Handbook of CELL and ORGAN CULTURE

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by

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PREFACE

The application of "tissue" culture techniques to the experimental study of a wide range of biological problems requires an effective means of instruction. Moreover, it is important that students of biology be familiar with the principles of cell, tissue or organ culture in vitro. Since 1953 the Departments of Bacteriology and Anatomy at The University of Michigan have jointly sponsored a course in the "Principles and Techniques of Cell and Organ Culture". This course has been designed to meet the requirements for training at the graduate and post graduate levels. Thus, selection of material for incorporation in this Handbook of Cell and Organ Culture* was made on the basis of considerable experience in teaching students with divergent backgrounds and different interests.

The organization of the exercises is such that they can be used for class instruction or as a guide for the technician or investigator. Introductory statements for each chapter and each exercise have been written with the intent of giving a brief statement of historical development and/or appraisal of the method of procedure. As a further aid, key references have been given to lead the reader into each subject area. In writing the handbook certain opinions had to be expressed and arbitrary decisions made only because of the vastness of the field and the lack of agreement on many matters by respected colleagues.

It is assumed that persons using the handbook have a basic knowledge of microbiological and histological techniques and terminology. To facilitate instruction and to ease the burden of the laboratory assistant, the materials listed for each exercise are those required for one individual to carry out the procedures. Moreover, it is expected that each student is familiar with the technique of sterilizing instruments by immersing them in ethanol followed by ignition. Finally, it is assumed that the laboratory has available the reagents and materials needed for histologic studies of cells.

It is hoped that this handbook will serve as a manual for class instruction and as a guide for the neophyte entering the field of cell and organ culture as well as a useful reference for the more experienced worker.

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GLASSWARE

Soft glass (soda-lime glass) is significantly soluble in water and salt solutions. Hard glass, which contains potassium carbonate, is more resistant to solubilization (etching) although this may occur at alkaline pH and at high temperatures. Consequently, exposure of glassware to adverse conditions, such as excessively stringent cleaning procedures or by use of strongly alkaline detergents, will shorten the effective life of laboratory glassware. Soft glass is satisfactory for growth of cells and for short term (1-2 weeks) storage of solutions, although hard glass is superior. For longer storage periods all solutions should be kept in hard glass bottles. Duraglass* and Neutraglass** are examples of satisfactory types of soft glass; hard glass is marketed under a variety of patented trade names such as Pyrex***, Kimax**, etc.

Toxicity of glassware is not an uncommon problem which often can be solved effectively by adequate cleaning procedures such as the one described below. In addition, toxicity or its absence is related to the biological properties of the cell strains employed. Unwashed glassware from commercial sources often is not toxic for established cell strains while even clean glassware may be toxic to especially sensitive cells. Detoxification frequently can be accomplished simply by first rinsing glassware with growth medium containing serum or some other biological product rich in protein.

PROCEDURE FOR CLEANING GLASSWARE

Nothing is more basic to cell and organ culture than properly cleaned glassware. Cells often are in direct contact with glass and are maintained with nutrient solutions having low buffering capacity and little or no detoxifying action.

Any of several detergents is satisfactory providing the cleaning and rinsing procedures are adjusted to the properties of the particular compound.

* ® Owen-Illinois Glass Co., Toledo, Ohio.
** ® Kimble Glass Co., Vineland, N. J.
*** ® Corning Glass Co., Corning, N. Y.

The following points may be helpful as a general guide.

Immerse all items in a solution of the cleaning agent immediately after use. Large items such as Erlenmeyer flasks, prescription bottles, etc. which cannot be conveniently handled in this manner should be kept tightly stoppered until ready for washing. These steps will prevent drying of materials on the glassware and will greatly facilitate subsequent washing. Vessels in which cells have been grown should be brushed before washing. Wax or grease should be removed from slides or other materials with carbon tetrachloride.

Most detergents clean more effectively when used as a warm solution. Boiling generally is not required but may speed the cleaning process and may actually be necessary for some items. To prevent coverglasses from sticking together during washing they should be dropped, one at a time, into a solution of cleaning compound which is boiling vigorously.

