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These laboratory notes are intended for research assistants without any previous training in microbiology. They apply specifically to requirements at Merlewood Research Statior , and, therefore, are not in any way an attempt to produce a comprehensive manual of microbiological procedures. An introduction is given to the general principles which must be acquired by anyone handling fungi and bacteria. In addition, general techniques, selected for our current research projects, are described with modifications for the facilities available at this Station. The information will be up-dated as new research programmes are developed and further equipment acquired.

Instructions for operating particular models of equipment, e.g., autoclaves, incubators and pH meters, are not included, but are available in an equipment file.

ACKNOWLEDGEMENTS

We are grateful to members of the soil ecology section, in particular to Miss S. Dawson and Miss S. M. C. Robertson for comments on the text. We are also indebted to Mr. A. D. Bailey, Mr. D. H. D'Arcy and Mrs. G. Howson for drawing the diagrams.

C O N T E N T S

I ISOLATION AND MAINTENANCE OF FUNGI AND BACTERIA

'Isolation' is often essential for taxonomic and experimental work on micro-organisms. In the microbiological sense, it is the process of separating a single species of micro-organism from its natural habitat and growing it by itself, without interference from other organisms, on a sterile substrate, i.e., in pure culture. The micro-organism can then be distinguished by its individual characters and propogated to provide experimental material. Only occasionally is a mixed culture of two species essential for survival or sporulation. Many fungi and bacteria live for years in culture, if competitors are excluded, and if the substrate is renewed periodically. A named collection of such cultures can be invaluable for comparison with freshly isolated and unidentified micro-organisms, although some diagnostic characters may be lost after long periods in culture.

Methods of isolating micro-organisms from natural substrates (soil, litter, air, water, etc.) are numerous. One of the simplest methods for a visible fungus on a leaf, for example, is to pick up the organism with a sterile needle and transfer it to a nutritionally favourable medium in a tube or plate. Dilution plates and Warcup soil plates, two widely used methods of isolating soil micro-organisms, are described by Johnson and Curl, (1972). Other more complex methods in use at Merlewood will be described in separate laboratory schedules. The basis of all selective isolation techniques is the provision of conditions particularly favourable to the organism concerned and as unfavourable as possible to its competitors. Thus they may involve:

1. traps or baits,

2. media nutritionally selective for certain species,

3. antibiotics or some chemical (e.g., rose bengal) to kill or inhibit contaminants,

4. a selective environment, e.g., anaerobic conditions or high temperature.

If the first transfer to a culture medium does not result in a pure culture, further cleaning must be carried out as described on p. 9. The final stages of purification are often the most difficult, since two species may be very closely associated, e.g., bacteria on fungal hyphae.

ROUTINE PRECAUTIONS

Throughout all processes of isolating and culturing, re-entry of unwanted organisms must be prevented and the worker protected from infection. The whole environment (air, the worker, laboratory bench, etc) is loaded with bacteria and fungi, most of which can grow on culture media. Therefore the following routine precautions should be taken:

1. Sterilize all instruments and materials which touch the culture.

2. Keep the surroundings clean to prevent contamination of sterile objects, i.e., hands should be washed before and after handling cultures; the bench swabbed with disinfectant, and the room, if heavily infected, should be sprayed (see Section III: Sterilization).

3. Work in a still atmosphere to prevent spores being carried into the culture vessels on air currents. For instance, in a laboratory, shut doors and windows and do not breath heavily at the critical moments. These precautions should be adequate for general culture work with test tubes and phials, which have relatively narrow mouths.

For more critical work, or when large surface areas have to be exposed, use a hood presprayed with thymol and then wiped out with alcohol. The disinfectant should be allowed to settle before the hood is used, and the observation window should be protected from the heat of a flame by an asbestos mat on a tripod.

For large scale experiments, an inoculating room, fitted with an air filter and presprayed with disinfectant, allows more mobility than a hood. It should not be used for routine subculturing, which can be 100% successful in the laboratory. Release of dry spores into the air which is likely to occur when subculturing certain fungi, e.g., the Penicillia, would be most undesirable in an inoculating room. Exposure of a clean agar plate for a few minutes will indicate the air spora present. The inoculating room at Merlewood is fitted with a wall filter, removing approximately 95% of spores with an average diameter of 2-3μ. The filter operates in conjunction with a Vent-Axia fan unit blowing air into the room The room is slightly pressurized to prevent ingress of dirt through the door. The door of the ante-chamber should be closed before the door of the inoculating room is opened, and both doors should be kept shut whenever possible. During the actual culturing operations, switch off the fan to reduce air movements, but restart it before opening the door.

Open culture containers for as short a time as possible and protect the apertures from contamination, i.e., culture tubes and phials should be held, with the mouths pointing downwards, at an angle of about 30° to the horizontal. Lids of Petri dishes should be lifted only far enough to allow an instrument or the mouth of another container to enter. When isolations are made from a mixed population of fungi on a culture plate, fast-growing colonies are sometimes excised, while they are young and non-sporing, to prevent them swamping slow-growers. Contamination can be prevented by holding the plate upside down while cutting out the agar.

5. Disposal of cultures. If possible, kill cultures by autoclaving before washing up to avoid contamination of the air with spores or infection of the worker. Most of the organisms handled at Merlewood are rarely pathogenic, but it is unwise to inhale heavy doses of spores or to come into direct contact with living cultures. Petri dish cultures may be disposed of in a disinfectant, but the agar should be cut out while the culture is held below the surface of the liquid, and rubber gloves should be worn. Further details are given in Section III. Cultures should not be disposed of without consulting the owner, and discards should be labelled as such whenever there might be doubt.

INOCULATION

Tools

Nichrome wire or a darning needle, mounted in a metal handle with chuck*, is used as an inoculating tool for transferring inocula to and from media. To inoculate fungi, nichrome wire can be flattened at the tip to a knife edge for cutting into agar, or the end may be straight or hooked for scraping the colony. For bacteria or liquid inocula, a loop of nichrome wire (internal diameter 2-4 mm) or pipette is used. A loop can also be used to make streak plates of fungi. Nichrome wire (24 s.w.g.) has the advantage that it cools rapidly after flame sterilization. Darning needles are more rigid and therefore easier to manipulate, but new ones should be heated before use to remove the metallic plating, which might flake on to the culture.

Procedure

An inoculating needle, including its chuck, is dipped into alcohol, the alcohol flamed off and the tip heated to redness in a Bunsen burner. Omit the alcohol treatment if there is a danger of spattering potential pathogens. Care should be taken not to burn the inoculum; the heat of the needle can be tested by touching a clear area of agar before touching the organism. Two inoculating instruments may be used alternately, one cooling, while the other one is in use. During cooling, plugs or caps of tubes (or other containers) are removed, care being taken to touch only the tops, and the mouths of the tubes are flamed. To inoculate fungi grown on solid media, a small fragment from the edge of a colony (the inoculum) is transferred on the tip of the needle to the new medium. Sporulating mycelium is preferable, since purely vegetative sectors of a colony often persist indefinitely in producing only vegetative growth. On solid media, this inoculum must be placed in close contact with the substrate. To obtain an inoculum of bacteria and yeasts, it is sufficient to touch just the edge of a well separated colony without scraping the surface; in this case, the smaller the inoculum carried over, the better is the result. This inoculum is then streaked on the surface of the agar (Fig. 2a,b). After transfer of the inoculum, the mouths of the tubes are flamed again, the plugs or caps are replaced, and the needle is immediately flamed to redness to prevent contamination of the laboratory with any spores or mycelium which may remain on the needle.

* Suppliers: (length 21 cm) Gallenkamp ,(12 cm) Dental Manufacturing Co.

