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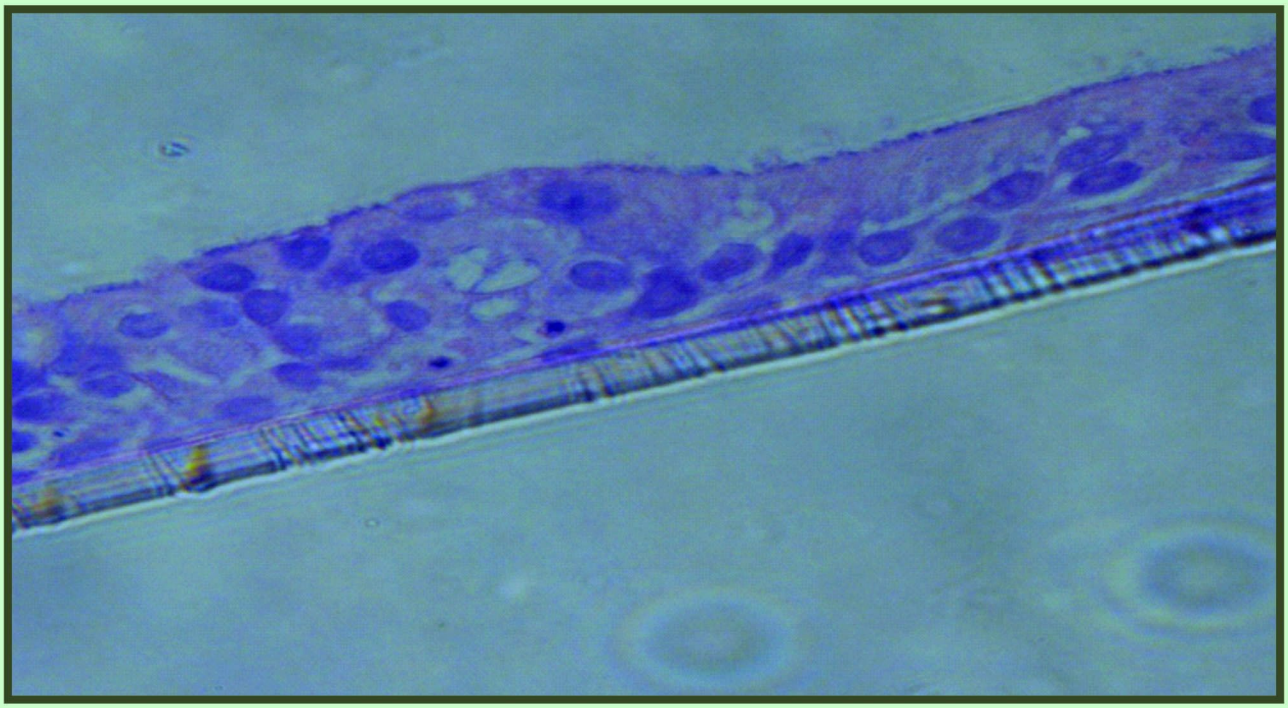
A Book on Experiments of Animal Biotechnology



JV'n Dr. Aziz Mohammad Khan

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INDEX

SAFETY IN LABORATORIES

General safety measures

Laboratory protection may seem to be a very boring subject at first glance, and the temptation may only be to read this portion superficially or not at all. The view of the issue, though, changes easily if you find yourself in the midst of a first or the victim of an accident, and ignorance can be dangerous or deadly by this time.

Laboratory safety equipment

Laboratories can be unsafe places to operate, and in the event of an accident, all users need to be mindful of the possible dangers and know what to do. It is necessary to become acquainted with the layout of the space and the location of the security equipment before beginning work in a new laboratory. The location of emergency exits, first alarms and extinguishers should be identified in order to be able to take suitable action in the case of a fire. It is also vital to know where the telephones are so that assistance can be called easily and to know the first aid box's location so that an injured person can get quick help.

In order for these utilities to be switched off in an emergency, the main gas and water taps and the switch for power should both be found.

Of course, the person in charge of the class can find out where the protective equipment is placed and therefore call attention to any particular dangers that are grounded in a real experiment.

Safety notices

The purpose of safety singing must also be understood to laboratory staff. Some of these, while others are in the shape of pictograms, are in plain English. In Britain and Europe, the sings is uniform in terms oflettering, diagrams and colour so that they can be easily recognized

Personal Protection

Goggles or safety eyewear are highly vulnerable to reagent splashes and safety spectacles.When doing any operation where there is a possibility, spectacles should always be worn.

When treating corrosive liquids such as strong oral acids, high duty gloves must be worn. The harmful nature of these compounds is visible, but the risks involved with skin contact with other chemicals are not necessarily noticeable.

Therefore, lightweight plastic gloves should be worn when measuring and treating chemicals to avoid the possibility of skin absorption.

Laboratory coats are not status badges, but are designed to shield the wearer from solvent splashes and material contamination. For a lab coat, cotton is a safer material than nylon because it has a great absorptive potential and is usually more resistant to chemical splashes.

About organisms.

These are not always mandatory for face masks, but they need to be worn where there is a chance of dust from.A chemical or micro-organism aerosol.

Dangers to avoid

The unintended movement of a compound to the mouth frequently results in toxicity, and this possibility can be significantly minimized by keeping three basic rules in the laboratory at all times.

1. No smoking
2. No eating and drinking
3. No mouth pipetting

Chemical hazards

Both chemicals should be considered and managed properly as potentially hazardous. It is important to prevent contact with skin and clothes, and even though a chemical is harmless, it should not be tasted or smelted.

On reagent tubes, hazard warning marks, which are black on an orange background, are present to warn of particular hazards that must be taken into account. Solutions for classroom reagents

can also be labelled by technical personnel and colored adhesive labels are available for this purpose.

Corrosive and irritant substances

A corrosive material is one that kills living tissue and is far too evident in the intrinsic hazards of heavy oralkalis acids coming into contact with the skin.

In the other side, an irritant causes local inflammation, but not bone and tissue damage.Hazards are more nuanced and not often appreciated in this situation. Occasional skin touch, for example, may indicate that the material does not have a noticeable effect. However, as in the case of certain organic solvents, prolonged exposure will unexpectedly give rise to irritation.