Rinsing is the most critical step in the cleaning procedure. All glassware should be rinsed a minimum of 8 times with tap water and twice with distilled water. If an automatic pipette washer is used, 10 cycles with tap water and 2 rinses of distilled water are recommended. Care should be taken to insure that each piece of glassware is filled and emptied completely with each rinse. For all general purposes the distilled water for rinsing may be either singly distilled or deionized water. For critical studies, such as the role of trace metals in nutrition or the action of carcinogenic agents, glass distilled water may be required.

ASEPTIC TECHNIQUE

The design of an aseptic technique depends on the needs of the individual and the demands of his problem. Although it is advantageous to have sterile transfer rooms or hoods, they are not required for many cell and organ culture procedures.

In that microorganisms are most commonly airborne on dust particles, it is essential that work areas be kept as free of dust as possible. Air turbulence should be avoided and for this reason the transfer area should be situated away from major traffic patterns.

Wherever it is feasible the use of screw-cap vessels will aid in the maintenance of sterility. This is particularly important when it is desired to pour from one container to another.

USE OF ANTIBIOTICS

The availability of antibiotics selectively toxic for many microbial agents has aided materially in the development and application of cell and organ culture techniques. The use of these materials in large scale tissue culture work or in the presence of known contaminated materials is certainly justified although the routine use of antibiotics in culture work is not without limitations.

The criteria most commonly used to determine toxicity of antibiotics are gross changes in morphology or growth patterns. It is quite clear that more subtle but significant effects may be missed. Thus, the current feeling that the commonly used antibiotics have little or no effect on tissue cells may not be justified.

A serious problem relates to the recent suggestion that routine use of antibiotics is responsible for the widespread occurrence of PPLO or L-forms of bacteria in tissue culture cell lines (1). The knowledge that penicillin may be used to establish L-forms from bacteria (2) is reason enough to suggest caution. Continued use of antibiotics also may mask bacterial or fungal infections and lead to a chronic or latent type of infection.

When the use of antibiotics is deemed necessary the probable type of contamination which may be encountered will determine which antibiotics to use. To control the widest range of bacterial contaminants it is common to employ either a broad spectrum antibiotic such as neomycin or a tetracycline or to use a combination of penicillin and streptomycin (p. 177). The latter are far less toxic but penicillin, as mentioned above, may induce L-forms. There is no effective antibiotic for control of the common fungi. Nystatin (Squibb) is moderately effective against yeasts but is both toxic and unstable and is not effective against the common filamentous mycotic contaminants.

Materials known to be contaminated and which must be added to cell culture systems, such as clinical specimens for virus isolation, are often treated with high concentrations of antibiotics for limited periods prior to use.

Once cultures become contaminated it is almost useless to attempt a "cure" with antibiotics. Time and effort are generally wasted. The infection may either be partially suppressed and break through as soon as antibiotics are removed, or stable L-forms may be induced. An exception is the use of tetracycline to free cell lines of PPLO or L-forms. This appears to be effective, at least in certain cases.

STERILIZATION PROCEDURES

Autoclaving, dry heat sterilization and filtration, as they are commonly used in microbiology, are adequate for most cell and organ culture materials. A few suggestions with regard to choice of technique and points of procedure are given below.

Autoclaving is the method of choice for most solutions and is often used for sterilization of glassware, particularly when heat penetration may be a problem. For some types of critical work it may be necessary to know the quality of steam supplied to the autoclave. Depending on water conditions various compounds are added to boilers to minimize rusting. Certain of these materials volatilize and become a source of toxic substances deposited within the autoclave. A filter unit is available* which can be placed in the steam line to avoid this difficulty. A more common and more serious hazard is failure to sterilize due to improper loading of the autoclave. Care should be taken to avoid overcrowding of materials.

