JOHNSTONE, K. I. (1953). *J. gen. Microbiol.* 9, 293-304.

Micromanipulation on an Agar Surface for the Isolation and Cultivation of Single Organisms

By K. I. JOHNSTONE

Department of Bacteriology, University of Leeds

SUMMARY: Organisms may readily be isolated and cultured separately by micromanipulation on the upper surface of an agar block. A micromanipulator is not essential, its place being taken by a second microscope with simple attachments. Illumination methods by dark-ground and phase-contrast techniques are described. A modification using a simple micromanipulator enables isolations to be made very rapidly. A device is described for making the necessary micro-instruments in a standard microscope.

The cultivation of single organisms or their spores by manipulation on the upper surface of an agar block has several advantages: (1) the organisms show clearly by dark-ground or phase-contrast illumination; (2) the microneedles, being above the agar surface, are constantly visible, centration is easy and accidents are minimized; (3) the selected organism is carried 0.5-1.0 cm. across a sterile agar surface, giving many opportunities to observe that it is single, and contamination from the other organisms is eliminated; (4) the position of the isolated organism is marked by melting pits in the agar around it (Johnstone, 1948), which, being visible to the unaided eye, enable the agar block to be dissected and the single organism to be carried on a portion of the block to any suitable medium for culture; (5) a micromanipulator is not essential but it can be used to accelerate the isolations. The disadvantage is that dry lenses only can be used, with a maximum useful magnification of $\times 600$, which is, however, adequate for most bacteria. The method has been successfully used for bacteria and their spores, the spores of fungi, amoebae and their cysts.

APPARATUS

The agar block. Either nutrient agar or a plain agar gel can be used, but it must be very clear, especially for dark-ground illumination. A 3×1 in. slide is mounted above a 3×1.5 in. slide, being separated from it at each end by a glass strip 2 mm. thick, the whole being clamped by wire paper clips and sterilized by dry heat in a Petri dish. After warming the slides to 60° , melted agar is run into the space between them with a bent capillary pipette and is allowed to set. The clips are removed, the upper slide is slid off and the agar slab is cut into blocks 2.5×1.0 cm. with a sterile stainless steel knife. Each block is lifted with the knife on to a sterile 3×1.5 in. slide enclosed in a Petri dish, with moist cotton wool wads to prevent drying and distortion of the plane agar surface.

A sterile Perspex cell (Fig. 1) enclosing an area 1.6×3.5 cm. protects the agar from dust and decreases evaporation. For methods 1 and 2 an aperture *A*

at one end admits the microneedle shaft, and the cover is of Perspex with a central raised collar *B* which just admits the nose of the 4 mm. objective. The cover slides in grooves in the cell, thus allowing movement of the objective relative to the cell and agar during manipulation in the lateral direction, whilst the whole cell moves on the slide during the short transverse movements. For method 3, the cover is replaced by a sterile rectangular cover-glass 1 × 2 in. and a second opening is provided for the marking needle, at the right-hand end of the cell, when a micromanipulator is used.

Fig. 1. The Perspex cell and cover. The enclosed area of the slide is stippled. *A*, aperture for microneedle shaft; *B*, collar admitting objective.

The manipulating microscope. This replaces a conventional micromanipulator and must have sensitive coarse and fine adjustments. The following attachments are used:

(1) *The nosepiece attachment.* A brass plug *A* (Fig. 2), with the R.M.S. objective thread, is screwed into the rotating nosepiece. The plug carries a brass arm *B*, rotating about the screw *C* and locked by the clamp nut *D*, so that the arm lies across the microscope stage with the socket *E* on the right-hand side when the plug is in the optical axis. A screw *F* clamps the microneedle-holder, or the marking needle, in the socket *E*.

(2) *The microneedle-holder.* A steel rod *G* (Fig. 2) fits into the socket *E* of the nosepiece attachment. The terminal portion *H*, 3 cm. long, is reduced to 2.5 mm. diam. and carries a socket with clamp screw *I* for the handle of the glass microneedle. A knurled brass collar *J* enables the holder to be rotated for correct orientation of the needle tip. The length from the optical axis to *I* should be approximately 14 cm.

