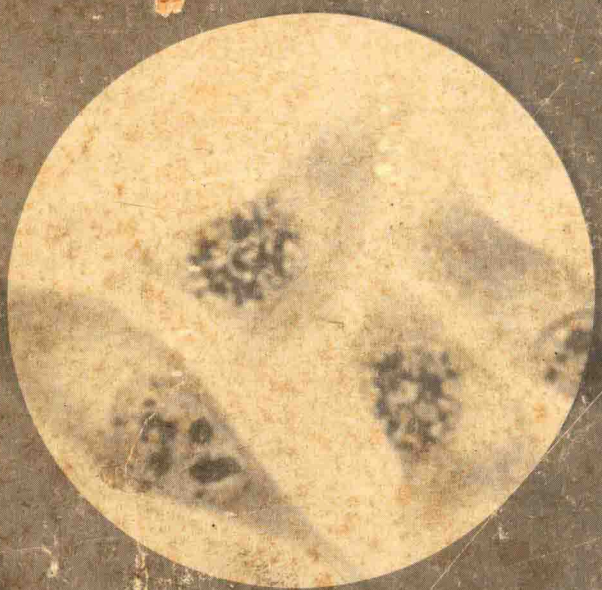
Handbook of CELL and ORGAN CULTURE



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Handbook of
CELL AND ORGAN CULTURE

by

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PREFACE TO THE SECOND EDITION

Development of techniques for the in vitro study of animal cells and tissues and the application of these techniques to a wide variety of problems has proceeded at a rapid pace during the past four years. Work has been particularly prolific in virology, radiobiology and cancer chemotherapy, while application has been initiated in animal cell genetics. In revising the Handbook of Cell and Organ Culture we have selected important new areas of application for illustrative purposes. The choice of new techniques to be included was made on the basis of their general usefulness to a variety of problems. A number of procedures presented in the first edition have been revised extensively on the basis of these new developments.

To further aid the investigator who wishes to use the book as a laboratory reference manual we have included several standard procedures which often are difficult to locate. The presentation of formulas for media and solutions also has been expanded. Wherever appropriate the formulas presented are those published by the original investigator. The literature citations have been expanded as well as updated and an index has been included to aid the user.

We wish to acknowledge the valuable contributions of many colleagues in the preparation of this volume, particularly: Dr. Richard Eidam, Dr. Raymond Hayes, Dr. Marvis Richardson, Dr. Helene Schneider, Miss Sheila Keefe, Mrs. Fannie Parker, Mr. Thomas Neumeier, Mr. Richard Giles.

Ann Arbor, Michigan
January, 1964

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PREFACE TO THE FIRST EDITION

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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GENERAL LABORATORY INSTRUCTIONS

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. Hard glass is marketed under a variety of patented trade names such as Pyrex*, Kimax**, etc. In case of doubt about the qualities of a particular glass product, check with the manufacturer.

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. Thus cationic, anionic and non-ionic detergents vary in their cleaning effectiveness and particularly in the ease with which they may be removed from

* Corning Glass Company, Corning, N.Y.
** Kimble Glass Company, Vineland, N.J.

glass. In some instances a minimum of 16-18 rinse cycles may be required to reduce the concentration of detergent to a non-inhibitory level though several available detergents require considerably fewer rinse cycles. Glassware washers which embody the principal of sonic agitation may permit use of lower concentrations of detergents and thus minimize these problems. However, the effectiveness of such apparatus is limited by the quantity and type of glassware to be processed.

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 handled conveniently 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.

Care should be taken to segregate glassware which has been treated with silicone from vessels used for growing cells in monolayers! Minute traces of silicone may inhibit cell attachment and the removal of residual silicone by rinsing procedures is difficult if not impossible. It is good practice to mark each piece of siliconed glassware with an "S" by etching it on the glass with a diamond pencil. Careful segregation of these items in cleaning, sterilization and storage should be adhered to.

It is extremely important to prevent glassware which has been in contact with certain toxic chemicals (formalin, picric acid, colchicine, methylcholanthrene, *etc.*) from being returned to the general glassware stock. In most instances it is not possible or practical to remove such agents from the glass.

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.

