed to develop unimpeded in a liquid substrate. Consequently, the most suitable cell material from a visual and handling point of view was the 5 to 7-day growth between coverslip and agar block that had developed in slide cultures incubated in the anaerobic drum. The small diphtheroid forms more common in the break-up of liquid growth were also employed. The type of each cell unit was charted as usual. The medium employed throughout was the stock (S) medium (glucose + heart broth + casein digest) of Erikson & Porteous (1953).

Individual cells were transferred by micropipette to:

- (i) small Durham tubes containing liquid broth;

- (ii) similar tubes containing the same medium with the addition of 0.2 % agar, melted and held at 45°;
- (iii) the same medium containing 0.1 % agar and which filled approximately 1 in. of glass tubing (c. 4 mm. diam.) held diagonally within a small screw-capped bottle by a layer of solid (2 %) agar medium at the bottom (see Fig. 2); after inoculation, during which operation the micropipette was plunged into the viscous medium inside the tubing and the contents washed out by repeated sucking-up and expulsion, the bottle was cautiously filled up to the lip and over the top of the tubing with sterile boiled water from which all air had been driven; and the metal cap screwed tightly down;
- (iv) the moistened surface of agar (2 %) blocks of medium on slide cultures, which were then placed in the anaerobic drum described in section (b), after being capped with sterile coverslips.

Cultures (i) and (ii) were incubated in an anaerobic jar in the ordinary way; and the anaerobic drum (iv) was also evacuated and filled with nitrogen or hydrogen; cultures (iii) were incubated directly. The temperature of incubation throughout was 37°, and the time of incubation 7–14 days.

Fig. 2. Screw cap bottle for anaerobic cultivation from a single cell. $\times 1.5$. a, 2 % agar medium; b, 0.1 % agar medium; c, sterile, freshly boiled water.

Results

Only 2.8 % of attempted single cell isolations of *Actinomyces israelii* were successful, and it required 2–3 weeks for the preparation of a mass culture from a single cell unit. The distribution of successes according to methods was as follows: in (i) none out of 37 attempted; (ii) 2 out of 43; (iii) 1 out of 33; (iv) 1 out of 60. The superiority of the semisolid to the liquid or solid medium

D. ERIKSON & F. M. MASSON—MODIFIED METHODS OF SINGLE CELL CULTIVATION.
PLATE 1

is evident. It is also noteworthy that relatively more profuse growth occurred more quickly in (iii), where the semisolid medium was contained in a continuous aqueous phase than in (ii), which was incubated in a gas phase. The necessity of making large numbers of slide cultures to watch the growth-stages of cells whose viability ratio is so low is also apparent. Yet, on the whole, by means of the combination of methods described here the proportion of successes was as high or higher than would be expected of an anaerobic organism well known as not easy in cultivation.

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EXPLANATION OF PLATE

- Fig. 1. Anaerobic drum, with outer lid removed, showing inner lid, lined with gauze at side, and internal scaffolding carrying slide trays. One large tray with moistened wadding is pulled out of position at bottom. Another similar tray fits into the top compartment.
- Fig. 2. Anaerobic slide-carrier drum, battened down and fastened to board, with lid containing the gas leads tightly screwed in place.

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