Toxic Compounds

Compounds are classified as toxic or extremely toxic based on the dosage needed to kill 50 percent of aHuman Population **(**LD50**).** The potential risks of swallowing a poisonous substance are evident, but the dangers of skin ingestion or inhalation are not always known.

Some compounds take a long time to become apparent before their toxicity, and this is particularly true of fircarcinogens and teratogens. This long-term toxicity is seen by several typical biochemical reagents, ninhydrin forexample is carcinogenic and thyroxin is teratogenic, an alternative can always be used whenever necessary, however if none is appropriate than special precaution must be taken while using these compounds.

Flammability hazards

Flammable compounds are those with a flash point, and when treating them, all naked fires in the laboratory should be extinguished, and Sparking fromelectrical equipment is less noticeable than a Bunsen burner, not just those in the immediate proximity of the substances, but can be almost as harmful. For this purpose, the refrigerator must not store organic solvents.

Oxidizing liquids may not be self-flamed, but when brought into contact with combustible material, they may spark a burn.

When such substances are to be used, the best precaution is to provide only the minimal volume needed on the bench and hold the key bulk far away from the work area in steel cabinets.

Explosives

Explosives are not handled in the usual biochemical laboratory as well, although certain general laboratory or reagents such as picric acid are volatile and must be treated with great care. Only minimal amounts of the substance can be used in the work area and ideally behind a protective barrier, as with flammable compounds. The mixing of two chemicals may also result in an explosion.

In order to deter a laboratory accident, those of themselves are harmless and knowledge of this is important.

EXPERIMENT 1

AIM: To prepare the cleaning solution to clean the glass wares.

Requirements: Balance, Erylyn Meyer flask (1 L), measuring cylinder, spatula, butter paper, potassium
dichromate, concentrated sulphuric acid, metal distilled water etc.

Composition for Cleaning Solution No:

Potassium dichromate

Water

Conc.sulphuric acid

Procedure

Approximately 800 ml of purified water stored in a sterile Meyer flask was dissolved with 60 g of potassium dichromate and combined with 60 g of potassium dichromate and mixed with 200 ml of concentrated water. H2SO4 is made of purified water up to a level of 1 litre. It is allowed to cool and use this remedy later.

This cleaning solution can be used to oxidize some organic material and to disinfect glassware such as test tubes, petriplates, pipettes, etc. It is possible to use the cleaning solution until it becomes a dark green solution.

Cleaning

The glassware is immersed overnight in the washing solvent and cleaned with soap and vinegar.

Rewashed in purified water and then in running tap water. Glassware was approved to remove water for further use and to dry in a hot air oven at 800C for 2 hours.

EXPERIMENT NO: 2

AIM : To prepare the materials required for various cell culture practices in sterile condition.

INTRODUCTION

The word regulation as applied here refers to the reduction of the overall microbial flora in numbers and or activity. The key purposes for the regulation of microorganisms and for the prevention of disease and virus spread, for the prevention of degradation or proliferation of unwanted microorganisms, and for the prevention of material decay and spoilage by microorganisms.

Various physical agents, physical processes or procedures may extract, prevent or destroy microorganisms.

Agents with chemical elements. There are a number of strategies and agents, they act in many different ways and each one behaves in many different ways.

Kits have application limitations of their own.

Steam under pressure: The most practical and efficient agent for sterilization is heat in the form of saturated steam under pressure. Steam under heat, as seen in Table 22-5, gives temperatures above those that can be achieved by boiling. In addition, it has the benefits of fast heating, penetration, and sufficient moisture, which promotes protein coagulation.

TYPES OF STERLISATION TECHNIQUES

AUTOCLAVE

An autoclave is considered a laboratory apparatus equipped to use steam under controlled pressure. In a microbiology or cell culture laboratory, the autoclave is an integral unit of equipment. With this apparatus, certain media, solutions, recycled cultures, and hazardous products are regularly sterilized. The autoclave is usually, but not always, worked at a pressure of around 15 lb/in2 (1210C). The time of service to attain sterility depends on the quality of the

sterilized substance, the container form, and the volume. For eg, 1000 test tubes containing 10 ml each of a liquid medium can be sterilized at 1210C in 10 to 15 minutes, 10 liters of the same medium stored in a single container to ensure sterilization will take 1 h or more at the same temperature.

BOILING WATER

It is difficult to sterilize hazardous products subjected to boiling water with confidence. It is true that all vegetative cells will be killed by exposure to boiling water within minutes, but for several hours, certain bacterial spores will survive this condition. Instead of sterilization, the process of exposing devices in boiling water for brief amounts of time is more likely to lead to disinfection (destruction of vegetative cells of disease causing microorganisms). Boiling water cannot be used as a form of sterilization in the laboratory.

DRY HEAT OR HOT AIR OVEN

Dry hearing or hot air sterilization is advised where steam under pressure is either unacceptable or impossible to make immediate and full contact with the sterilized materials. This refers to many laboratory glass objects, such as petri dishes and pipettes, as well as oils, powders, and the like. A special electric or gas oven or even a dry kitchen oven can be the apparatus used for this form of sterilization. For laboratory glassware, for sterilization, a 2h exposure to a temperature of 160^0C is necessary.

ULTRAVIOLET LIGHT

All emission from 150 to 3900 A contains the ultraviolet part of the spectrum. The highest bactericidal efficiency has wavelengths of 2650A. While sunlight's radiant energy is partially made up of ultraviolet radiation, most of the shorter wavelengths of this sort are filtered out by the atmosphere of the earth. As a result, ultraviolet emission is limited to the span of around 2670A to 3900A at the surface of the planet. We may infer from this that sunlight has microbicidal potential under some conditions, but to a restricted degree.

In using this means of killing microorganisms, an important practical factor is that ultraviolet light has very little potential to penetrate matter. A significant proportion of the light is emitted even as a thin film of glass filters. Therefore, only microorganisms on an object's surface when they are specifically subjected to ultraviolet light are vulnerable to death.

FILTRATION

The microbiologist has been available for several years with a number of filters that can extract microorganisms from liquids or gases. Such filters are made of various materials-an asbestos pad in theSeitz filter, Berkeloid diatomaceous earth, Chamberland-Pasteur porcelain filter and sintered porcelain

Glass disks in other filters. In these bacteriological filters, the mean pore diameter varies from roughlyone to multiple microfilters; most filters, depending on the average size of the pores, are available in several grades.Other variables, such as the filter's electric charge, the quality of filtration can be influenced by the electric charge borne by the species, and the essence of the fluid being filtered.