(3) *The microneedle.* The most useful type for the isolation of bacteria, bacterial spores and organisms of similar size has (a) a handle 2 cm. long and 1 mm. in diameter, drawn from 6 mm. soft glass rod by hand; (b) a shaft 2.5 cm. long and 0.1–0.2 mm. in diameter, drawn out over a microburner and inclined slightly downwards relative to the handle so as to clear the agar

Fig. 2. The nosepiece attachment (left) and microneedle-holder (right).

Fig. 3. The platinum marking needle and mount.

surface except at the tip; (c) a tip, inclined to the agar surface at an angle of 30° and $1\text{--}5\mu$. in diameter at the end. (The microburner is simply a minute coal-gas flame burning at the end of a vertical fine capillary tube.) Simple tips can be made merely by touching the end of the shaft momentarily against an electrically heated platinum filament (36 s.w.g.) at bright red heat and by drawing away at an angle. They are best made, however, under microscopic observation as described below.

(4) *The marking needle.* The steel rod *A* (Fig. 3) fits into the socket of the nosepiece attachment and carries a brass block *B*, with two steel rods *C* and *D*, 2.5 mm. in diameter, one of which (*C*) is electrically insulated by a plastic or ebonite sleeve *E*. Into the ends of *C* and *D* are soldered thick platinum wires *F* (24 s.w.g.) 2 cm. long, which carry the heating element *G* made from two fine platinum wires (36 s.w.g.), one end of each being welded into the

hooked and flattened end of the corresponding thick wire by tapping the joint with a steel rod whilst at white heat in the blowpipe flame. The free end of one wire is hooked around the other at *H* and welded to it, the terminal 2 mm. of the second wire being bent down at an angle of 45° to form the needle tip. The sides of the heating element should be 7 mm. long. Connexion is made to the steel rods at *I* with fine silk-covered wire from a 3 V. transformer (*a*) directly, to raise the 'V' of the element to bright red heat, thus sterilizing the tip by conduction, and (*b*) through a resistance such that the tip is just sufficiently warmed to melt the agar, thus forming the locating pits without causing spluttering of the agar.

Sterilization of the apparatus. The Perspex cell, cover-glass and microneedles are sterilized by exposure for 6 hr. to the vapour of commercial formalin in a desiccator, the upper section being warmed by a 6 V., 3 watt electric bulb to prevent condensation on to the articles. The apparatus is then stored in sterile Petri dishes overnight, when all formaldehyde vanishes.

The observing microscope. This must have a rigid mechanical stage, free from backlash, and preferably an inclined eyepiece since the stage must be horizontal. A 4 mm. objective is used with a maximum N.A. of 0.70 to give sufficient working distance for the microneedle to operate. This aperture is reduced to 0.60–0.65 for dark-ground illumination, or when a long-working-distance attachment is used. A 16 mm. objective is centred on the nosepiece relative to the 4 mm. lens and is mounted on an extension tube to make it par-focal with the long-working-distance attachment if this is used.

Method 1. Dark-ground illumination with short working distance

This is the simplest method, the organisms being well illuminated and appearing larger than by other methods. The disadvantages are (1) the excessive glare from the needle tip often obscures the organism; (2) the intense illumination may cause condensation on the objective due to local heating of the agar, but viability of the cells is usually unimpaired if ultra-violet light is filtered out. Two condenser systems are satisfactory: (*a*) the long-working-distance dark-ground condensers (e.g. the dry 'Zonal' of Watson); (*b*) an achromatic condenser can be adapted to give a long working distance of 3 mm. to focus through slide and agar block (Goldie, Gordon & Johnstone, 1948).

Method 2. Phase-contrast illumination with short working distance and optional dark-ground illumination

The advantages are (1) the microneedle appears without marked glare; (2) the intensity of illumination and heating of the agar are much reduced; (3) organisms can be selected which show marked positive phase-contrast and these (type *A* of Pulvertaft) are more likely to be viable (Pulvertaft, 1952). A 4 mm. phase-contrast objective with adequate working distance is used and a phase-contrast condenser with at least 3 mm. working distance. Alternatively an achromatic condenser modified to give this working distance can

be used, provided that the lower focal plane is accessible for the insertion of an annulus to register with the phase-plate of the objective. If two concentric annuli are used, phase-contrast illumination is obtained by partially closing the iris diaphragm, thus obscuring the outer annulus; dark-ground illumination by obscuring the inner annulus with a patch stop and opening the iris. This transition is valuable during manipulation of organisms. A similar transition is obtained by different means with recent types of condenser by Leitz and Zeiss-Winkel. The inner annulus automatically gives dark-ground illumination with the 16 mm. lens.