DISPOSABLE PLASTICWARE AND GLASSWARE

Sterile, disposable units including pipettes, tubes, culture dishes and flasks are available currently. Many of these items are fabricated of non-toxic plastics and the plastic surfaces permit the growth of most tissue cells in a highly satisfactory manner. Optical quality of plastic flasks often exceeds that of glass vessels of comparable price. A number of inexpensive, disposable glass products are also marketed including tubes and pipettes.

The variety of plastic or glass disposable items is being expanded rapidly. Considerations other than availability in selecting plastic versus glassware are: a) comparative cost (including cleaning of ordinary glassware and replacement), b) avoidance of toxicity due to chemicals and detergents when using disposable items, c) limitations of plastics relative to their intrinsic physical and chemical properties. (In the latter category is a rather severe limitation on use of common fixatives and staining reagents).

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.

Whenever it is feasible the use of screw-cap vessels or of caps which fit well over the mouth of the vessel (*e.g.*, Morton Closures^{R}*) will aid in maintaining sterility. This is particularly important when it is necessary to decant 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 and hazards. The decision to use antibiotics in tissue culture studies should be made only after a careful evaluation of the necessity and of the consequences.

* Bellco Glass Co., Vineland, N.J.

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. Additionally, the apparent lack of toxicity for one or more cell strains in a test series may have little meaning when extrapolated to other cell strains and/or other experimental conditions.

A serious problem relates to the 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 tetracyline or to use a combination of penicillin and streptomycin (p. 242). The latter are far less toxic but penicillin, as mentioned above, may induce L-forms. Though the common antibiotics effective against bacteria have little or no activity against the common fungi several antifungal agents are available. Among these nystatin and amphotericin B have demonstrated value.

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 or kanamycin to free cell lines of PPLO or L-forms (3, 4). This appears to be effective, at least in certain cases.

The most commonly employed antibiotic combination for control of bacterial contamination is penicillin (50 units/ml) and streptomycin (50 μg/ml) with or without the addition of nystatin (30 μg/ml). Other antibiotics frequently used are tetracycline (5 μg/ml), neomycin (25 μg/ml), kanamycin (50 μg/ml) and amphotericin B (2. 5 μg/ml). It is to be emphasized that these antibiotics and particularly the dosages listed are only examples and are not recommended necessarily for any particular situation. For serum containing media it will often be necessary to approximately double these amounts. To rid cultures of PPLO or L-forms, much higher concentrations of tetracyline and kanamycin are required.

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 serious hazard is failure to sterilize due to improper loading of the autoclave. Care should be taken to avoid overcrowding of materials.

A temperature of 121°C applied for 20 minutes is recommended for most materials. If all air in the autoclave has been replaced by steam this temperature will be achieved at a pressure of 15 lbs/sq. in. It is imperative that timing be commenced only when the temperature has reached 121°C! For materials such as glucose, which are unstable at high temperatures, the temperature may be lowered providing the time interval is increased accordingly. In the case of steam sterilization of screw cap containers it is imperative that the caps be left quite loose until after the vessels have been removed from the autoclave and allowed to cool! This will prevent production of a vacuum which can result in pulling contaminated air into the vessels as they are opened for use.

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. A minimum of 170°C for 2 hours is necessary. 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.

* Selas Corporation, Dresher, Pa.

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 generally is not an important factor as long as the integrity of the filter is maintained. With membrane or asbestos filters, clarifying pads may be inserted in series before the sterilizing pad to speed the filtration procedure. For small amounts of material a Swinney adapter* which fits any standard hypodermic syringe is quite advantageous. Both asbestos and membrane filter pads are available to fit these adapters.

Asbestos and membrane filters are expendable, thus eliminating the problem of cleaning. Asbestos pads or membrane filters may develop leaks if not used properly and asbestos pads 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 absorption to the pad. Membrane filters generally have a more rapid flow rate than asbestos pads unless very viscous or turbid suspensions are being filtered. In the latter cases clarifying prefilters are useful. In contrast to asbestos pads, absorption on the filter and contribution of inorganic ions are minimized with membrane filters.

Unglazed porcelain filters should be 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 thoroughly, 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 slowly 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

* Becton, Dickinson, Co., Rutherford, N.J.
** Selas Corporation, Dresher, Pa.