A new kind of filter has been created in recent years, called the membrane or molecular filter, whose pores are of a fixed uniform and precise scale. Membrane or molecular filters are made of cellulose esters which are biologically inert. They are developed as circular membranes with a thickness of approximately 150μm and contain millions of very uniform microscopic pores. Filters of this form can be generated with known porosities ranging from approximately 0.01 to 10μm. Membrane filters are used widely to sterilize fluid products in the laboratory and in industry.

In order to classify and enumerate microorganisms from water samples and other products, they have been tailored to microbiological procedures. By applying a negative pressure to the filter flask by using a vacuum or water pump, or by placing a positive pressure on the fluid in the filter chamber, it is customary to push the fluid into the filter and thereby push it in. Precautions must be taken to avoid degradation of the filtered content following completion of filtration.

EXPERIMENT 3

AIM : To prepare desired medium for the given Animal cell culture.

PRINCIPLE

In a liquid culture medium that consists of a mixture of vitamins, salts, glucose, amino acids and growth factors, all animal cells can be produced. Calf serum is also a readily usable source of growth and attachment factors. To prevent bacteria from developing, antibiotics are applied. Under these conditions, cells will expand to form a single layer on the culture vessels at physiological pH (7.4) and at body temperature (37oC).

MATERIALS REQUIRED

Medium

Adult bovine serum

Membrane filter (Millipore 0.45μ)

Sterilize

Double distilled water 1000 ml

1 litre measuring cylinder

100 ml measuring cylinder

1 litre filtration flask

Medium storage bottles

Other Glasswares

Method:

Sterilize the laminar air flow by UV irradiation for 45 minutes before using it.

1. In a 1000 ml measurement cylinder, take 500ml of sterile double distilled water.

2. Switch the contents of the powdered medium to a measuring cylinder of 1 litre and apply 3.7 gms of

NaHCO3 in the lack of an incubator of CO2.

3. To remove the powdered medium, blend thoroughly, and add penicillin /streptomycin/gentamycin.

4. Fill the cylinder with a double purified water blend of 1 litre and pass it to a sterile 2 litre flask and mix. The medium's pinkish red colour reveals the usual pH spectrum.

5. Assemble the set-up for filter sterilization and perform the filtration under negative pressure.

6. using a 100 ml measurement cylinder, prepare 400 ml of medium containing 10% adult bovine serum and store it in a 500 ml sera lab bottle.

7. Move the residual medium into large glass bottles without serum.

8. Store the medium in the refrigerator, dispose of the membrane used and immerse the glassware used for washing in water.

9. For different types of experiments, different kinds of media are used.

10. The components of the various media forms are given in the tables below.

EXPERIMENT 4

Objective- To prepare Dublbacco's buffer salt solution

Principle: A synthetic blend of inorganic salts known as a physiological or stabilized salt solution is the basis of all media used in tissue culture (BSS). The salt solution originally defined by Sydney Ringer was used to derive all physiological salt solutions (1885). The fifth one Tyrode's solution was a balanced salt solution primarily intended to promote the metabolism of mammalian cells. In order to achieve improved buffering salt solutions and to avoid calcium precipitation, many improvements have been made since then.

The function of a salt solution is:

·To maintain the medium within physiological pH range.

·To maintain intracellular and extra cellular osmoticbalance.

·Modified with a carbohydrate, such as glucose serves asan energy source for cell metabolism.

Materials required

Tissue culture grade water

1N Hydrochloric acid

1N Sodium hydroxide

INORGANIC SALTS COMPOSITION

Ingredients	mg/L
Calcium chloride dihydrate	133.000
Disodium hydrogen phosphate	1150.000
Magnesium chloride hexahydrate	100.000
Potassium chloride	200.000
Potassium phosphate, monobasic	200.000
Sodium chloride	8000.000

Procedure:

1. Weigh all the inorganic salts and apply constant, gentle stirring in 900 ml of tissue culture grade water. Please do not heat the water.
2. To dissolve the powder entirely, it is important to lower the pH to 3.00 with 1N HCL.
3. Using 1N NaOH, change the pH to 0.2-0.3 pH units below the ideal pH until the powder is fully dissolved, as the pH continues to increase during filtration.
4. With tissue culture grade water, render the final volume up to 1000ml.
5. Immediately sterilize the solution by filtering into a 0.22 micron orless porosity sterile membrane filter, using positive pressure rather than vacuum to reduce carbon dioxide loss.
6. The desired quantity of sterile solution is aseptically dispensed into sterile tubes.
7. Store the liquid solution at room temperature and in dark until use.

Condition of media to be maintained

Appearance
Off-white to Creamish white
Solubility
Clear solution at 9.8gms/L
pH
7.00 -7.60
Osmolality (mOsm/ Kg H2O)
280.00 -320.00
Toxicity test
Passes
Endotoxin content
NMT 1EU/ml

Storage and Shelf Life:

1. At room temperature, all powdered salt mixtures and formulated salt solutions should be processed. Apply before the expiry deadline. Any powdered salts may display certain signs of oxidation / degradation in certain situations, considering the above suggested storage conditions. The difference of color, change in appearance and occurrence of particulate matter and haziness after dissolution may suggest this.

2. Preparation of condensed solutions is not advised, since concentrated solutions may be precipitated by salt complexes of poor solubility.

3. Sterile supplements should be added to the sterile solution as needed, observing all precautions for sterility. The solution's shelf life would depend on the quality of the supplements.

To the approach applied.

Experiment 5- To prepare Hank's salt solution

Objective- To prepare Hank's salt solution

Principle: A synthetic blend of inorganic salts known as a physiological or stabilized salt solution is the basis of all media used in tissue culture (BSS). The salt solution originally defined by Sydney Ringer was used to derive all physiological salt solutions (1885). The fifth one Tyrode's solution was a balanced salt solution primarily intended to promote the metabolism of mammalian cells. In order to achieve improved buffering salt solutions and to avoid calcium precipitation, many improvements have been made since then.

Hanks' Balanced Salts are engineered to balance oxygen, so no combination of CO2 air is needed.