Method 3. Phase-contrast illumination with long working distance

A 4 mm. phase-contrast objective is carefully centred within a reflecting attachment (Newton), which lengthens the working distance to 1 cm. The advantages are (1) all manipulations can be done under a sterile cover-glass, greatly decreasing air contamination; (2) the inclined tips of the microneedles can be lengthened, thus raising the shafts well above the agar, to which they may otherwise adhere by capillarity if lowered too far, thus spoiling the agar block; (3) with a micromanipulator the marking needle can be kept in position close to the microneedle throughout the isolations, thus saving much time in recentring the needles.

The disadvantage is the great loss of light in the mirror system, necessitating a powerful lamp (e.g. a 12 V., 60 watt bulb with concentrated filament) and an aplanatic lamp condenser. The beam should be cooled either by a thick water trough or a 8 mm. thickness of Chance's ON 20 glass, to avoid heating the agar. This is the method of choice.

TECHNIQUE

(1) *Inoculation of the agar block*

A faintly turbid suspension of the organisms is made in sterile distilled water. A sterile brass protecting screen 1 cm. high, carried on feet so that its lower edge just clears the agar surface, is placed across the agar block, one-quarter of the length of the latter from the right-hand end, with sterile forceps. This catches droplets of the suspension liberated as the water column between the loop and the agar breaks after inoculation, which may otherwise contaminate the adjacent sterile agar surface. A small (1 mm.) loopful of the suspension is drawn across the right-hand end of the block, without touching the plane agar surface with the loop. Contact is maintained by the column of water between loop and agar, and the surface is not deformed. The fluid evaporates or is absorbed and the screen is removed. Organisms from the left-hand margin of the inoculum are used for manipulation, being readily identified as a well-defined line (Fig. 4a). The sterile cell and cover are placed over the block.

(2) *Setting up of the agar block and centration of the microneedle*

The slide, with block and enclosing cell, is placed on the mechanical stage, the condenser is focused and the left margin of the inoculum is located with

the 16 mm. objective. The 4 mm. objective is then lowered through the cell cover (or simply rotated into the axis for method 3) and the margin of the inoculum is centred in the field. The objective is then raised in methods 1 and 2 for insertion of the microneedle.

Fig. 4. (a) A single organism being drawn away from the edge of the inoculum. The cone of water around the needle tip *A* is shown by the broken line. Direction of movement of the agar is shown by the arrows. Method 3. No apparent image reversal: 4 mm. phase-contrast objective. (b) The positions of the locating pits *C* and *D* in the agar surface relative to the isolated organism *B*. The arrows show the alternative courses taken by the marking needle. The microneedle has been removed. Image reversed: 16 mm. objective.

The manipulating microscope is placed on the left of the observing microscope and, by sliding it on the smooth bench, the sterile microneedle carried in the needle-holder is introduced through the aperture in the cell. The tip is lowered by the coarse adjustment of the manipulating microscope until just above the agar surface and is centred in the field.

(3) *Manipulation of the organism*

The objective and microneedle are lowered alternately in stages, *first* the objective and *secondly* the microneedle, using the coarse adjustments, until the organisms come into focus. The needle is then lowered with the fine adjustment until it just touches the agar, when it draws a cone of water from the gel (Fig. 4*a*). With methods 1 and 2 the image is reversed, but with method 3, owing to the additional reversal in the mirror system, objects appear to be in their actual positions and manipulation is simplified.

The principle, based on that of Koblmüller & Vierthaler (1933), is that if the needle tip *A* (Fig. 4*a*) is lowered over an organism, it will float in the cone of water around the tip and can be carried for 1 cm. or more across the sterile agar by operating the mechanical stage, thus moving the agar relative to

the needle as indicated by the arrows in Fig. 4*a*. The needle point remains centred in the field, its height being constantly regulated by the fine adjustment of the manipulating microscope. Should the organism escape from the cone, the needle is raised, the agar is moved back, the needle is lowered over the organism and progress continued. To assist recognition of the escape of the organism, the needle point should be set slightly to one side in the field. The organism is constantly presented in different positions and the presence of a second organism evident.