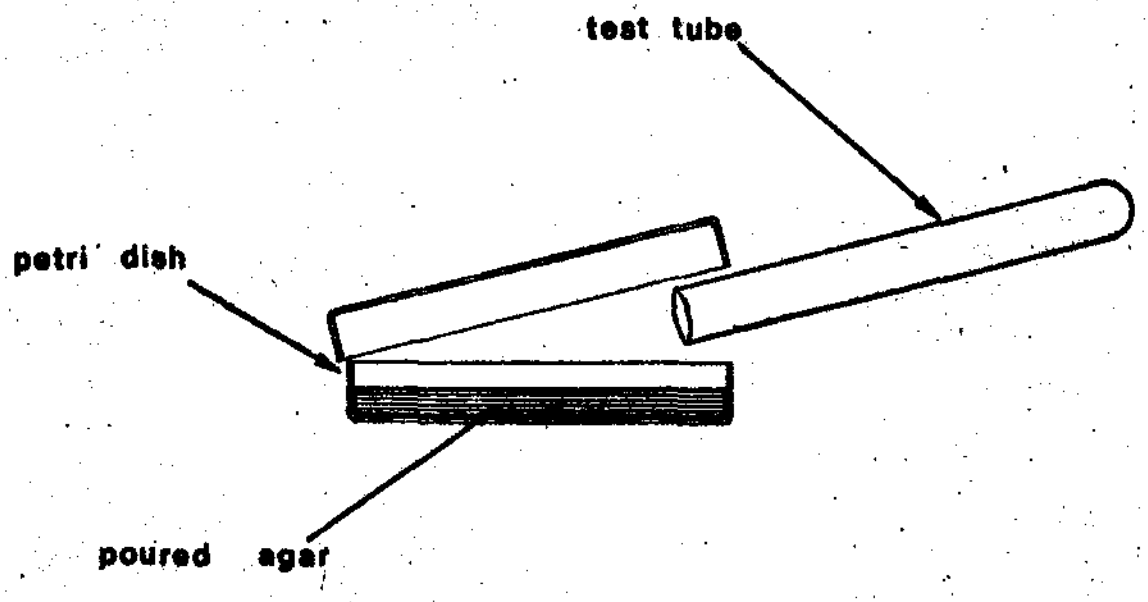

Fig 1. Pouring agar from test tube into Petri dish

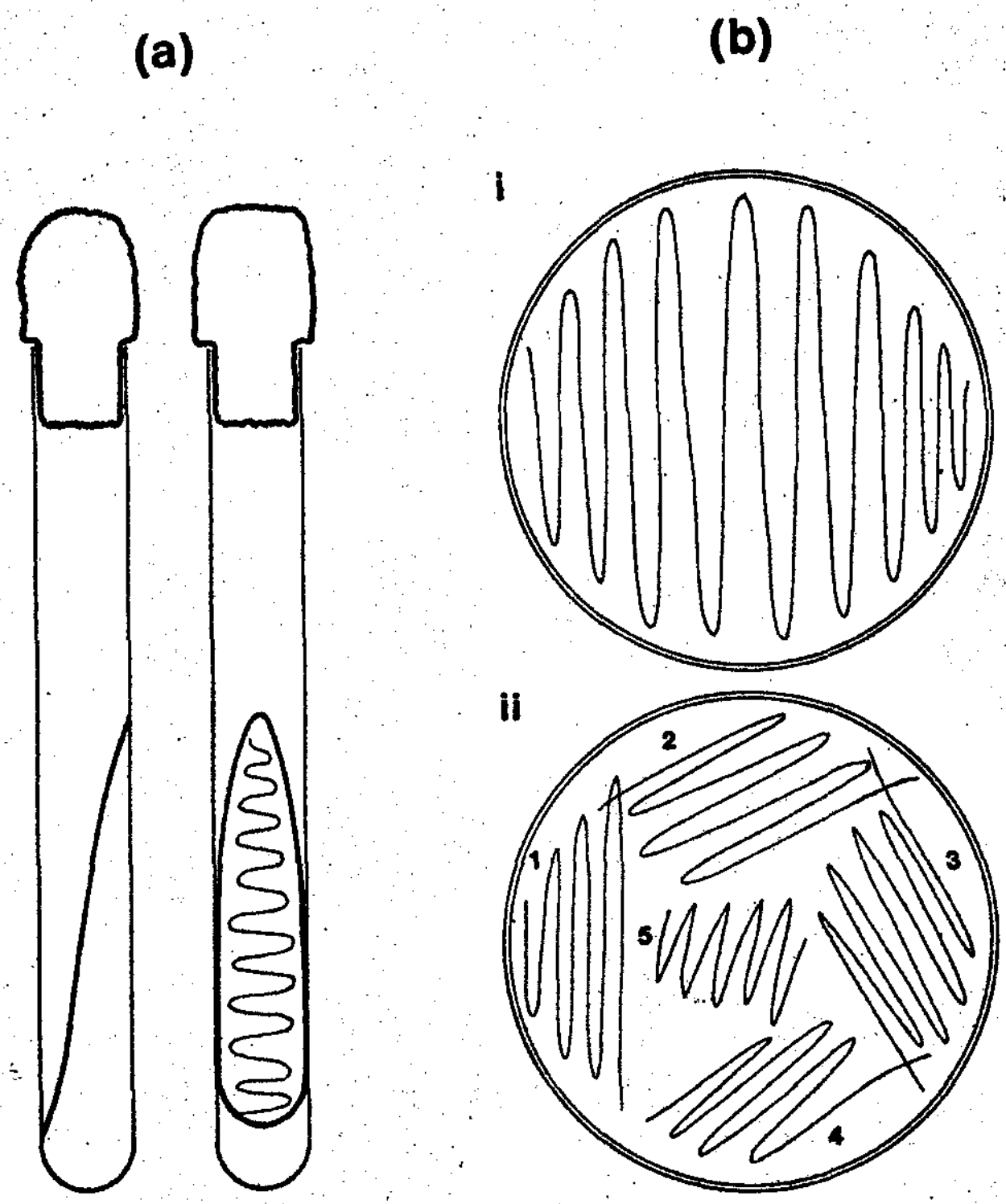

Fig. 2. Streaking micro-organisms on solid media

(a) for a slope culture in test tube

(b) for a Petri dish culture, methods i and ii.

In method ii, the numbering 1-5 indicates sequence of streaks

TYPES OF CULTURE

Plate cultures

These are required for many morphological and experimental studies, but are too short-term for storage. A thin layer of agar or similar solid medium is poured into Petri dishes (Fig. 1,), and allowed to set before, or after, inoculation. Ten to fifteen ml of medium are normally used for each dish, and can be poured from individual tubes or phials, or in larger amounts from conical flasks.

Slope cultures

Agar medium (5-7 ml) is poured into plugged test tubes or McCartney bottles (phials) and allowed to set in a sloping position after sterilization. The slope, or slant, provides a relatively large agar surface in a narrow container. For fungi, this enables the various stages of development within the colony to be observed.

'Dry slopes' in test tubes are more convenient than phial cultures, described below, if the organism has to be examined frequently. They are easier to handle when the culture has to be renewed (i.e., subcultured or 'subbed'), contamination during subculturing is rare, and the colony can be examined through the glass under a low power microscope.

Non-absorbent cotton wool plugs should be used (see p38). While the agar is setting, support the plugged end on a clean rod about 0.5" (1 cm) diameter, so that the medium extends up the tube to within about 1" (2.5 cm) of the plug. Care should be taken not to wet the plug; if wetting does occur, discard the tube, otherwise the plug will shrink and agar on it may become infected. After cooling, agar slopes contain condensed water, so the tubes must be stored and handled in the vertical position to prevent the fluid from flowing over the agar surface or wetting the plug.

To inoculate a test tube slope, hold the culture tube and the tube containing the new slope between thumb and first finger, and between first and second finger of the left hand respectively, with the plugged ends inclined downwards. Fungal growth is usually more typical, and the slope is covered more quickly, from a single spot inoculation at the centre than from a streak inoculation. In general, a fungal inoculum should be taken from the apex of the colony unless the culture is very dry or appears unhealthy. Care should be taken to prevent the inoculum touching the hot glass during these operations.

All tube cultures should be labelled with the culture number, date and medium. Labels should be attached just above the apex of the agar slope and below the plug, so that the whole of the colony can be examined; labels nearer the mouth of the tube are likely to burn during flaming.

Slope cultures should be packed loosely and stand upright in wire baskets or tube racks, in clean, dry and airy conditions, preferably in the light if they are fungal cultures. Growth is often restricted if the tubes are stored horizontally, this is thought to be a carbon dioxide effect. Normal room temperatures are usually suitable, but, for rapid development, the cultures may be incubated at 25°C, the optimum temperature for growth of most fungi, or at 25-30°C, the optimum for most bacteria. A short plastic cover over a wire basket of slopes on an open bench will protect the plugs from dust, without restricting circulation of air through the sides of the basket; it should be swabbed periodically with methylated spirits.

Oiled slopes in phials ('wet slopes') are recommended for long-term storage of cultures. Oil restricts desiccation without complete cessation of respiration. Routine subculturing is therefore reduced from intervals of 3-6 months to 1-2 years or more, but inocula from oiled slopes always carry over some oil, so that a second transfer must be made to obtain growth of normal appearance.

The slopes are prepared and inoculated in a similar manner to dry tube slopes. The phials are screw-capped McCartney bottles (J. 669 10 oz. 'Universal Containers') with aluminium caps; the rubber liners should be removed as they dissolve in oil. Growth of fungi is better in these wide mouthed bottles than in the narrow mouthed type, sometimes used by bacteriologists. Ten ml agar medium per bottle are autoclaved and slanted with the cap left slightly loose until cool. A short slope is preferred to ensure complete cover with oil. During inoculation, the interior of the cap must face downwards to prevent contamination.

The oil is medicinal liquid paraffin of British Pharmacopoeia quality, obtainable from Boots. It must be sterilized, at 15 lb/in^2 for 15 minutes, in individual doses, since air, displaced by the oil, carries with it airborne spores, which would adhere to the mouth of a dispenser and contaminate the next culture. Some batches of oil are cloudy with water vapour after autoclaving; usually, this can be removed by incubation at 30°C for two days.

The sterile oil is added to a culture when good growth and, if possible, sporulation have been obtained. The surface level of the oil should be about 1 cm above the tip of the slope, and further oil should be added, during storage, before the tip of the culture is exposed, or desiccation will occur very rapidly. Onions (Booth, 1971) states that the depth of 1 cm is fairly critical, because the oxygen transmission by layers of mineral oil in excess of 1 cm becomes less favourable.

The oiled cultures should be stored at room temperature in clean conditions at low atmospheric humidity. Baird & Tatlock's wooden drawer units used at Merlewood are suitable containers; they keep out dust, but allow air to enter, and the wood absorbs water vapour given off by the cultures. Lower storage temperatures are not always satisfactory; at 4°C some fungi are killed, and at 8°C contamination may occur by growth of fungi in condensation on the bottles and under the caps (Anon. 1960), but 7°C has been found to be a suitable temperature for bacterial cultures.

Oiled cultures should always be prepared in duplicate, because they are more susceptible to contamination during preparation than dry tube cultures. The lids can be numbered, but labels with full details should be stuck at the base of the bottle, on the reverse side of the slope, so that the colony is not obscured.

Liquid cultures

These are set up in conical flasks, medical flats (medicine bottles), or similar containers. A thin layer of liquid medium is placed in each container; the depth should not exceed 1 cm for aerobic organisms. After sterilization, the medium is inoculated with a small piece of culture, or with a suspension of bacteria, fungal spores or homogenized mycelium. Medical flats are incubated in a horizontal position to increase surface area of the medium. More uniform growth of liquid cultures is obtained by use of shake culture. This is a means of improving aeration of liquid cultures and, with fungi, results in growth in the form of pellets.

Damp chambers (Keyworth, 1951)

This method is used only for fungi. The increased humidity of a damp chamber culture induces many species to fruit on plant material, so that they can be identified *in situ* or isolated more readily. Many fungi which never grow on agar media can be obtained by this technique.