The Controlled Salts mixture of Hanks with phenolred is intended for use with cells that are kept in a less CO2 or CO2-free environment.

The function of a salt solution is:

·To maintain the medium within physiological pH range.

·To maintain intracellular and extra cellular osmoticbalance.

·Modified with a carbohydrate, such as glucose serves asan energy source for cell metabolism.

Materials required

Tissue culture grade water
1N Hydrochloric acid
1N Sodium hydroxide

Sodium bicarbonate

Sodium bicarbonate solution

INORGANIC SALTS COMPOSITION

Ingredients	mg/L
Calcium chloride dihydrate	185.410

Disodium hydrogen phosphate	48.000
Magnesium sulphate anhydrous	97.720
Potassium chloride	400.000
Potassium dihydrogen phosphate Anhydrous	60.000
Sodium chloride	8000.000

OTHERS

D-Glucose	1000.000
Phenol red sodium salt	11.000

Procedure:

1. Weigh all the inorganic salts and apply water with constant, gentle stirring in 900 ml of tissue culture grade water. Air, do not heat it.
2. For 1 litre of solution, add 0.350gms of sodium bicarbonate powder or 4.7ml of 7.5 percent sodium bicarbonate solution and mix until dissolved.
3. Using 1N HCl or 1N NaOH, change the pH to 0.2-0.3 pH units below the target pH, so the pH continues to increase during filtration.
4. Make up the final volume of tissue culture grade water to 1000ml.
5. Immediately sterilize the solution by passing into a sterile membrane filter with a porosity of 0.22 micron or less, using positive pressure to reduce carbon dioxide loss rather than vacuum.
6. The desired quantity of sterile solution is aseptically dispensed into sterile tubes.
7. Hold the liquid solution at room temperature and in the dark before it is used.

Condition of media to be maintained

Appearance

White to light pink, homogenous powder

Solubility

Clear solution at 9.8 gms/L

pH without Sodium Bicarbonate

6.20 -6.80

pH with Sodium Bicarbonate

7.00 -7.60

Osmolality with Sodium Bicarbonate (mOsm/Kg H2O)

280.00 -320.00

Endotoxin Content

NMT 1EU/ml

Toxicity Test

Passes

Storage and Shelf Life:

1. At room temperature, all powdered salt mixtures and formulated salt solutions should be processed. Apply before the expiry deadline. Any powdered salts may display certain signs of oxidation / degradation in certain situations, considering the above suggested storage conditions. The difference of color, change in appearance and occurrence of particulate matter and haziness after dissolution may suggest this.

2. Preparation of condensed solutions is not advised, since concentrated solutions may be precipitated by salt complexes of poor solubility.

3. Sterile supplements should be added to the sterile solution as needed, observing all precautions for sterility. The solution's shelf life would depend on the form of supplements applied to the solution.

Experiment 6: Objective:To determine the optimum pH of trypsin.

Ingredients: Trypsin, Water bath**,** Gelatin, photographic film, Tris -buffersolutions of pH around 2.0, 4.0, 6.0, 8.0, and 10.0

Introduction

Trypsin is a protease of sorts. In the small intestine, this enzyme is active and can break down proteins into amino acids. Various enzymes can have varying levels of maximal pH. The enzymes work better at the optimal pH. The operation is the best. Excessive hydrogen or hydroxide ions can break the ionic bonds at a lower pH or greater pH. This alters the enzyme's structure. It also affects the shape of the active site. The catalytic activity is decreased by this. A protein called gelatin has a photographic film that coats its surface. A whitish stain will surface if it is removed.

Principles

In this experiment, we put a strip of photographic film into a solution of trypsin. The protease can digest the gelatin coating to turn it whitish. To change the ambient pH, separate buffer solutions may also be applied to the solutions. The gelatin's degree of digestion represents the enzyme's operation. The optimum pH should, thus, be determined.

Procedure

1. Set up and change the temperature to 37 ° C in the water bath.

2. Pipette one mL of pH 1.0 buffer solution into a test vessel.

3. Connect a strip of photographic film to the solution and place it in a 5-minute water bath.

4. Pipette 1 mL of the solution of trypsin into a further test vessel. For 5 minutes, put it in a water tank.

5. Pour 1 mL of trypsin solution into a film-based test tube.

6. Record the time it takes for the gelatin to dissolve entirely.

7. Repeat the experiment with pH about 2.0, 4.0, 6.0, 8.0, and 10.0.0 buffer solutions.

Precautions

1. Since temperature is a factor influencing enzymatic operation, the temperature must be kept steady.

2. It is easy to scrape and destroy the gelatin layer, and should be treated with caution.

3. It is necessary to bring the enzyme and the film into the water bath for 5 minutes before mixing. This causes the optimal temperature to be achieved by both substrates and enzymes before combining.

4. It is important to combine the tubes from time to time.

5. If the gelatin coat lasts for longer than 1 hour, we should conclude that infinity is the time to hit the maximal pH.

Experiment-7- To disaggregate tissue by the technique of cold trypsinization

Isolation of Animal Material (Tissue)

Attempts should be taken during laboratory work to ensure that animal products are not infected. Animal products should be managed while glassware and media are sterilized. Before that a balanced salt solution (BSS) is required. This solution consists of 1000 penicillin units and 0.5 mg per ml of streptomycin or neomycin. The tissue to be grown should be properly sterilized with 70 per cent ethanol and surgically removed under aseptic conditions before culture animal materials are washed aseptically in BSS to prevent contamination. To deter pollution, efforts should be made. Thus, the isolated tissue is either preserved in the freeze or immediately used.

Disaggregation of tissue

Some of tissues consist of cells which are tightly aggregated. Tissue like epithelium is impregnated with Ca^{2+} and Mg^{2++} ions that provide integrity to it. Therefore, for getting primary culture it is necessary that tissue must be disaggregated either mechanically or using enzymes or chemicals so that cell suspension could be obtained. The cells in suspension grow to produce primary culture.