For filamentous organisms which will not follow the needle, alternative methods are used: (1) a hooked microneedle encircles the organism at the middle of its length and drags it along (Johnstone, Crofts & Evans, 1950); (2) the normal tip is used to lift the filament from the agar surface and to deposit it on a sterile area. It is then straightened and examined to ensure that no other organisms are present, is again lifted and carried to its final position where it is located in the usual way.

(4) *Marking the site of the isolated organism*

When at least 5 mm. from the inoculum, the position of the organism is marked by melting two or more pits in the agar surface around it. For methods 1 and 2, the microneedle is raised slightly and, with the 16 mm. objective, the organism is located by dark-ground illumination close to the tip of the needle. The microneedle is withdrawn from the cell and the needle-holder is replaced by the platinum marking needle, which is sterilized by the heating current applied for 2 sec. The needle tip is centred in the field, is pressed slightly into the agar surface to one side of the organism (Fig. 4*b*) and the *warming* current is applied for 1 sec. to melt the agar. The needle is raised and the tip is moved, as shown by the arrows, to avoid possible damage to the organism, to the other side of the field where the process is repeated. The marking needle is removed and the organism is re-located with the 4 mm. objective. The stage verniers are read and the block is returned to the original reading of the lateral movement, when the edge of the inoculum is found.

Each selected organism is treated in the same way, but each is taken from a different point on the edge of the inoculum and to a greater distance from the inoculum. Finally, a line of pits marks the limit of the inoculated area. The same microneedle can be used if kept in a sterile tube when withdrawn from the cell.

(5) *Cultivation of the isolated organisms*

Method 1. The entire block, which must contain the requisite nutrients, is incubated in a sterile Petri dish with wads of moist cotton wool to maintain the humidity. The dishes must be insulated from the warm floor of the incubator, either by suspension with cords or by cork insulation. Otherwise marked drying of the agar will occur. Every 2 hr. the agar is cooled (to avoid condensation on the objective) and examined under the Perspex cell on the same mechanical stage. The organisms are located (i) by the stage verniers; (ii) by the locating pits, which move with the organisms during drying and

distortion of the block. Viable organisms usually appear as microcolonies after 8 hr. When the colony contains about thirty organisms, the sterile marking needle is lowered into the colony under observation with the 16 mm. objective, is raised and inoculated to a solid medium either by pressing the tip lightly into the surface, or by encircling the tip with a loopful of sterile broth, which washes off many organisms and is spread on the medium.

This method originated from that of Ørskov (1922), when random-smear blocks were used and it was essential to watch the development of colonies from selected organisms to avoid contamination from adjacent organisms. The method is tedious and now unnecessary except for observation of the mode of fission of the organisms, or when separation of the members of the clone arising from the organism is required (Zelle, 1951; Zelle & Lederberg, 1951), for both of which a heated stage is desirable. The method is unsuitable for anaerobes.

Method 2. The agar block is cut immediately to the left of the line of pits, thus separating the inoculated area, using a small knife made by hammering out thick platinum wire (20 s.w.g.) mounted in a needle-holder. Each pair of pits, with the organism, is cut out in turn with the sterile knife and transferred as a 3 mm. cube of agar to the surface of a 'slope' of solid medium. For an aerobe, the cube is laid with the organism exposed. The pits can be seen and the developing colony observed to arise between them after incubation, if the organism is viable, with a hand-lens or dissecting microscope. For an anaerobe, the organism or spore is trapped between the cube and the medium to facilitate anaerobiosis. Growth appears at the edges of the cube face after incubation in the anaerobic jar.

Method 3. The block is dissected and each cube is transferred to a tube of fluid medium containing the required nutrients. This method should be avoided when handling organisms (e.g. staphylococci) not readily distinguishable from dust contaminants, which may occasionally gain access to the medium during the manipulation of the tubes. This is a good method for anaerobes.

The risk of dust contamination is very slight, provided that draughts are avoided and that the manipulations during which the agar block is momentarily exposed to the air are done deftly and quickly. Several isolations are always made on each of a number of blocks to ensure the selection of several viable organisms and the resulting growths are carefully compared.

*A modified technique using a micromanipulator
to expedite the isolations*

The chief delay is in the replacement and recentring of the microneedle after each use of the marking needle. This is eliminated by mounting the marking needle in a micromanipulator on the right-hand side of the observing microscope. It enters by a second opening in the right-hand end of the cell and, with the long-working-distance attachment, can be kept in position close to the microneedle and below the cover-glass during the isolations. Both needles are centred once only for each agar block.