The base of a Petri dish is covered with a filter paper (9 cm diameter) and the inner rim lined with rolled asbestos or a strip of Carlson Ford filter paper*. This thick and highly absorbent paper retains water for several weeks at room temperature, so that frequent watering of the chambers is unnecessary. A base lining of Carlson Ford paper, however, makes the substrate too sodden and reduces fungal growth. The chamber is autoclaved dry, and the fresh material to be examined, such as a leaf or root, is then placed in the centre, taking the usual precautions to prevent entry of contaminants which might grow on the filter paper. The chambers are watered from a sterile dispenser by wetting the paper rim, so that water diffuses inwards to the centre of the dish. Free water should not come into contact with the specimen. Incubation is usually carried out at room temperatures in the light. Examinations should be made within 2 days, but the plates can be kept for months to allow fruit bodies of Basidiomycetes to develop.

*Obtainable from T. Barcham Green Ltd., Hayle Mill, Maidstone, Kent, in 25" squares.

CLEANING TECHNIQUES

Fungi

1. Repeated transfers of an inoculum are made from the edge of a colony to fresh medium, usually in a Petri dish, until all the contaminant has been left behind. Use of tap water agar, addition of an antibiotic (see recipes), and/or inoculation onto the base of the Petri dish before pouring the agar is often helpful. The method is successful only if the organisms spread out at different rates from the inoculum. If the fungus is sporing, a streaked inoculum may separate out single spores from which clean colonies will develop.

2. Hyphal tip isolation. A single, clean, hyphal tip in a Petri dish colony is located under the microscope. The objective is then replaced by a sterilized dummy, consisting of a metal tube with a cutting edge, which is lowered to cut out the hyphal tip on a block of agar. The block is removed with a needle to fresh sterile medium.

3. Raper's ring method for fast growing fungi contaminated with bacteria is advocated by several text books, but we have not been successful with this technique.

4. The dilution method for single spore cultures is described by Smith (1969).

Bacteria

1. Pour-plate method. An inoculum from a culture, or a loopful of bacterial suspension, is transferred to a tube of molten agar medium. This tube is shaken,* the loop sterilized, and a loopful of the inoculated medium taken to another tube of agar, which is also shaken. The agar from both tubes is then poured into Petri dishes. Well separated colonies should result in the second dish at least.

2. Streak-plate method. An inoculum from a culture or a suspension is streaked successively on the surface of one or two plates of solidified sterile agar, using an inoculating loop. The aim is to disperse the organisms as much as possible. There are various ways of doing this, as shown in Fig. 2b. The loop may be flamed between each plate, the inoculum then being taken from the streak on the previous plate.

* i.e., by rubbing between the palms of the hand.

MAINTENANCE OF TYPE AND VIRILITY

The aim should always be to preserve the species, in a culture collection, true to type, with the same characters (when transferred to a particular medium) as when they were first isolated and described. General storage conditions for 'dry' and 'wet' slopes have been given on page 6 .

The frequency at which subculturing should be carried out depends on the particular organism and on the storage conditions, particularly temperature. Cultures should be transferred before the agar has shrunk far from the glass, and never left to dry out completely. Some fungi, which do not spore freely, will not survive for 6 months, even in a refrigerator, without subculturing. This may be due to 'staling' of the culture by toxic waste products. Spores of some fungi remain viable for years, while others, e.g., certain common Mucorales, have a viability of less than 6 months. In general 'dry slope' cultures kept at 18-20°C should be subcultured within 4-6 months and 'wet slopes' within 2 years.

Media

Cultures for identification are often grown on relatively rich media, e.g., potato dextrose agar for most fungi, Czapek-Dox agar for Penicillia, and nutrient agar for bacteria. These produce a particularly characteristic type of growth and colouring. Comparison of the gross morphology of large numbers of cultures is carried out most easily on such a medium, although weaker media may be necessary for development of particular features, e.g., fruit bodies of fungi. For long-term storage of fungi, a natural and less sugary medium should be used such as weak potato carrot agar, but a change of diet should be given from time to time to preserve full vigour. On sugary media, many bacteria and fungi gradually decline and cease to spore, although there are exceptions requiring high sugar concentrations for survival. Most soil bacteria grow well and store satisfactorily on tryptone-soya agar.

Light

If a change of medium fails, an increase of light will sometimes reverse a decline of fungi. Many species are stimulated by light; some require regular exposure to diffuse daylight, others require bright sunlight.

Some organisms undergo change by genetic mutation and a sector of the colony develops with several characters differing from the original. Abnormal conditions are often responsible. The new form should be separated before it swamps the 'parent'.

In spite of careful culturing, some species decline after long periods in culture. Fungi, e.g., Chaetomium and Fusarium, often produce large quantities of floccose mycelium, differing in colour from the original; other fungi may become entirely conidial losing all vegetative mycelium. In any collection, loss of virility of a small number of cultures is inevitable without detailed knowledge of nutritional requirements.

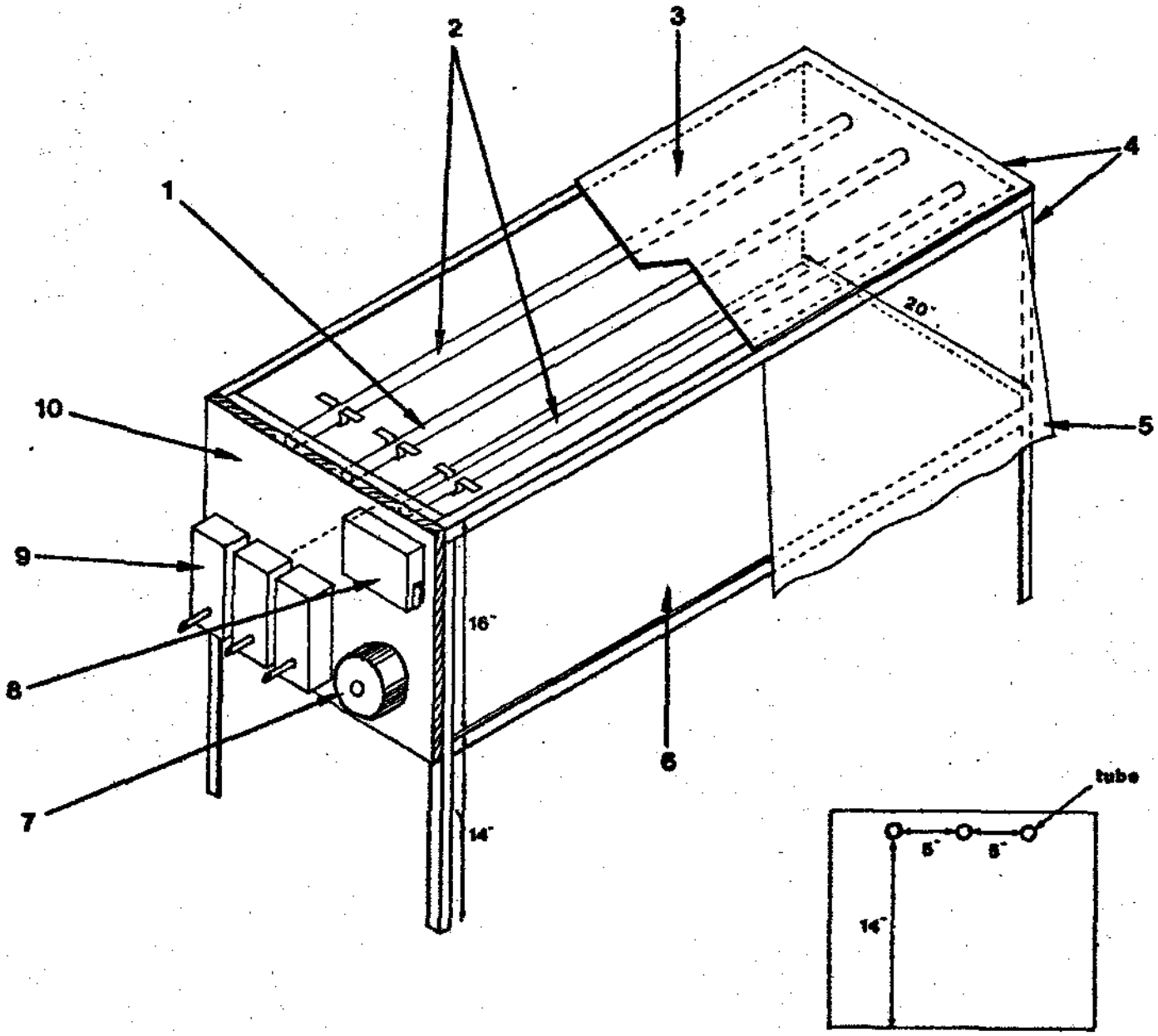

Fig. 3. 'Black light' apparatus

1. One 4 ft. 'black light', TL/40/08*.

2. Two 4 ft., 40 watt 'natural' fluorescent tubes.

The three tubes are parallel, 5 in. apart and 14 in. above the platform. They are supported by Terry clips and supplied with electricity via end caps.

3. Hard-board roof, under which the lights are mounted.

4. Dexion frame, 30 in. high.

5. Curtain.

6. Hard-board platform for cultures.

7. Sangamo, 24 h dial, synchronous time-switch to give 12 h of darkness and 12 h of light.

8. Main switch

9. Chokes and starters (one per tube).

10. $\frac{3}{4}$ in board to hold control gear.

* Obtainable from Phillips Electrical Co. Ltd., Central Stores, Beddington Farm Way, Purley Way, Croydon, Surrey.

GENERAL TECHNIQUES FOR INDUCING SPORULATION

Fungi

Change of medium. Sporulation will often occur if a fungus is transferred to a weak shallow medium, e.g., potato extract, potato-carrot or tap water agar, on which vegetative growth is reduced. Mortierella species will generally spore prolifically when transferred from agar to tap water. A small portion of the agar culture with well developed aerial mycelium is floated on sterile water in a watch glass until sporing mycelium has grown over the surface of the water. Good results have not been obtained with Merlewood tap water, which is chlorinated, therefore water from other sources should be used. Alternaria species often spore on the thin layer of agar obtained when a slope is rotated rapidly just before setting.