(i) Physical (mechanical) disaggregation. The tissue after careful removal from a given spot is aseptically kept in a sieve of 100mm sieve (Fig.1). It is put in a sterile Petri dish containing buffered medium with balanced salt solution. The cells are alternately passed through sieves of decreasing pore size. (50mm and 20mm mesh). If desired the process is repeated to get more disaggregation of cells. The debris remaining on sieves are discarded and medium containing cells is collected. The cells are counted using a haemocytometer. If necessary, medium is diluted by serum to raise the level of cells to 10^4 cells/ml. The other methods of mechanical disaggregation of cells are forcing the cells through syringe and needle or repeated pipetting. Although the physical method is quick and cheap yet it damages many live cells.

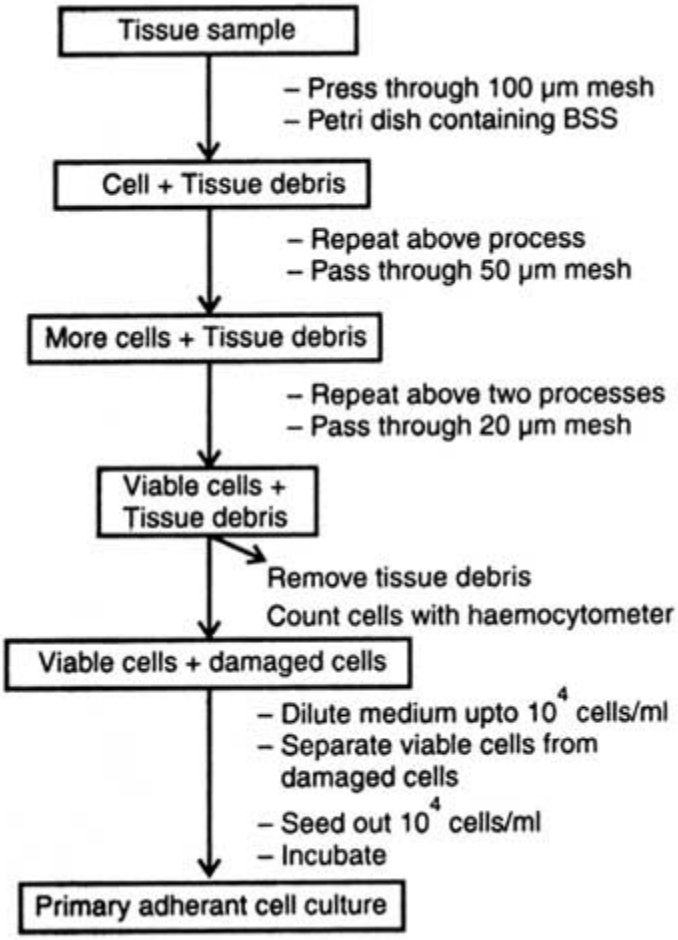

Fig.1 Physical disaggregation of tissue by sieving (diagrammatic).

The viable dissociated cells are now termed as 'primary cells'. When the primary cells are seeded on culture medium in high density, these grow well. Thus the primary viable cells of primary culture are called adherant culture and the cells adherant cells. Moreover, at this stage some of the non-viable cells if growing along with adherant cells can be separated out by using the second medium. Similarly, primary culture can also be grown in suspension. In suspension the non-viable cells be removed from primary disaggregates by centrifugation using Ficoll and sodium metrizoate. In this way viable cells are separated from non-viable cells.

(ii) Enzymatic disaggregation. Enzymes are also used for dislodging the cells of tissue. By using enzymes a high number of cells is obtained. Moreover, in embryonic tissue a high number of undifferentiated cells with least extracellular matrix is found. Therefore, disaggregation of embryonic tissue occurs more readily than that of adult or new borns. In addition, in fragile tissue such as tumours the chances of cell death and cell recovery are more than the normal tissues. There are two important enzymes used in tissue disaggregation, collagenase and trypsin.

(a) Use of collagenase. Collagenase is used for disaggregation of embryonic, normal as well as malignant tissues. The intracellular matrix contains collagen; therefore, collagenase disaggregates normal and malignant tissues. Moreover, the epithelial cells can be damaged by it but the fibrous tissues remain unaffected. Moreover, the epithelial cells can be damaged by it but the fibrous tissues remain unaffected.

The crude collagenase also contains non-specific proteases. First, the biopsy tissues are kept in medium containing antibiotics. Thereafter, the tissue to be disaggregated is dissected into pieces in basal salt solution containing antibiotics (Fig. 2). The chopped tissue is properly washed with sterile distilled water and transferred in complete medium containing collagenase. After five days of treatment the mixture is pipetted so that the medium may get dispersed.

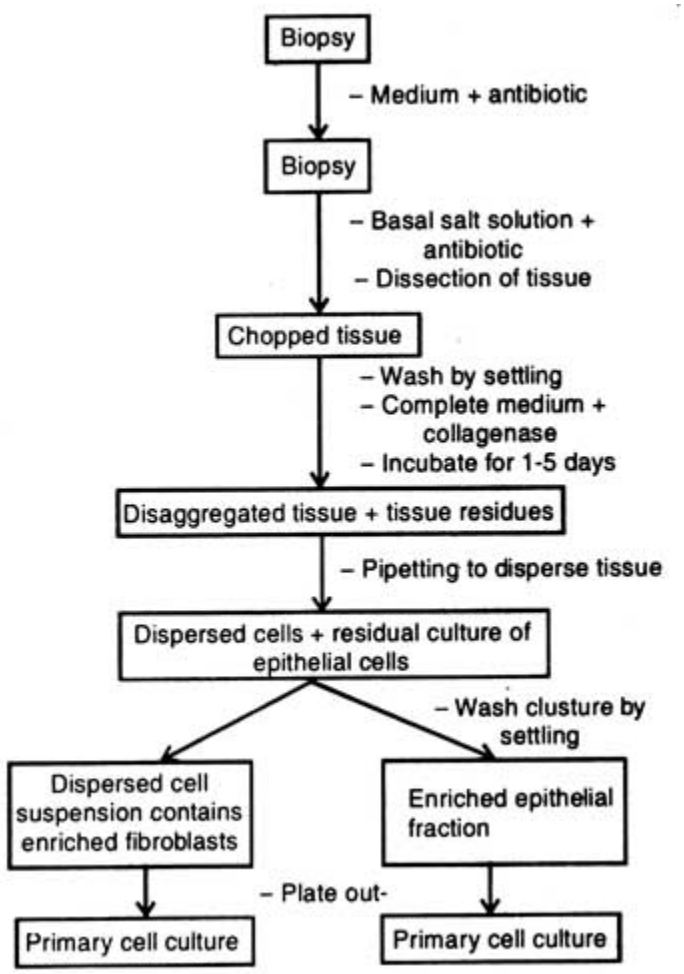

Fig.2 Tissue disaggregation by collagenase.