Using the Singer micromanipulator (Barer & Saunders-Singer, 1948), the marking needle can be mounted in a rocking device held in the micromanipulator and operated by a pneumatic capsule and rubber bulb. The micromanipulator then controls the marking needle in the horizontal plane only and the rocking device causes the warm needle to dip into the agar to a fixed depth and to return, thus forming the pits very rapidly and uniformly.

A device for making microneedle tips in a standard microscope

Of methods for making needle tips under microscopic observation, those of Schouten (1907, 1984), Péterfi (1924) and Thaysen & Morris (1947) require both right- and left-handed micromanipulating devices and that of Hilson (1952) a single instrument. The 'microforge' of de Fonbrune (1937) is self-contained and very complete, but costly. The present method (Johnstone, Crofts & Evans, 1950) requires no micromanipulator, the two attachments for the microscope require only simple lathe work for their construction and the movements of the microscope supply all necessary adjustments except for rotation of the small stage carrying the microneedle.

Fig. 5. A device for making micro-instruments: (a) plan and back elevation of stage attachment; (b) plan and elevation (with partial section) of substage attachment.

(1) *The substage attachment* carries the microneedle on a small stage mounted on a pillar above the microscope stage, but screwed into the substage. Raising and lowering of the needle are therefore effected by the focusing movement of the substage and centration by its centring movement. An adaptor is fitted into the condenser mount carrying R.M.S. female thread. A hollow pillar *A* (Fig. 5b) with R.M.S. male thread at its lower end screws into the adaptor, passes upwards through the mechanical stage and carries the rotating stage *B* and a finely ground cover-glass *C*. Light from the microscope mirror passes up the pillar and illuminates the cover-glass, thus providing a diffused

illumination for the needle. The pillar should be matt black inside and the bearing for the stage should be carefully fitted.

At its edge, the stage carries a rotating brass turret *D*, with clamping nut *E*. The steel needle-holder *F* is adjustable in the turret, being locked by the screw *G*. The microneedle handle *H* is lightly clamped in the holder by a small screw and is adjusted so that the needle shaft is supported almost at its end by the crutch *I* fitted to the turret. The crutch is made by grinding away a portion of the eye of a no. 9 crewel needle, bent to shape, and overcomes the flexibility of the shaft during the pull exerted by the heating element. Centration of the tip is effected roughly by the adjustments of the turret and needle holder and finally by the substage centring movement. Rotation of the stage *B* enables the needle tip to be presented at any angle to the direction of pull of the heating element, thus varying the inclination of the tip. (The stage *B* may be graduated in 6° divisions as illustrated, reading against a zero mark on the central pillar, to enable exact reproduction of a given needle tip.)

(2) *The stage attachment* carries a platinum heating element on a base-plate fitting into the mechanical stage, thus giving motion in two dimensions to the element by operating the stage controls. A brass plate *A* (Fig. 5*a*) 3 × 1 in. forms the base, being cut away in front to allow for the pillar of the substage attachment. The base illustrated fits the attachable 'Service' mechanical stage (Watson). Steel pillars *B* and *C* are mounted in brass sockets, with insulating sleeves, on the base-plate and brass blocks *D* and *E* are clamped on the pillars by locking screws *F*, enabling the element to be adjusted for height. Steel rods *G* and *H* project forwards from the blocks above the rotating stage and hold a platinum heating element *I*, made as described for the marking needle, but with a simple V-shaped element (86 s.w.g.). Electrical connexions are made to the bases of the pillars with fine silk-covered flexible wire *J* from a 3 V. transformer, with a rheostat and a foot-switch in the circuit.

Protection from mechanical damage and from over-heating can be given to the 16 mm. objective by fitting a brass cell to the front of the objective, with a protecting cover-glass and a connexion to a filter pump, by which means a current of cool air is drawn across the front lens.