Light. Short exposure to sunlight or ultra-violet rays often stimulates production of spores or fruit bodies, although heavier doses may kill mycelium and spores. Several fungi, e.g., Trichoderma, will often spore in a few days if they are placed on a sunlit bench. If this fails, the fungus should be exposed for about 2 weeks to UV in a 'black light' apparatus as recommended by the Commonwealth Mycological Institute (C.M.I.), (Fig. 3). Plate cultures should develop in an incubator for about a week before exposure to alternating 12 h periods of UV lighting and darkness. Although disposable plastic Petri dishes allow greater penetration of UV, we have had more success with glass plates in which desiccation is less of a problem.

Change of temperature and treatment with red-heat. Sudden changes of temperature, e.g., from cold-room temperatures to 25°C in an incubator, will sometimes induce sporulation, but the method is not usually as successful as a change of medium or lighting. Stroking a culture with a red-hot needle, however, often has spectacular results. This simple technique is particularly successful with some Trichoderma species which produce green spore heads along the singed furrow.

Sporulation on slide cultures. When identifying fungi, it is often essential to see how the spores are borne. The spores of many fungi are all too readily detached from the mycelium when transferred from a culture to a liquid mount. Various methods, have therefore been devised to induce fungi to spore on a microscope preparation, so that they can be examined in situ under a microscope without disturbance. The slide culture technique (Riddell, 1950) is one of the most useful of these.

Agar medium is poured into a Petri dish to a depth of about 2 mm (14 ml per dish). Graph paper is placed under the dish, and a 1 cm square of the set agar is cut out with a sterile scalpel and transferred to the centre of a slide, previously dipped in alcohol, flamed and cooled. The slide is then placed on a tile wiped with alcohol and protected by the lid of a Petri dish. Each vertical side of the block is inoculated centrally with a minimum amount of the fungus, and then a large No. 0 sterile cover slip (22 x 26 mm) is placed over it. The cultures are supported on two glass rods in a Petri dish lined with filter paper, soaked in a 20% solution of glycerine; only the dish itself need be sterile. The glycerine keeps the agar moist, but not too wet as would water, and the culture dishes do not usually require rewetting, if kept at room temperature for 7-14 days. Meanwhile, the slide cultures can be lifted out for examination under a microscope.

Many fungi will grow out over the slide and cover slip and sporulate freely. At an appropriate stage of growth, two preparations are made, one from the slide and one from the cover slip. The cover slip is carefully removed from the agar block and a drop of 95% alcohol added to wet and kill the fungus growing over it; when most of the alcohol has evaporated, the mountant is added (lactophenol with or without cotton blue, or 2% trypan blue) and the cover slip is lowered on to a slide. The agar block is then carefully removed from the slide and discarded so that a slide preparation of the fungus can be made in a similar manner. The preparations are sealed as described on p. 17, but care should be taken not to cover the fungus, which is attached close to the edge of the mount.

A simpler alternative method has been tried with reasonable success. Two cover slips are placed in a Petri dish before sterilization Agar is carefully poured into the dish, so that the cover slips remain in place on the base of the dish. Before inoculation, a circle of agar is removed from the area above the cover slips, using a flamed cork borer slightly smaller than the diameter of the cover slip. The cork borer must be cooled first. The plates are inoculated and the area of the cover slips observed until a thin film of growth occurs with good development of sporing structures. The cover slips are then removed, quickly flamed on the top surface to remove condensation, and mounted as usual. The use of two cover slips allows for breakage in mounting.

Bacteria

Sporulation of bacteria, i.e., Bacillus or Clostridia species, is enhanced in media containing trace elements. Soil extract, which also supplies trace elements, has a similar effect. The inclusion of sugar in a medium inhibits sporulation.

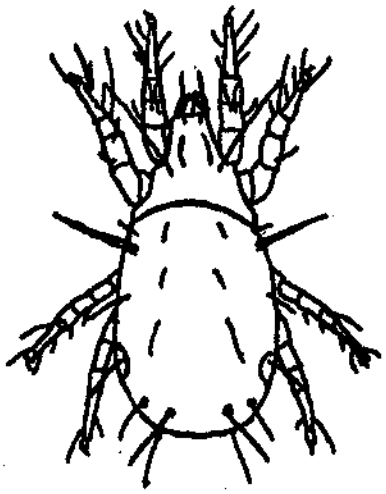

Fig. 4. A fungal mite. Actual size = 0.5 mm.

MITES

All fungal cultures should be protected from mites (Fig. 4). A laboratory may escape an invasion for years, and then, almost over night, a culture collection can be lost. Simple preventive measures, described below, are therefore worth while. Most fungal-feeding mites are barely visible to the naked eye and are not usually noticed until damage has been done. Lids of Petri dishes and cotton wool plugs do not keep them out; paraffin waxed plugs were once in vogue, but mites can tunnel through the wax. They crawl from one culture to another carrying contaminants, particularly Cephalosporium which is almost impossible to eliminate from other fungi. New laboratories with damp walls, and cupboards and incubators, in which a relatively high humidity can build up, are particularly vulnerable. In a mass outbreak, a whole building can be infected from a source in about a week, and mites can be found with the aid of a lens even on clean glassware. They gather on finger prints, which are a food supply for the fungi brought by the mites, each print becoming a minute grazed colony.

Prevention

1. Constant vigilance.

2. General cleanliness. Mites and their eggs are carried on shoes and hands and are also blown about in dust. It is difficult to exclude all sources of infection, but books, files and packing materials should be kept off the culture bench. Cupboards and benches should be wiped down regularly, giving particular attention to cracks and crevices.

3. Strict exclusion of all plant materials (moss, litter, wood shavings, toad-stools, soil, etc.) from a laboratory where cultures are kept. Deep enamel trays should be used and washed immediately after use, if brief examination of such materials is necessary.

4. Storage of cultures in dry airy conditions at room temperatures. It is better to keep dry slope cultures on a laboratory bench than shut away in a cupboard. Mites move and breed slowly at low temperatures, but dampness is a greater risk than high temperatures.

5. Vaseline traps. Movement of mites between Petri dishes can be impeded by smearing tiles and trays with Vaseline.

6. Use of repellants. Yellow, unrefined, tractor vaporizing oil (T.V.O.) contains an impurity which repels mites without ill effects on fungi. The only local supply is at the Penrith depot of Texaco. Baskets of cultures can be stood on corks above shallow trays of Vaseline and T.V.O., the latter renewed weekly, but air attack is still possible.

Dried herbarium specimens can be protected with a few crystals of paradichlorbenzene or naphthalene, but these repellants can cause sterility and mutations in living cultures.

7. Separation of culture baskets from one another, particularly those containing Penicillia, which are especially attractive to mites, will slow down an invasion.

8. A few months quarantine of direct isolates from plants and of cultures from other laboratories is usual.

Signs of attack

1. A characteristic earthy smell, if many cultures are infected.

2. Mouldy plugs or labels.

3. Cultures appear grazed and may be growing over the glass and plug from spores carried by the mites, or a culture may just look 'untidy' with churned up agar.

4. Fungal contaminants in the culture. The cause of these should always be investigated.

5. Mites with their pellets and eggs often congregate at the base of the culture tube between the agar and the glass. The adults are usually visible with a 10x lens.

Remedies

All cultures should be examined through the glass under a low power microscope. Clean fungi should be removed, and infected cultures destroyed by autoclaving or subcultured repeatedly. Re-examination over a long period is necessary to allow for hatching of eggs. Cupboards should not be re-used, because further outbreaks from eggs in the crevices sometimes occur over a period of years. Infected cultures can be fumigated in an air-tight box with paradichlorbenzene, but there will be a risk of adverse effects on the fungi. Formaldehyde may be used to fumigate a laboratory after removal of living material.

SLIDE MOUNTS

However carefully living cultures are maintained, some will deteriorate or die. Slide mounts are therefore prepared as soon as possible after isolation.

Slides and cover slips for mounts should be clean and grease-free. They should be stored in alcohol to remove grease, and wiped on a clean linen cloth before use.

Fungi

Booth (1971), reviews methods of observing fungi in the living condition, including slide chambers for time-lapse photography.

For non-living preparations, lactophenol is the standard mycological mountant used at Merlewood, because measurements in taxonomic works usually assume the use of this medium. This is a liquid mountant which does not harden and it must be sealed to hold the cover slip in place. We use crude nail varnish*, which does not react with lactophenol, to seal these mounts. Two coats of clear nail varnish ('Microscope slide sealing compound wax', O/N 126012; OD 36) are followed by one coat of orange varnish ('Microscope slide sealing compound red', O/N 126012; EX 337). Many seals described for lactophenol mounts react eventually with the lactophenol, so that the specimen is obscured by a mass of crystals. The above varnish, recommended by the C.M.I., has so far stood the test of time, but further coats are required for long storage periods.

Bacteria

Living bacteria can be observed using phase - contrast microscopy. To observe motility, use young, 24h, cultures. Apply a smear of Vaseline to the edge of the well in a cavity slide. Then place an inoculating needle in a liquid culture or into condensation water at the bottom of a slope, and touch the centre of a cover slip to produce a small drop of the culture. Invert the cavity slide over the cover slip, and press gently to make a seal with the Vaseline. Turn the slide over carefully, and then the hanging drop should be suspended in the centre of the hollow chamber of the slide. If properly prepared and sealed, the drop is protected from evaporation and air currents, thus avoiding any external influence on the movements or drifting of the organisms within the drop.

For permanent mounts a smear preparation is made. Pass the microscope slide through a Bunsen flame three times. Place a drop of distilled water on the slide, and take a small inoculum from the culture to mix with the drop of water. Spread the drop in a thin even film over the slide and allow to dry in air. Again pass the slide, film side up, rapidly through a Bunsen flame to 'fix' the smear. Just enough heat should be applied, so that the slide can be tolerated on the back of the hand.