Dry heat sterilization is effective for glassware unless rubber or teflon-lined caps or other rubber or plastic parts are involved. Heat transfer is slow and the timing of sterilization must be made from the time the material in the oven reaches the required temperature. Again, proper loading of the oven is essential. For electric ovens 170° C for 2 hours is convenient. If a manually operated gas oven is used 190° C for 30 minutes is adequate.

Filters may be used to remove bacteria, yeasts and molds from solutions but do not remove most viruses or PPLO and L-forms of bacteria. Four types of filters are commonly employed for this purpose, namely, asbestos pads, membrane filters, sintered glass and unglazed porcelain. Mean effective pore diameter of the filter should be 0.5 μ or less. Effective filtration cannot be attained if the filter is allowed to become saturated with organisms. Therefore, the amount of a material which can be filtered safely will depend upon the numbers of organisms it contains and the volume of material to be filtered.

Viscous or protein containing solutions such as serum generally require pressure filtration. This speeds up the filtration process and also prevents foaming. In using pressure, however, it is necessary to determine the maximum safe pressure for the filter. If large amounts of solids must be removed from solution sedimentation by high speed centrifugation or a prior filtration through a clarifying type unit may be required. Rate of filtration is generally not an important factor as long as the integrity of the filter is maintained.

Asbestos and membrane filters are expendable, thus eliminating the problem of cleaning. The latter are more expensive and filter more slowly. Asbes-

* Selas Corporation, Dresher, Pa.

tos pads may develop leaks if not used properly and also may contribute inorganic ions, particularly Mg^{++}, to the solution being filtered if not previously washed. This is generally an important consideration only when small volumes are being filtered. With small volumes an appreciable amount of material may be lost due to adsorption to the pad.

Unglazed porcelain filters have the advantage that they may be readily checked for cracks or imperfections by measuring bubbling pressure. This is done by placing the filter element in a cylinder containing distilled water and connecting the filter to a sensitive compressed air source. Selas 02 filters* have a bubbling pressure of 25 lbs/in^2 while the 03 filter* has a bubbling pressure of 35 lbs/in^2. If air bubbles appear before these pressures are attained a crack or flaw in the filter is indicated. Cleaning of a porcelain filter is done by rinsing first with 2% $NaHCO_3$ or 2% NaCl to remove residual proteins. The filter candle is then soaked overnight in concentrated HNO_3 or dichromate solution, rinsed, dried at 110° C and then heated in a muffle furnace with a rise of 160° C/hr. until the temperature reaches 675° C. Hold the filter for one hour at this temperature, and then allow it to cool to room temperature inside the furnace. Reverse flush the filter with distilled water to remove ash, soak it in distilled water several hours and check bubbling pressure as indicated above.

Sintered glass filters are best cleaned with hot sulfuric or nitric acid to remove organic materials. Reverse flush the filter but avoid using pressure in excess of 15 lbs/in^2. Wash them thoroughly in distilled water and rinse with ethanol and acetone to remove water. With both glass and porcelain filters care should be taken to avoid sudden temperature rise if the filter is damp as production of steam may crack the filter.

TESTS FOR STERILITY

All media and solutions to be used in cell or organ culture work must be carefully pretested for sterility by obtaining a representative sample. On the other hand it is equally important to recognize the limitations of sterility tests. Few, if any, test media are as sensitive growth indicators as the tissue cultures themselves. Moreover, due to statistically predictable difficulties involved in obtaining an adequate sample, one cannot be certain that a particular batch of medium is sterile unless the entire batch is consumed in testing. A practical rule of thumb is to take 1% of the specimen for a sterility test.

Routine testing procedures should be designed to include the normal range of aerobic and facultatively anaerobic bacteria as well as yeasts and molds.

* Selas Corporation, Dresher, Pa.

In view of the recent evidence of PPLO and/or bacterial L-forms in cell strains, tests for their presence also should be made at frequent intervals. If serum or other natural products are used they should be checked for the presence of viruses.