When the whole treatment is left for some times, the residual clusture of epithelial cells settles on bottom of test tubes. Clustures present in test tubes are washed by settling or the dispersed cell suspension is

made free from the enzyme collagenase by centrifugation. Suspension consists of enriched fibroblast fraction which is plated out on medium. Similarly, the clusture which is washed by settling consists of enriched epithelial fraction. It is also plated out on medium.

(b) Use of trypsin. Use of trypsin for disaggregation of tissue is called trypsinization. However, the enzyme trypsin in crude form is commonly used for embryonic tissue because many kinds of cell can tolerate it and different types of tissues are significantly affected. Besides, serum or trypsin inhibitors *(e.g.* soybean trypsin inhibitor) can neutralize its residual enzyme activity only in serum-free medium. On the basis of role of temperature on trypsin, activity is of two types, cold trypsinization and warm trypsinization.

Cold trypsinization. The tissue sample to be disaggregated is chopped into 2-3 small pieces and kept in small sterile glass vial (Fig.3). If necessary, these may be washed with sterile distilled water. The pieces are removed from vials, dissected keeping in BSS, The whole content again transferred in glass vial is placed on ice and soaked in cold trypsin for 4-6 hours. This allows penetration of enzymes in tissue. Further, trypsin is removed and tissue is incubated at 36.5°C for 20-30 minutes. The vials that contain tissue pieces, 10 ml of medium containing serum is added and cells are dispersed by repeated pipetting. The cells are counted by using haemocytometer. Cell density is maintained to 10^4 cell/ml with dilution in growth medium. These are plated and incubated for 48-72 hours for cell growth.

Warm trypsinization. Similar to cold trypsinization, the tissue sample is chopped into 2-3 pieces (Fig. 6.2B) and washed in distilled water keeping in glass vial. The pieces are transferred into 250 ml flask containing 100 ml warm trypsin (36.5°C).

The content is stirred for 4 hours, thereafter, pieces are allowed to settle. The dissociated cells are collected at every 30 minutes. This facilitates the minimum exposure of cells to warm enzyme. The process may be repeated by adding fresh trypsin back to pieces and incubating the contents. The trypsin is removed by centrifugation after 3-4 hours during which complete tissue may be disaggregated. The glass vials containing dispersed cell pellets in medium are placed on ice.

After different trypsinization time, samples are pipetted, cells counted using haemocytometer and cell density maintained to 10^4cells/ml. The cells are plated on medium and incubated for 48-72 hours for cell growth.

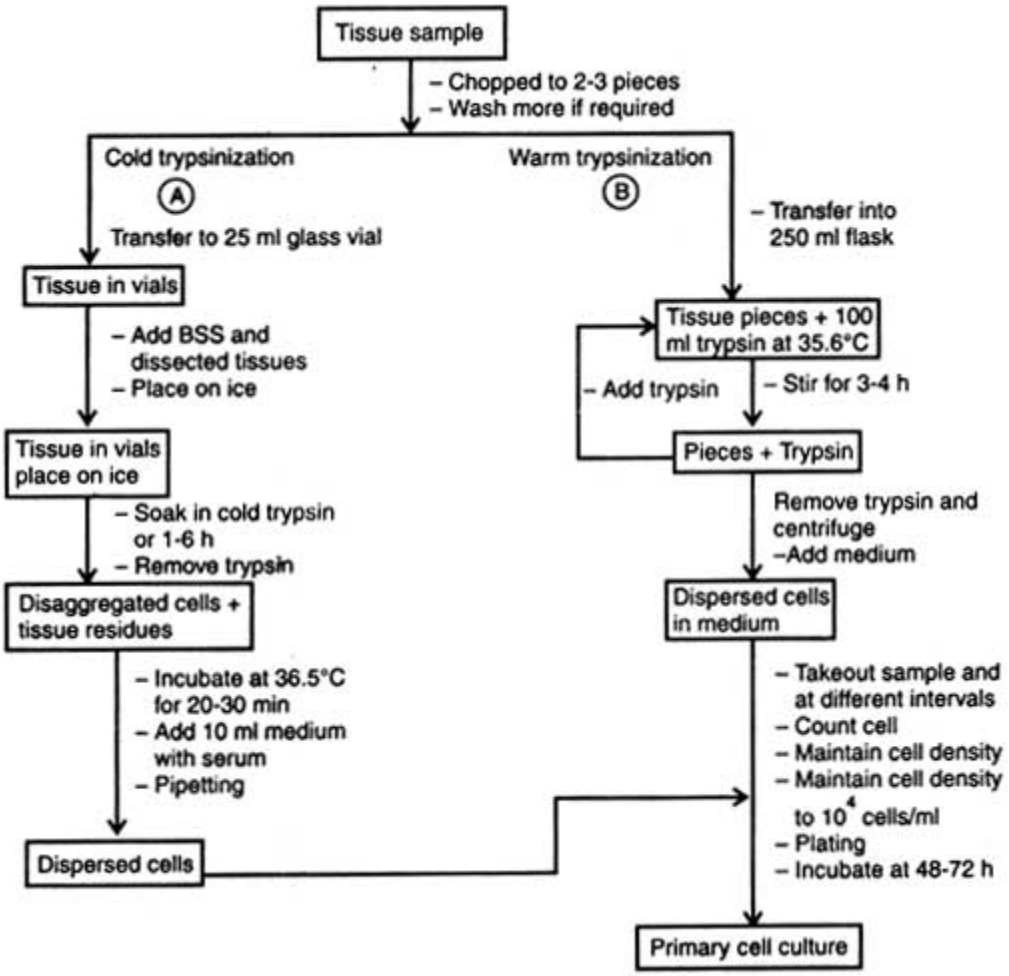

Fig.3. Diagrammatic representation of primary cell culture by disaggregation in trypsin.

EXPERIMENT 8

AIM: To separate the serum and plasma from the given blood samples.