After cooling, the slide can be stained with the Gram stain, or other stains as appropriate. The recipe for the Gram stain varies and should be checked with the worker concerned. The preparations can be observed directly under oil immersion or mounted in Euparal.

See general text books for other mountants, seals or stains used in microbiology.

* Obtained in bulk from W. Harland & Co., Building Paints Division, Merton, S.W.19.

DRIED HERBARIUM CULTURES OF FUNGI

Dried cultures of Ascomycetes and Sphaeropsidales with fruit bodies are useful for reference, because the spores can be re-examined after the culture has been soaked and teased out. The following method (Anon. 1960) is recommended:

Tap water agar (1.5%) is poured on to the smooth side of 4.5" squares of hardboard. The culture to be dried down is removed from the Petri dish, test tube or McCartney phial and placed on the melted agar; slope cultures must be flattened first by slicing off some agar. Old, dry tube cultures can be loosened by heating a little water in the bottom of the tube. The cultures are protected and allowed to dry. After 2-5 days they are ready to loosen with a razor blade, peel off and trim. They are stored in envelopes with a few crystals of paradichlorbenzene in the container as a protection against mites.

Freeze-drying of larger fungi is discussed by Onions (Booth, 1971).

II CULTURE MEDIA

Culture media are used as a nutritive substrate, on which to isolate and grow micro-organisms under artificial and controlled conditions, after their removal from the natural habitat.

A good medium should:

1. Simulate the natural environment.

2. Permit satisfactory growth.

3. Be reproducible.

In practice these three points cannot be completely satisfied in any one medium and a compromise is made according to the purpose of the experiment. It is, for instance, virtually impossible to reproduce exactly the natural conditions under which micro-organisms flourish. Also, to obtain satisfactory growth a far richer source of pure nutrients is normally used. However, micro-organisms show a considerable degree of adaptability and on the whole, suitable artificial media can be found. It seems highly unlikely that any one medium, containing all the necessary substances, will be found for the culturing of all organisms; so many kinds of media are used, each more or less specific for a particular purpose or organism. Thus, general media are used to culture as many organisms as possible, selective media to culture only one type or group of organisms.

The basic needs of all organisms include water, food (nutrients), and a suitable physical environment. Nutrient substances necessary for growth and reproduction include carbon (C), nitrogen (N), hydrogen (H), oxygen (O), sulphur (S), phosphorus (P), calcium (Ca), sodium (Na) and various metal salts and vitamins in low concentrations. The proportions of these elements needed by different organisms, or for the same organism, for different physiological processes are not the same. Also, some organisms obtain compounds of carbon or nitrogen, or the latter in its elemental form, from the air, and for these microbes either carbon or nitrogen is excluded from the medium. Others require carbon or nitrogen in various forms, from simple inorganic to complex organic compounds. Autotrophic organisms are those which grow in the absence of organic carbon, while heterotrophs need it.

The organic and inorganic consituents of media are mixed in the correct proportions to satisfy these various requirements.

Media are made up as liquids or as a solid gel with agar or gelatin; the solid condition aids manipulation and observation of cultures.

THE CONSTITUENTS OF CULTURE MEDIA

Water

Tap water contains many accessory compounds and varies in composition from one locality to another, and it is often chlorinated to inhibit growth of harmful bacteria with the result that media made with it would be subject to variation in constitution and may be bacteriostatic. For most scientific purposes, tap-water is therefore purified by distillation or de-ionization processes. Water treated in one of these ways should be used in preference to tap water, unless otherwise specified, for the preparation of culture media (tap water is used in some cases to provide trace elements).

Nutrients

1. Organic carbon and nitrogen are supplied as fresh, or treated, natural products. Extracts of potatoes, carrots, soil etc., make good, easily prepared media. They are usually cooked to extract the nutrients and may be supplemented with other constituents.

Sugars and other carbohydrate constituents are added as sources of organic carbon, or in order to test the ability of an organism to degrade them. Common sugars used are glucose (dextrose) and sucrose. Starch, cellulose, pectin, glycerol are polysaccharides frequently used.

Meat extracts, e.g., Lablemco, supply nitrogen and other food materials. They stimulate activity and accelerate growth of micro-organisms.

Peptones are meat digests and their most important function in culture media is to provide an available source of nitrogen. Forms of peptone include tryptone and casein hydrolysate.

Yeast extract is an excellent stimulator of growth. It is a complete food and also a rich source of the B vitamins and is used to supply these factors in culture media.

2. Inorganic nutrients are supplied as mineral salts, particularly the chlorides, phosphates, and sulphates amongst the acid radicals, and calcium, potassium and sodium amongst the bases. Nitrogen is supplied in the inorganic form as nitrates, nitrites, or as ammonium salts. Carbonates are sometimes added to check development of acidity.

3. Trace elements, vitamins and other growth factors, are also needed for the successful cultivation of organisms. The failure of an organism to grow on a certain medium can be due to the absence of one or more of these essential growth substances. They may be added as trace element, or vitamin mixtures, but in most complex media sufficient quantities are present as contaminants of the other chemicals used, particularly those of natural origin. Soil extract solution performs a similar function.

Constituents which adjust the physical state of media

1. The pH of culture media is important because organisms live only between certain limits of acidity and alkalinity. To adjust the pH to the required level, dilute solutions of potassium hydroxide or hydrochloric acid, are added, (see pH schedule). The pH of all media should be known. In general, bacteria prefer neutral, and fungi, acid pH.

Buffers are substances which increase the ability of a solution to resist pH changes. Organic compounds or phosphates may be added as buffers, but in complex media the ingredients used provide sufficient buffering capacity.

2. The osmotic pressure of a medium must be in equilibrium with the cell contents of the organism, or it will burst due to unequal pressures. Sodium chloride is often added to adjust the osmotic pressure to the correct level. Standard recipes for media should provide suitable osmotic pressure so no adjustment is needed.

Indicators of physical or biochemical state

Acid/alkaline indicators, and other chemicals, which change colour under particular conditions, are used in media to demonstrate biochemical changes, or to indicate physical state, e.g., anaerobiosis. The materials used are normally non-nutritive.

Solidifying agents

Agar is used to solidify media. It is a substance extracted from certain sea-weeds. Although of a carbohydrate nature, it is attacked by only a very few organisms and is regarded as an inert constituent of media. It is a hydrophilic colloid, which dissolves in water and becomes liquid at 98°C, and then remains liquid until cooled to approximately 40-45°C, when it solidifies. The modern refined agars give satisfactory gels at a concentration of about 1.2% and the medium is sufficiently clear for most purposes to make filtration unnecessary. Semi-solid media can be prepared down to 0.05%.

The dried powder dissolves in water when heated at 100°C for 1 hour. Agar media should not be adjusted to a pH lower than 5.8 prior to sterilization, or the agar will be hydrolyzed and will not set on cooling. For lower pH the agar can be adjusted after sterilization by adding sterile acid, or a higher concentration of agar can be used. Agar media must be cooled to 45°-50°C, before adding micro-organisms or heat sensitive constituents. The concentration of agar used in solid media results in a correct moisture content.

Organisms are sometimes grown on other solid substrates, these include gelatin, silica gel (an inorganic gel), potato slices or damp chambers (p 8), etc.

Gelatin is soluble in boiling water, and solidifies on cooling to form a transparent gel. A firm gel is formed at 15% concentration. It is rarely used as a substitute for agar in the preparation of solid media because it is attacked and decomposed by many organisms and it melts at temperatures normally used for incubation (i.e., above 20°C). It is added to media to test the ability of organisms to attack it and cause liquefaction.

TEMPERATURE, AERATION AND HUMIDITY

Micro-organisms are limited to particular ranges of temperature, outside which they will not grow; these vary with different species and varieties. Optimum growth is obtained within a more narrow range. Incubators are used to maintain uniform conditions, at the required temperature during growth, or for examining growth at different temperatures.

Oxygen is required by all organisms and is normally obtained from the air. Aerobic cultures are sufficiently aerated in Petri dishes and in containers with cotton wool plugs, (if they are not too tight). Anaerobes are inhibited by gaseous oxygen and obtain it in combined form. Anaerobic incubators are available for growth in various gas mixtures; for small numbers of cultures, anaerobic jars can be used.

The moisture content of air is normally satisfactory, but it can be increased by placing a dish of water in an incubator, or by using a controlled humidity oven. Booth (1971, p 43) discusses the use of graded solutions of salts, glycerol or sucrose to achieve specific humidities in incubators.

TYPES OF CULTURE MEDIA

Classified on composition

1. Synthetic or defined media are composed of compounds of known chemical composition. They may be composed entirely of inorganic salts, or be a mixture of inorganic salts and organic compounds. The exact chemical composition of all ingredients is known so that two batches of the same medium can be duplicated to a high degree of accuracy. Synthetic media are also employed where it is desired to ascertain what effect an organism will have on a certain compound. The nutrient requirements of organisms may be accurately determined only by the use of synthetic culture media, carefully prepared in specially cleaned glassware.

2. Non-synthetic or natural culture media, include natural ingredients of variable chemical composition, (e.g., vegetable or meat mixtures and digests). Many of these are now obtainable in a dehydrated form and have a fairly uniform constitution. It is, however, impossible to prepare two identical lots of the same media from different batches of the ingredients.

A mixture of synthetic and natural compounds, is often used.

Classified on function

1. General media are used to grow as many types of micro-organism as possible on one medium. Examples are,

for bacteria:	Lablemco agar
	Tryptone-soya agar
for fungi:	Czapek-Dox agar
	Malt agar

2. Selective media are used to isolate particular types of bacteria or fungi and are made up so that only the required organisms are able to develop properly and thus can be selected and isolated.

3. Biochemical test media are used to test the different biochemical activities of micro-organisms. Many types may grow on these media, but they are devised so that changes caused by particular enzymes can be easily detected e.g., liquefaction of gelatin.