For detection of bacteria, yeasts and molds both brain heart infusion broth* and fluid thioglycollate*,** should be inoculated in duplicate. One tube of each medium should be incubated at room temperature and the other set at 35° C for a minimum of one week. In cases where contamination cannot be recognized readily in the sterility tests, serial passage may be helpful. To increase the chances of detecting contaminants in tissue culture medium the entire batch may be incubated overnight and then tested for sterility. It is wise to make a Gram stain of any contaminants which are isolated and to keep a record of the types of organisms found. This may be helpful later in tracing the source of contamination.

To test for presence of L-forms or PPLO, plate 0.1 ml of material on the surface of each of several plates of PPLO agar (3) in 4 cm petri dishes. If cultures are being tested, it is advisable to use the medium from a heavy monolayer or from the plateau phase of growth of suspension cultures. If L-forms or PPLO are present in such cultures they will be in the supernatent medium in numbers which can be detected. Cultures must be incubated in 5% CO_2 and 95% N_2 at 35° C (or 37° C). After 5-7 days examine the surface of the plates with a hand lens or a dissecting microscope for the presence of colonies, which range in size from 50-300 μ, and which characteristically have a "fried-egg" appearance. Staining of the colonies with methylene blue-azure is an aid in identification (4). For confirmation a colony may be dug from the agar, crushed on a slide and observed with the phase contrast microscope.

Presence of viruses in serum, ascites fluid, etc. may be checked by testing the materials on one or two cell strains of known broad sensitivity, e.g., Rhesus monkey kidney cells. Evidence of cytopathology, as compared to control cells, is taken as presumptive evidence of a viral agent (or primary toxicity). Specimens showing such activity should be discarded. For routine purposes it is not worthwhile to attempt the identification of an agent or to differentiate between toxicity and a viral agent.

PREPARATION OF MEDIA AND SOLUTIONS

Materials of at least reagent grade should be used for preparation of media or solutions. For most purposes deionized water will serve quite adequately. However, it should be recognized: (a) that many organic compounds are not removed by ion exchange resins, (b) that the quality of the water used to charge the resin will affect both the quality of water produced and the effective "life"

* Baltimore Biological Laboratory, Baltimore Md. or Difco Laboratories, Detroit, Mich.

** Should not contain methylene blue which is toxic for some organisms.

of the resin and (c) certain bacteria may grow in the resin and contribute toxic materials to the water. Glass distilled water is more satisfactory but also may be more difficult to prepare in adequate amounts for large scale work.

pH CONTROL

In most cell or organ culture systems pH control is provided by the carbonate-bicarbonate system. To maintain the proper pH level with such a system it is necessary to use tightly stoppered vessels or to gas with 5% CO_2. In most cases the closed system is adequate. pH control is particularly difficult when chemically defined media are used since the added buffering capacity of serum or other proteins is not available.

Adjustment of pH of solutions and media may be accomplished, with maintenance of isotonicity, by use of 0.3 N NaOH and 0.3 N HCl. Excessive use of HCl, however, will drive off CO_2. pH may also be raised by the addition of isotonic $NaHCO_3$ (1.4%) or lowered by equilibration with gaseous CO_2. It is advisable to add phenol red to all solutions in a concentration of 0.01-0.02 grams/liter to facilitate approximation of pH.

REFERENCES

1. ROTHBLAT, G. H., and MORTON, H. E., Detection and possible source of contaminating pleuropneumonialike organisms (PPLO) in cultures of tissue cells, Proc. Soc. Exper. Biol. & Med., 100: 87-90 (1959).
2. KLIENEBERGER-NOBEL, E., Origin, development and significance of L-forms in bacterial cultures, J. Gen. Microbiol., 3: 434-443 (1949).
3. BARILE, M. F., YAGUCHI, R., and EVELAND, W. C., A simplified medium for the cultivation of pleuropneumonialike organisms and the L-forms of bacteria, Am. J. Clin. Path., 30: 171-176 (1958).
4. DIENES, L., Morphology and nature of the pleuropneumonia group of organisms, J. Bact., 50: 441-458 (1945).