A method for making simple needle tips

With the microscope stage horizontal, the tip of the shaft is centred in the field of the 16 mm. objective and the heating element is brought almost to touch the shaft and is focused accurately. The needle shaft is brought into sharp focus by the substage rack and pinion, being then inclined by rotation of the stage to give the required tip angle (normally 30° with the horizontal). The stage must be turned so that the traction from the element forces the needle shaft into the crutch (Fig. 6*a*), thus eliminating the flexibility of the long shaft. The foot-switch is closed and the rheostat adjusted to bring the element to red heat. On bringing the point of the element into contact with the end of the shaft, the glass fuses on to the platinum and, on withdrawing the element quickly, the glass is drawn out to form the needle tip. By controlling (*a*) the temperature of the element, (*b*) the rate of pull, and

(c) the time at which the current is cut off by the foot-switch, the length, steepness of taper and terminal diameter of the tip are controlled. A short tip tapering rapidly is ideal as flexibility in the tip must be avoided. It is a great advantage to prepare the tip with a double bend (Fig. 6*b*), thus giving the needle shaft good clearance above the agar surface, if the working distance will permit.

Fig. 6. Making a microneedle tip: (a) the end of the shaft *A*, held in the crutch *B*, fused on to the platinum element *C* which is moved as shown by the arrow; (b) the completed tip showing the double bend, steep taper and angle of inclination (30°). Image reversed: 16 mm. objective in both (a) and (b).

Hooks and loops are formed from straight tips by stroking the end of the glass filament with the platinum element at a low temperature until the required curvature is obtained. Directions for making many micro-instruments are given by de Fonbrune (1949), for which the above attachments can be used. When traction by gravity is required (as in the making of micropipettes), the microscope is used in the horizontal position, with the inclined eyepiece turned upwards. An air-blast, as described by de Fonbrune, is not essential for the simpler operations, but can be added as a separate attachment if required.

REFERENCES

- BARER, R. & SAUNDERS-SINGER, A. E. (1948). A new single-control micromanipulator. *Quart. J. micr. Sci.* 89, 439.
- DE FONBRUNE, P. (1937). *Micromanipulateur pneumatique et micro-forge pour la fabrication des micro-instruments*. Société Industrielle d'Imprimerie, 10 rue Vallier, Levallois-Perret, Paris.
- DE FONBRUNE, P. (1949). *Technique de micromanipulation*. Paris: Masson et Cie.
- GOLDIE, W., GORDON, M. & JOHNSTONE, K. I. (1948). Mutation in a single-cell culture of *Corynebacterium diphtheriae*. *J. Path. Bact.* 60, 369.
- HILSON, G. R. F. (1952). A simple and inexpensive microforge. *J. gen. Microbiol.* 7, 175.
- JOHNSTONE, K. I. (1943). A simple technique for the cultivation of organisms from single cells. *J. Path. Bact.* 55, 159.

- JOHNSTONE, K. I., CROFTS, J. E. & EVANS, D. G. (1950). Single cell culture of *Cl. welchii* type A morphologically changed by penicillin. *Brit. J. exp. Path.* 31, 562.
- KOBLMÜLLER, L. O. & VIERTHALER, R. W. (1933). Über ein Gerät zum Isolieren von Keimen auf der Oberfläche fester Nährböden ('Plattenmanipulator'). *Zbl. Bakt.* (1. Abt. Orig.), 129, 438.
- ØRSKOV, J. (1922). Method for the isolation of bacteria in pure culture from single cells and procedure for the direct tracing of bacterial growth on a solid medium. *J. Bact.* 7, 537.
- PÉTERFI, T. (1924). *Mikrurgische Methodik*. Abderhalden, *Handbuch der biologischen Arbeitsmethoden*, Abt. 5, Teil 2, Heft 5, 479.
- PULVERTAFT, R. J. V. (1952). The effect of antibiotics on growing cultures of *Bacterium coli*. *J. Path. Bact.* 64, 75.
- SCHOUTEN, S. L. (1907). Methode zur Anfertigung der gläsernen Isoliernadeln, gehörend zu dem Isolierapparat für Mikroorganismen. *Z. wiss. Mikr.* 24, 258.
- SCHOUTEN, S. L. (1934). Der Mikromanipulator. *Z. wiss. Mikr.* 51, 421.
- THAYSEN, A. C. & MORRIS, A. R. (1947). The preparation of microtools for the micromanipulator. *J. gen. Microbiol.* 1, 221.
- ZELLE, M. R. (1951). A simple single-cell technique for genetic studies of bacteria. *J. Bact.* 61, 345.
- ZELLE, M. R. & LEDERBERG, J. (1951). Single-cell isolations of diploid heterozygous *Escherichia coli*. *J. Bact.* 61, 351.

(Received 1 April 1953)