4. Ionically balanced solutions, such as Ringers and physiological saline, are of the correct density to keep organisms alive, but contain no food materials for growth. These solutions are used, when high dilutions or washed cell suspensions are made up for counts and experimental work. Under these conditions, the natural materials which maintain correct osmotic pressure become too dilute.

MEDIA PREPARATION

Recipes for media are filed on cards and a colour code for cotton wool plugs is available. Manuals containing recipes include, Ainsworth (1971), Booth (1971), Difco (1972), Fred & Waksman (1928), McLean & Cook (1958), Oxoid (1969).

Manufactured media in dehydrated form, obtainable from Difco, Oxoid and other firms, are useful, when small quantities of media are required.

Media should be made up quickly and sterilized the same day, if possible. To avoid microbial growth, unsterile media should not be left in the laboratory, or at a warm temperature, after preparation is complete, but should be placed in a refrigerator or cold room.

Media should be prepared in the required quantity for current work, as reheating or prolonged storage affects the chemical nature.

Glassware for storage of media

Pyrex or some other heat resistant glassware is normally used.

1. Large conical flasks (1 litre upwards): to store bulk quantities of sterile media, e.g., soil extracts, amoeba medium, Ringers solution and saline, if to be used in these quantities.

2. 250-500 ml. conical flasks: to store solid media, and liquid media for shake cultures.

3. McCartney bottles: to store liquid and solid media in small quantities for individual cultures.

4. Medical flats: to store liquid media, (not usually solid medium because it takes too long to melt and the containers often crack).

5. Boiling tubes: to store liquid paraffin for soil cultures, or small quantities of agar media.

Containers for slopes

1. McCartney bottles: the rubber liner to the cap is removed to permit access of air.

2. Test tubes: Usually Pyrex 6" x 5/8" rimless type. Rims increase the risk of contamination, make packing in baskets difficult and frequently crack when flamed.

Procedure

1. Collect glassware and ensure that it is clean. Measuring cylinders for quantities required, large flasks to mix complete medium properly and containers required to store the medium.

New glassware contains insoluble salts which would alter the constitution of media, and may be toxic. It must therefore be thoroughly washed before use.

2. Measure out the water, distilled, unless deionized or tap water is requested.

3. Weigh out (see schedule on Balances) agar, if required, and add to half the water in the large flask. Heat in a steamer or water bath at 100°C, until the agar is completely dissolved. Shaking to disperse undissolved agar during this process will assist rapid solution. The time (1 hour or more) will depend on the quantities involved.

4. Weight out and dissolve all components, in separate amounts of water, leaving some to rinse out the beakers used. Crush and stir ingredients with a clean flat-ended glass rod. A magnetic stirrer is also available. Gentle heat may be required to dissolve some substances.

5. Mix the autoclavable ingredients together, rinse out containers used and add all these liquids to the large flask (no more than half full) and mix thoroughly.

6. Determine the pH (see pH schedule). Adjust if necessary.

7. Transfer aliquots to suitable containers, which should not be more than 2/3 full, to allow for boiling or frothing during sterilization. Aliquots should be correct quantities for slopes, or individual cultures, or of a suitable amount to avoid repeated opening in use. A semi-automatic dispenser can be used for tubes or large numbers of flasks. Before using for agar, the working parts of the apparatus should be warmed with boiling water or placed in the steamer. After use, hot water should be passed through to completely clear all tubes or narrow bores of the tap. The apparatus can be sterilized and thus used to dispense sterile medium in an inoculating room or under a hood.

For larger quantities a measuring cylinder is used, or where the amounts are not critical, it can be poured by eye into suitable containers. For smaller quantities, or more accurate dispensing, a pipette can be used.

Media must not be allowed to get on to the rims of containers, or contamination may occur at a later stage. If any insoluble ingredients (e.g., $CaCO_3$) are included in the recipe, the medium must be kept well mixed at all stages of preparation and distribution to containers.

8. Close containers with caps, or cotton wool plugs, as required (see p. 38). Screw-caps should be left half unscrewed at this stage, to avoid explosion in the autoclave.

Label all containers with type of medium, date of sterilization, and the initials of the person using it.

9. Sterilize according to the recipe. Under-treatment may not ensure sterility. Over-treatment may change the physical or chemical nature of the medium.

10. Leave in the autoclave to cool, if possible, to avoid the sucking in of unsterile air during cooling. When cool, tighten screw tops.

11. Store the sterile medium in a cold room if not required immediately.

12. Any sterile ingredients needed to complete the medium or to adjust pH, are usually added immediately before use.

13. If any sterile medium is opened and partially used, it should be discarded or clearly marked that it has been opened. Remains of media, and used flasks, should be placed in disinfectant to prevent subsequent growth of organisms.

14. Label all containers, or crates containing test tubes.

LABELLING

Scotch Pressure Sensitive Tape labels are recommended for cultures in tubes, phials and plates: adhesion is good, but the labels are easy to remove from the glass; they should be marked with an H lead pencil to prevent smudging if they get damp.

Chinagraph wax pencils are used for temporary marking of the lids of McCartney bottles and glassware; tie-on labels for flasks and medical flats, and diamond pencils for permanent marking of glassware.

Coloured plugs are useful for distinguishing media. Coloured cotton wool is available for this purpose. It is best to add a small quantity to a white plug as the colours sometimes 'run'.

III STERILIZATION

To sterilize means to kill, or completely remove, all forms of life. For experimental and culture work on micro-organisms, sterile media and apparatus are essential, and subsequent contamination by live organisms on dust in the air, or on other surfaces, must be prevented. Where complete sterilization is not feasible, methods which involve the partial killing or inhibition of microbial growth are used. For detailed accounts of all types of method see Sykes (1965). Death may, or may not, involve disintegration of the organisms.

Scrupulously clean conditions and the correct use of techniques for sterile culture work and disposal of discarded cultures, ensure that cultures remain uncontaminated and the worker protected from accidental infection. Any glassware used for media should be rinsed out with hot water immediately after emptying.

Organisms differ in their resistance to heat and other sterilizing agents, and any procedure must allow for the most resistant forms.

The amount of treatment required will be affected by the following:

1. Concentration and age of cells. A more concentrated suspension will need longer or more drastic treatment.

2. The size of the container is important in any heat or other physical treatment. The treatment must reach the whole of the material for the required time.

3. Organic matter of any form and particularly fatty materials and mucilage, protect the cells against sterilization. This is one reason why soil is difficult to sterilize.

To disinfect means to partially kill, or only inhibit, micro-organisms, usually certain undesirable forms, such as human pathogens or food spoilage organisms.

Various methods are employed to kill, or remove organisms, according to the nature of the article to be sterilized and the use to which it will be put. Each method has its own particular use with well defined limitations. Two main processes are involved.

1. Killing of organisms by: Some form of heat, dry or moist.
   Other physical methods including radiation.
   Chemical methods.

2. Mechanical removal by filtration.

HEAT

Of all methods of sterilization heat, and particularly moist steam under pressure, is the most simple and reliable method and therefore the most frequently used. Death by dry heat is primarily an oxidation process, while moist heat causes coagulation of cell proteins. The vessels used should be loosely packed to allow good circulation of hot air or steam.

Dry heat

1. The flame of a Bunsen burner will kill organisms almost instantly and is used for many purposes. A Spencer type burner (Fig. 5) is best since these give a larger and hotter flame. The ring is adjusted to give a strong blue flame.

Inoculating needles and other instruments are heated to red heat in a Bunsen flame and allowed to cool before use. Care must be taken to flame all parts which may contact the sterile container in use.

The mouths of culture tubes are also flamed, after opening, to kill organisms, which may have settled on the rim during storage. Similarly flaming is used when joining up any apparatus, e.g., for filtration. The capillary tips of pipettes may also be quickly flamed before use.

Needles, scalpels and other instruments may be sterilized by dipping them in industrial alcohol and then burning off the spirit. This process can be repeated several times. Since the alcohol burns in direct contact with the organisms, a lower heat is necessary. This method is therefore better than direct flaming for articles which may be damaged by red heat, e.g., glass rods and forceps, but is less efficient, and 'spattering' of live organisms may occur.

2. Hot-air ovens (see schedule on Ovens), heated by gas or electricity, can be used to sterilize dry glassware. A temperature of 160°C for 1 hour gives complete destruction of organisms. Articles are wrapped in aluminium foil, placed in special metal canisters, or in any container, which with its cap will withstand heat. Newspaper wrapping must not be used. For powders or other solid materials, the greatest surface area possible should be exposed and containers should not hold more than 30-60 g of solid material.

Moist heat

Moist heat, or steam, is used to sterilize media and any apparatus that may be damaged by dry heat.

Boiling water kills most vegetative organisms, but does not kill bacterial spores. A temperature of 80-100°C for 10 minutes kills all vegetative organisms and most fungal spores and is referred to as pasteurization. Flowing steam and steam under pressure are far more effective.