INTRODUCTION: The fluid portion of blood that suspends blood cells is blood plasma. Blood plasma The average amount of blood is about 60 percent. Mostly consisting of water, it comprises dissolved proteins, glucose, coagulation agents, mineral ions, hormones and carbon dioxide (plasma being the main component for excretory product transportation). Plasma is the surnatant substance produced by the centrifugation of the anti-coagulated blood. A suitable quantity of anti-coagulant including heparin, oxalate or EDTA is used for the blood. In order to prevent clottage, this preparation should be combined sooner than later. The blood serum is the fibrinogenless blood plasma or other coagulation causes.

PROCEDURE:

•Blood Plasma Preparation

1. Draw blood into the centrifuge tube with around 1.8 mg potassiumEDTA per millilitre of blood. To ensure the right number, make sure to take the whole volume.The ratio of blood to anti-coagulant.
2. Carefully invert the centrifuge tubes 10 times to combine the blood and anti-coagulant and store before centrifugation at room temperature.
3. Samples can automatically undergo centrifugation. This should be carried out byOut in the room for a minimum of 10min at 1000-2000rcf (usually 1300rcf)Temperature. Temperature Do not interrupt the centrifuge from using breaks.
4. It would have three layers of plasma, leucocytes (Buffy coat), and erythrocytes from top to bottom.
5. At room temperature, gently aspirate the supernatant (plasma) and pool it in a centrifuge tube. Take caution not to disrupt or move any cells to the cell layer.
6. Examine plasma for turbidity. To extract residual insoluble matter, turbid samples should be discarded and centrifuged and aspirated again.

•Blood Serum Preparation-

1. Draw all of the blood into centrifuge tubes with no anticoagulant in them. Draw the amount required for use approximately 2.5 times.

2. Incubate for approximately 60 minutes at room temperature (30-45 degrees) in an upright position to facilitate clotting.

3. 1000-2000rcf Centrifuge. Do not interrupt the centrifuge from using a split.

4. Aspirate the supernatant (serum) cautiously and swim into a centrifuge channel, Take caution not to disrupt or move any cells to the cell layer. Using a clean one

For each channel, a pipette.

5. Inspect the turbidity serum. It is essential to centrifugate turbid samples and

Aspirated to extract insoluble matter again.

OBSERVATIONS: In the centrifuge tube containing blood and anti-coagulant, three layers were clearly seen. From the floating layer over the clotted blood, serum was extracted.
RESULT : At the end, a bright yellow liquid color is found on the supernatant.

EXPERIMENT 9

Aim: To immobilize enzyme usingagar/agarose/ polyacrylamide gel.

Equipments and Materials

Agar/agarose/bisacrylamide and acrylamide, salivary amylase, petri plates, spatula, beaker, hot plate, Spstarch, iodine solution.

Method

Amylase enzyme, immobilized by polyacrylamide, agar/or agarose gels form of gel trapment. Agar/agarose (0.25, 0.5, 1.0, 1.5 and 2.0 percent) and polyacrylamide (7.5, 10.0, 12.5, 15 and 20 percent) are used for enzyme trapping and the trapped enzyme is tested for repeated cycle conversion of the substrate into the substance.

Entrapment in agar/agarose gel

The agar/agarose solution is heated separately to liquify the agar at concentrations of 0.5, 1, 2, 3 and 4 percent. Gel is permitted to cool to 45-50o and then the same volume of amylase is applied to it in the phosphate buffer (25 ml) such that the final agar concentration is 0.25, 0.5, 1, 1.5 and 2 percent, respectively. The agar-amylase mixture is then allowed to solidify and cut into small blocks on the glass plate and is used for repeated cycles to transform starch to maltose.

Entrapment in polyacrylamide gel

The most widely used substance for entrapment is polyacrylamide gel. Method given by Freeman and Aharonowitz for cell trapping by modified polyacrylamide gel. 7.5 g acrylamide, 0.5 g bisacrylamide, 50 mg ammonium persulfate was applied to 25 ml of phosphate buffer, pH 6.8, for preparation of 15 percent gel, and combined to dissolve these solids. Then 25 ml of solution with amylase is added. Properly blended and 50 µl TEMED attached. Gently combined and poured into glass Petri dishes or vertical electrophoresis machine gel casting to achieve the uniform and ideal thickness gel (with thick gels, problem was of diffusion of substrate). Polymerization is achieved for 1 h at room temperature. The gel is broken into small parts and

suspended for further use in a phosphate buffer of 0.1 M. Similarly, 3.75, 5.0, 6.25 and 10.0 g of acrylamide was applied to the final concentration of 7.5, 10.0, 12.5, and 20 percent for the preparation of other gel concentrations.

Measuring the enzyme activity

The activity of amylase is assessed by digesting 5 ml of 1% starch solution to the achromic stage. A variation in the color of the iodine solution from blue to colorless indicates the time needed for the conversion of starch into maltose. For trapped enzymes in agar or polyacrylamide gels, and even for free enzymes as a control, the time of conversion of starch into maltose is noted.

EXPERIMENT NO: 10

PRIMARY CELL CULTURE

Aim : To perform primary cell culture technique using chick embryo under aseptic condition.

Introduction:

The development of in vitro animal cell culture techniques has proved to be useful for the study of cell structure and function under controlled conditions. In addition, cultivated cells find important applications in the development of vaccines, in the production of hybridoma and in chromosome karyotyping. If it is sufficiently distributed, nearly every tissue can be cultured, however high rates of cell culture success are more reported for embryonic and tumor tissues rather than normal adult tissues.

Cultures starting fresh from tissues are referred to as primary cultures. A procedure for the dissemination of

Below are the main cultures of mouse embryo cells that can be adopted for the culture of other embryonic tissues obtained from various organisms. Normaltissue-derived primary and secondary cultures also have a finite life cycle close to their in vitro life. However, by passing this definitive lifetime, certain cells from a large population aresecondary cultures are immortalized with an ability to differentiate forever and these are called cell lines. In society, several cancer cells have the capacity to fragment forever. Virus-transformed normal cells and chemical carcinogens have become continues cell lines.

Principle

From large tissue volumes, primary cultures are normally prepared. Therefore, a number of distinct cells are present in these cultures. For primary cultures, embryonic tissues are chosen because the embryonic cells can be quickly disaggregated to have more viable cells. Because their survival rate is considerably smaller, the quantity of cells used in the primary culture should increase.