Steam under pressure (see schedule on Autoclaves) is the usual method used. When water is boiled under pressure, temperatures higher than 100°C are reached and this moist heat is a very efficient sterilizing agent and can be used for many purposes. The pressure usually employed is 15lb/in^2 and at this pressure water does not boil till it reaches 120°C. Fifteen minutes exposure to this temperature kills (with few exceptions) all forms of organisms, including spores. Sufficient time must be allowed for the replacement of all air by steam (in the non-automatic autoclaves), otherwise the correct temperature will not be reached. At the end of the holding time, the steam cools and water

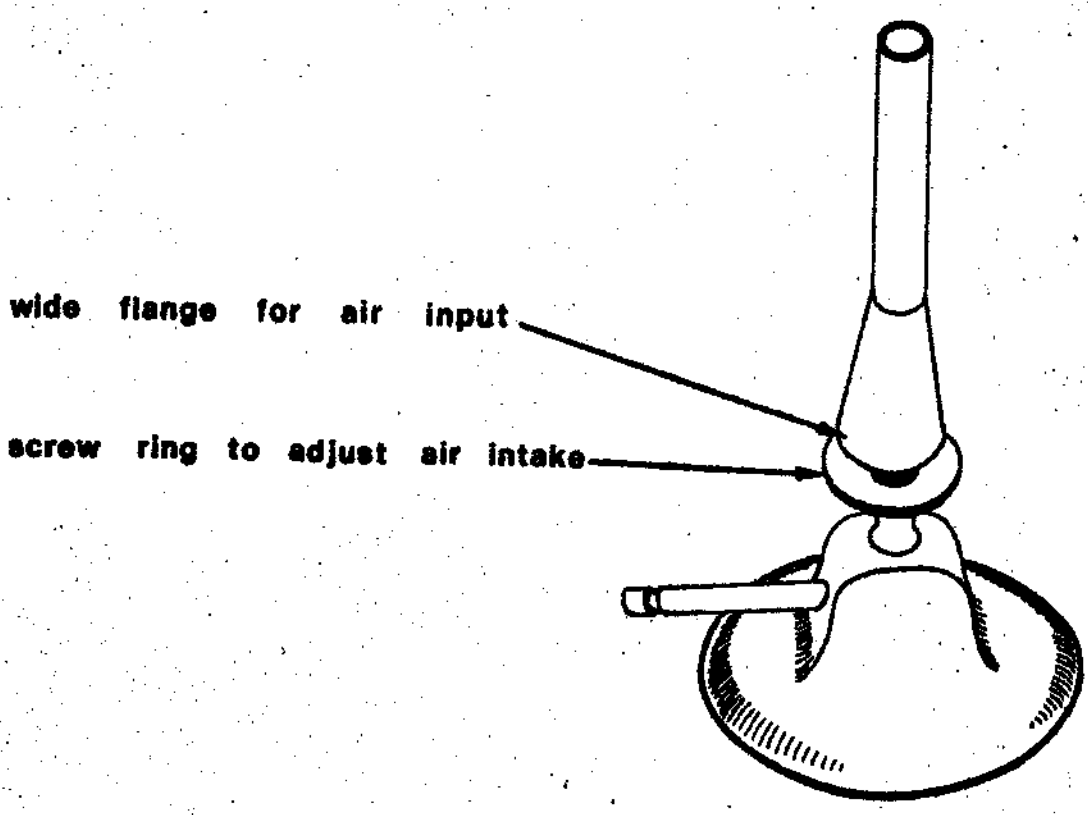

Fig. 5. Spencer-type Bunsen burner

will condense on to articles; all plugged articles must therefore be covered with some absorbent paper to prevent wetting of the plugs. Completely sealed containers must not be used because they may explode.

Since the temperature used is lower than that used for dry heat sterilization, the method can be used to sterilize anything which will withstand heating to at least 115°C, such as:

1 Plugged glass containers.

2. Screw-top containers with rubber liners (without black rubber liners if to be used for cultures).

3. Discarded cultures, e.g., sporing fungal cultures, before washing up.

4. Media, with the exception of some sugar media, or media containing vitamins, which would be decomposed by heat. Sugar media can be sterilized as concentrated solutions at pH 7.0 at 10 lb/in^2 for 15 minutes. Any variation in pressure or holding time is indicated on the media recipe card.

5. Soil placed in thin layers in flat containers such as Petri dishes.

Intermediate steaming at 100°C (Koch sterilization) is occasionally used for heat sensitive materials.

The material being sterilized by this method, must be capable of supporting germination of spores and quick microbial growth. It is placed in a steamer (see schedule on Steamer), for 20 minutes to 1 hour, on each of three successive days. One exposure kills only the vegetative organisms but the spores then germinate, being in a favourable medium, and are destroyed in the vegetative form during subsequent heatings. The method can be used for sterilizing media containing sugars or gelatin which are decomposed by higher temperatures, or for sterilizing soil. It is not always effective against very resistant spores and may be more harmful to ingredients than a short autoclaving process. Usually therefore, the latter is preferred.

IRRADIATION

This method is often less harmful to the material being sterilized, than heat, but complete sterilization is not always achieved. High energy ionizing radiations such as X-rays and gamma-rays are lethal to living cells and have good penetrating power. They affect the metabolism of the cell, but some of the enzyme functions may continue after the cell ceases to be viable.

For a comparison of the effects of different sterilization treatments, including radiation, on leaf litter see Howard and Frankland, (1972).

Ultra-violet light

Light of a certain wave length within the ultra-violet region (2000-4000 Angstrom) is lethal when it makes direct contact with living organisms. Plate glass, or water, will absorb ultra-violet light and because the waves have low penetrating power, the reverse side of any object will not be affected. These facts thus limit its use in many situations. The optimum wave length for maximum killing is 2,600 Å, and even Bacillus spores can be killed in 2 hours by a 15w lamp at 2" distance with continual stirring, if only a thin layer of suspension is exposed.

An ultra-violet lamp is sometimes used for partial sterilization of air, in rooms and hoods, or for apparatus. Ultra-violet rays are damaging to the eyes, any pair of glasses will give some protection against reflected rays, but NEVER look at the ultra-violet source.

X-rays

These have been used to kill soil animals, and their eggs, in the partial sterilization of plant litter. A total of 0.02 Mrad of X-rays in 3 separate doses seems to be sufficient for this; the position of the samples was changed between doses to facilitate penetration.

Gamma-rays

These rays, of very high penetrating power, resemble X-rays but are of shorter wave-length and are emitted from an element at only one or two fixed wave-lengths.

They are even more effective than X-rays, and gamma-radiation is now frequently used to sterilize such materials as soils and plant tissues, which are difficult to sterilize by other methods without considerable structural damage. A package irradiation service is supplied by LRC International Ltd., in collaboration with the Atomic Energy Research Establishment at Wantage, Berks. A total dosage of 2.5 Mrad from ^{60}Co source has been found to be satisfactory for 2-3 g samples of air-dry soil and plant litter. Samples are irradiated in plugged flasks or sealed polythene bags packed in standard cardboard boxes supplied for use in the irradiation plant.

CHEMICALS

Many strong chemicals will effect sterilization, but are not normally suitable.

Disinfectants

Disinfectants are used in the laboratory to prevent infection and to maintain general cleanliness, e.g., for wiping benches or for spraying air. When air is sprayed, the liquid droplets also bring down dust thus greatly reducing this source of contamination. Cultures should be removed from the area being sprayed.

Terms used for disinfectants are as follows:

1. Bactericides or fungicides (i.e., suffix-cide) kill vegetative organisms, but not all spores. With good agents a 99-100% kill is achieved and is satisfactory for many cleansing purposes. Proprietary products with the suffix-cide may not, however, reach this standard.

2. Bacteriostats or fungistats (i.e., suffix-stat) are substances which only prevent or inhibit growth, without killing all the organisms.

The same substance may show either property at different concentrations.

Hibitane, Chlorosan (a hypochlorite) and Lysol (a phenol) are powerful disinfectants commonly used in the laboratory, as for example to soak glassware which has contained culture media or micro-organisms, to wipe surfaces or to kill cultures accidentally spilt by the worker.

The concentrated solutions supplied are diluted as follows,

Hibitane (supplied as 5% solution): 0.1% (see I.C.I. Handbook)

Chlorosan: 0.2 - 0.3%, i.e., 20-30 cc per 2 gal. bucket

Lysol: 5%

(Panacide: formerly purchased, is no longer recommended because fungal spores on cultures are not killed.)

At these concentrations the above materials are bactericidal and fungicidal.

The neat solutions of Chlorosan and Lysol are toxic chemicals, the former is a strong oxidizing and bleaching agent, and the latter is corrosive and causes burns. For safety, two people should handle the drums of these materials. Hands should be washed after handling and any spilt liquid must be diluted. Hypochlorite-type materials should not be allowed to contact any phenol-type disinfectant, since chlorine is produced on mixing.

Thymol is used as an air spray and to wipe benches before work is begun and on completion. Two concentrations are used: 0.1% in 1% alcohol, or 2% in 95% alcohol (see recipes file). The first is used as a spray and to wipe benches. The second is stronger and is used for inoculating rooms or for more complete sterilization, but the room must not be entered for 30 minutes as the vapour is harmful.

Alcohol (70-95%) is used to swab benches, 95% to dip instruments prior to flaming. Microscope slides and cover glasses are stored in alcohol mainly to remove grease.

Propylene oxide gas is used to sterilize soils and similar materials. It is an inflammable and highly toxic substance but completely evaporates from the material after treatment. For use see Howard & Frankland (1972).

Formaldehyde can be used as a fumigant, to destroy fungal spores and mites (Smith 1969). The room is fumigated overnight.

FILTRATION

The passage of gases or liquids through sterile filters removes organisms from them . . Solutions of heat-sensitive compounds, and media for critical experiments, are better sterilized by this process.

Filtration is not the mere mechanical removal of particles of a certain size, by slightly smaller pores, but depends on physico-chemical factors affecting adsorption; e.g., an earthenware filter consists mainly of magnesium and calcium silicates, and according to the electric charge on a particle so it will be adsorbed, or be able to pass through. The maximum pore size of any bacterial filter is always greater than the sizes of the particles it can remove.

Liquids

In the filtration of liquids, acidity promotes adsorption, and the presence of protein or tissue, the temperature, pH, and viscosity of the solution will all affect the duration and effectiveness of sterilization. The method can only be used for liquids whose ingredients are in complete solution. Single ingredients are usually sterilized by filtration in a concentrated solution, and then added to the rest of the medium which has been sterilized by usual methods. Since the soluble products of metabolism are left, the growth of any organism during preparation of the medium must be avoided.

As fluids do not readily pass through filters by gravity it is necessary to use positive or negative pressure. Negative pressure (suction) from a vacuum pump is the most convenient method of filtration (Fig. 6), the fluid being drawn through the filter into a previously sterilized container, usually a Buchner flask, which is a conical flask of thick glass with a side-arm. An air filter is used to sterilize incoming air, and all outlets or side-arms of flasks, are plugged during prior sterilization by autoclaving.

The smallest negative pressure that produces satisfactory filtration should be used, starting with a small pressure and gradually increasing as filtration proceeds. The time of filtration should not be prolonged, or small motile flexible organisms may pass through the filter. A high negative pressure must be avoided, as small particles are rapidly forced into the pores and clog the filter.

For ordinary purposes a negative pressure of 100-200 mm of mercury is usually sufficient.

A disadvantage of the negative pressure method is that the filtered fluid has to be transferred to another container and contamination may occur. If positive pressure from a compressor is used, the fluid can be filtered directly into any type of container required.

A sterility check must always be made on the filtered material. Solutions known to be badly contaminated should be filtered twice, using a fresh sterile filter the second time.

Membrane filters were developed as a method of counting bacteria in liquids, but are also used to sterilize solutions by filtration. Special filter holders are available. To sterilize the membranes, they are placed between sheets of filter paper in a Petri dish, and autoclaved. They are destroyed after use, so that no cleaning is involved. Oxoid membrane filters are commonly used (see Oxoid manual), but a wide range of porosities can be obtained from other firms.

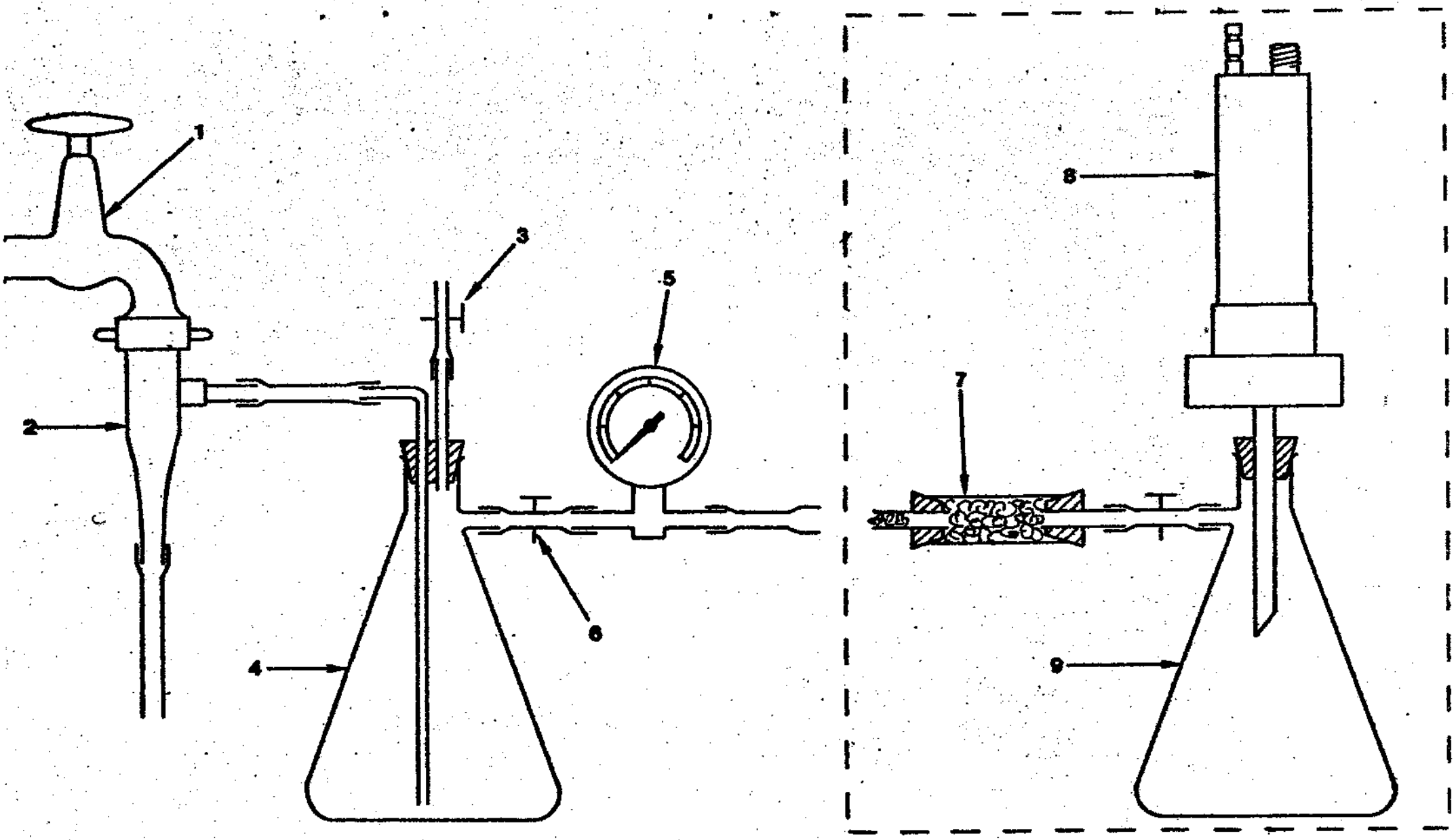

Fig. 6. Diagram to show assembly used for filtration. Parts to be sterilized enclosed by broken line.

1. Water tap; 2. Suction pump screwed onto tap; 3. Screw tap to release vacuum during use; 4. Liquid trap; 5. Vacuum gauge; 6. Screw tap to hold pressure if tap suction turned off; 7. Air filter; 8. Membrane filter holder; 9. Receiving flask.

Sintered glass filters are available in five porosities, the finest of which (no. 5) will remove bacteria.

A number of older filters are seldom used now, e.g., candle filters, or asbestos Seitz filters. The latter are liable to yield alkaline compounds or traces of iron, have undesirable adsorption properties, and often shed fibres into the medium.

Air

Cotton wool is a most effective air filter and is used to plug test tubes, pipettes and other open apparatus, or is packed in a flask or open cylinder to remove organisms from flowing air.

Special air filters are available and can be fitted to air lines or rooms.

METHOD OF RESTRICTING GROWTH IN MEDIA AND CULTURES

Low temperatures

A proportion of organisms will die when frozen, the proportion depending on the species and the suspending medium. Bacterial spores are little affected. Low temperature nearly always arrests the growth of micro-organisms and some form of cold storage is essential for storage of culture media and of samples prior to examination.

Desiccation

Some cells will die when dried, although drying has little effect on spores. Few micro-organisms will grow in a dry environment, so that drying alone is a useful method of preservation and prevents spoilage of materials.

EQUIPMENT FOR STERILE CULTURE WORK

Petri dishes

These dishes, sometimes called plates, consist of two glass dishes which fit into each other. This provides a chamber which remains free from contamination over long periods. Petri dishes are packed in biscuit tins, or metal canisters, for sterilization in a hot-air oven. Once the tin has been opened, the dishes should no longer be regarded as sterile. Individual dishes can be wrapped in foil before sterilizing and kept in the foil until used. Petri dishes can also be sterilized in the autoclave, if needed quickly. For this they are placed in special metal canisters provided with holes in the lid, which must be aligned during autoclaving to allow access of steam. The lid is turned to close the canister after treatment.

Test tubes and flasks

The interior of open test tubes, flasks, medical flats, etc., must be carefully protected from contamination by organisms in unsterile air during the preparation of media. They should therefore be kept covered, until plugged or closed in some way.

Cotton wool used for plugs must be dry and non-absorbent; absorbent cotton holds moisture after steam sterilization which allows passage of organisms and subsequent growth of moulds. Long-fibred cotton wool, free from short broken fibres and dust, is desirable.

Plugs should be about 2" (5 cm) long, with 0.75" (2 cm) protruding, and a tight fit with plenty of 'body', so that they retain their shape when pushed in and out repeatedly, and thus prevent dust from working down into the tube. It should be possible to hold the tube by the plug without supporting the tube. In a mite attack (see p.15), tubes with the shorter plugs are usually the first to be contaminated, as spores have a shorter distance to travel before they drop into the culture. There are two methods of making cotton wool plugs. They have separate devotees, but the end result is what matters. To plug a test tube:

Method 1. Tear off a piece of cotton wool about 4" (10 cm) square, fold two opposite edges into the centre, and roll up across the folds; the shape will be retained after sterilization.

Method 2. Tear off a rectangular piece of wool, fold in the edges to make a piece 2.5" (6 cm) long and of a width twice the diameter of the tube, fold again lengthwise, lay the wool across the mouth of the tube and push its centre in by means of a glass or metal rod.

The size required for larger articles can be found by trial and error.

Foam plastic plugs ready cut to shape are now available and are quite suitable for short term cultures. They are inserted into test tubes by means of a pair of straight, flat-sided forceps.

Metal caps of various types are also used for short term cultures. They act in a similar way to a Petri dish in excluding contamination. Their main fault is that they are too easily dislodged in use and must be carefully matched to the size of individual tubes.

When tubes or flasks have to be stored for some time, the tops of the crates may be loosely covered with foil, polythene sheeting or paper. Covering of plugs too tightly may cause moisture to be held in the plugs and thus allow contamination.

Pipettes

Before sterilizing, pipettes are plugged with non-absorbent cotton wool at the mouth end. A small pointed instrument is used to do this and the plugged top is then passed through a flame to remove protruding cotton wool. Plugging, prevents contamination of the sample by the worker and reduces the chance of the worker getting a mouthful of a sample being pipetted. Safety pipettes or a suction bulb should be used for any dangerous materials, either chemicals or pathogenic organisms.

For autoclaving, plugged pipettes are placed in large glass tubes with a cotton wool stopper; or in metal canisters for sterilization by dry heat. A plug of cotton wool pushed to the base of the container will prevent breakage of the tips of the pipettes. They can also be wrapped in foil or paper, individually, or in small groups. If sterilized in the oven, the plugs will char but the pipettes remain dry. As for Petri dishes, paper should not be used in oven sterilization, and only metal canisters provided with an outlet should be used for steam sterilization.

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