Materials Required:

13 – 14 days pregnant mouse / 8-10 days old embryonic eggs.

100 ml beaker – 1

2 pairs of scissors

A pair of bent scissors

2 big forceps

Petriplates – 2 pairs

100 ml conical flasks

Small funnel covered with

cheese cloth – 1

10 ml testubes cotton plugged – 4

Trypsinization flask – 1

Growth medium (M.199 with 10% ox serum)

Calcium, Magnesium free – phosphate buffered saline (PBS).

METHODS

MOUSE EMBRYO FIBROBLASTS

For cultivation, mouse embryos aged 13-15 days are required. Swiss mice were held for the purpose of having these

Mating and the gestation cycle were periods when the day of genital plug discovery was designated as the first day of growth.

1. Sacrifice by cervical dislocation of the pregnant rodent. Put the beast in its supine position.

2. With 70% ethanol, swab the abdomen and split it open around the midventral axis.

3. Drop the uterine horns and pass them to a PBS-containing beaker. Move to hood of flow Instantly.

4. Inside the flood, pass the uterine horns into PBS-containing petriplates and break open theuterine horns to remove embryos.

5. Wash the embryos and cut the head, visceral organs and appendages with PBS.

6. Shift the remains of the embryos with a pair of bent scissors to another petriplate containing tiny amounts of PBS and mince thoroughly.

7. The minced tissue is moved to a PBS trypsinization flask containing 40 ml of 0.25 percent trypsin.

8. For 30-60 mins, swirl the contents at 37oC.

9. Add 5ml of serum-containing medium at the end of the above time and stir the contents for 2 more minutes to inactivate the activity of trypsin.

10. Using sterile cheese cloth to filter the cell suspension and catch the filtrate into a 100 ml conical flask.

11. Centrifuge the filter for 10 mins at ~ 1000 rpm.

12. Pour the supernatant out and resuspend 5 ml of medium with the pellet. Distribute to all 120cm2 culture bottles in equal proportion and incubate at 37oC.

Chick Embryo Fibroblasts

The CHF culture protocol is the same as for MEF. Remove embryos from 8-10 day old embryos temporarily, split the shell with the aid of foreceps and pass the embryo into PBS-containing petriplates and adopt steps 5 to 12 of the culture of mouse embryo fibroblasts (see above). While chick embryo cells develop in the same medium, if 1 percent chicken serum is also added, they will grow faster. You should substitute Millipore-filtered goat serum (10 percent) for the culture of the above cells instead of calf serum.

EXPERIMENT NO 11

PREPARATION OF ESTABLISHED CELL LINES

Aim: To develop secondary growth or established cells from primary culture by repeated subculture.

Principle

Cells which derive from a primary culture subculture are called cell lines. Primary cell lines sometimes divide for a long time at a very high rate and may be transmitted repeatedly. At times, few cells can change in such a way that they acquire a different morphology, expand and multiply faster. For a long period, these cells can be cultured and can also be permanently subcultured in vitro. These lines of cells are called existing lines of cells.

Materials required

Monolayer cells(chick embryo)

Beaker

TC bottles

Trypsinization flask

Growth medium

Phosphate buffer saline

Pasteur pipette

Trypsin

Methods

Take the Tc bottle, which holds a fully shaped cell monolayer.

2. Discard the old medium and use PBS to wash the monolayer three times.

3. Add 0.25% trypsin to 5-6 drops and cause the drops to extend over the entire monolayer.

4. To make it possible for the cells to fall off the substratum, wait a minute and shake the container vigorously.

5. Then apply 5ml of medium containing serum until the cells start to come off. Pasteur Flush withto dislodge the cells that adhere to the glass surface, the pipette. Divide the suspension of cells into two,Incubate the bottles at 37oC.

6. To extract the dead cells along with the old, replace the new medium after three days Medium.

7. To generate proven cell lines, the above steps should be repeated 70 times.

Result

Cell lines may undergo extensive changes in their properties through repetitive subculturing, i.e. the cells may expand in clumps rather than in monolayers, the orientation of the cells can be abnormal. These cell lines are said to have been modified, and they are most usually neoplastic. It is also understood that the cell lines produced have odd chromosome numbers.

EXPERIMENT NO -12

STAINING OF ANIMAL CELLS

Aim : To ensure the differentiation of live cells from dead cells by Giemsa stain method.

Introduction

In order to provide fast quantitation of live cells, a variety of critical staining processes have been established. Most commonly, a large proportion of cells are destroyed as tissues are spread to collect cells. So, before going on, the proportion of living cells needs to be identified. The metabolic activity of cells (e.g. staining with methylene blue) relies on certain staining techniques, while others using trypan blue or erythrosineB rely on the cells' membrane transport properties. Living cells exclude these stains (do not pick them up).

These critical staining techniques are very random and reservations should be used. The easiest way to assess viability is to plate (propagate) the cells and later count the cells or nuclei to estimate the plating quality. This strategy is based on the fact that it can reproduce only viable cells.

Vital Staining

1. In a container, hold 0.5 ml of dilute cell suspension and add 0.1 ml of 0.4% erythrosin B or Trypan Blue to the tube.
2. Under the microscope, observe a decline. For erythrosine B or Trypan Blue, the dead cells are stained red.

Preparation of Erythrosin B Stain

Dissolve 0.4 g of Erythrosin B in 100ml sterile PBS.

Giemsa staining

Preparation of 10X Giemsa Stain

1. Add 20ml of glycerol to 0.3g of Giemsa powder and store at 56oC for 2 hours in a water tank.
2. Then apply the methanol to 20ml and blend properly. Keep it on RT for 7 days, then filter.

Staining Method

1. Monolayer with PBS Rinse

2. Fixed with 10ml of 3:1 Methanol: ethanol or acetic acid

3. Drain-fixer and dry air

4. Dilute Giesma 1 to 10 (10ml + 90ml H_2O) and dye for 30 min with 20 ml of diluted stain.

5. Discard the stain, rinse with water several times, eventually with tap water, dry air and look beneath with a microscope.

Observation

The viable cells appear unstained and the dead cells look red, arising from manual disruption (chopping) or chemical disruption (tripsinisation). The cell membrane is weakened by disaggregation, so that the dye does not pass into normal cells in the damaged cells.

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