Uttar Pradesh Rajarshi Tandon
Open University

CPLT-02

Printed and Published by Dr. Arun Kumar Gupta Registrar, Uttar Pradesh Rajarshi Tandon Open University, 2020.

Printed By: Chandrakala Universal Pvt. Ltd. 42/7 Jawahar Lal Neharu Road, Prayagraj.

RIL-037

UNIT 1 AN OVERVIEW OF BIOLOGY LABORATORIES

Structure

1.1 INTRODUCTION

In Unit 1 of LT-1 course, you have learnt about the design and essential requirements of a good laboratory (abbr. singular lab, plural labs). In this unit we will concentrate on the features and requirements specific to biology labs. Special features of physics and chemistry labs will be discussed in the respective courses.

Undoubtedly, biology labs are remarkable and interesting places to visit even for a layman. There are a variety of animal and plant specimens, charts, displays, models of heart, lungs and brain and other vital organs, and above all a full-size human skeleton. The overall sight of a biology lab is intriguing.

As students we look at a lab with a different perspective. We observe the features that interest us the most and the ones that cannot go unnoticed. We also take note of the things that our teachers point out to us. Unfortunately, we are in a hurry to complete the experiments and cannot afford to get completely involved in it even if we wish to. There is always the pressure of the theory component and we have to do a tight time-budgeting for getting good marks. But for a technician or a research scholar it is very important to be familiar with every aspect of a laboratory where he/she is placed, so that he/she can contribute to its organisation, management and maintenance.

This unit provides you with an overview of the biology labs of schools and colleges; ancillaries to it are discussed in detail in the following unit. Since no two labs are alike, we also guide you to what all to look for in a biology lab.

Objectives

After studying this unit you should be able to:

- describe the typical features of biology labs of schools and colleges,
- list the essential rooms/units of a biology lab,
- describe the basic features of the main lab,
- investigate the infrastructure and the facilities available in the lab of your study centre, and

- suggest the features that could possibly be introduced to enrich a biology lab.

Study Guide

Before you work through this unit, it is important to study Unit 1 of LT-1 course which is basic to this unit. It would be best to read this unit while sitting in a biology lab or to make a visit to it on the day you plan to work on it.

1.2 GENERAL FEATURES OF BIOLOGY LABORATORIES

You must have had the experience of working in a biology lab of your school or college. Can you recall its features? You may list them below.

A biology lab in a school or college consists of a few rooms. In schools generally there is a main lab, a store and probably an office. In colleges there are separate labs for botany and zoology. Since many new branches of biology have come up, there may also be separate labs for Physiology, Biochemistry, Microbiology and Tissue Culture as well. In addition, colleges generally have a preparation room, microtomy room, museum, herbarium and animal house. Some colleges may also have additional facilities such as cold room, darkroom for photographic work, culture room, balance room, instrument room etc. There may also be a poster room and a projector room. The list does not end here because special rooms are made keeping in view the interest of the staff and the availability of space.

Like houses, one laboratory is different from the other in size, design, layout and also in the facilities and organisation. The lab space increases with the level of education so also the number of rooms (units). The lab space in a college is decided taking into account the variety and number of courses which will be on offer and the probable enrollment. Accordingly, the rooms of a laboratory are planned. In almost all the labs the maximum space is allotted to the room(s) where experiments are to be performed. You may recall from Unit 1, LT-1 course that we opted to call this working room the **Main Lab**. There may be more than one main lab in a college.

Biology experiments require outdoor facilities such as garden plots, forest trail, aquarium, animal house, vivarium and green house. Some enthusiastic teachers make provision for campsites, farm sites, vegetable garden and fruit garden. There are open ground laboratories for experimentation. In all probability you will not find all the elements in one college. Our experience shows that in India most public schools and colleges have the minimum facilities i.e. a main lab, a store and an office and in some cases even these may not be well maintained and utilized.

From the above account we hope that you have a fairly good idea of the general features of biology labs. In the following sections we will discuss the main lab, preparation room and store. Before you read further, work on the following SAQ.

SAQ 1

a) List the essential units of a biology lab of your school.

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b) What could be the other units of a biology lab?

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c) In the following statements fill in the blank spaces with appropriate words.

i) The outdoor facilities for a biology lab are ....., vivarium, garden plots and .....

ii) In colleges there could be separate labs for ....., ... and Microbiology.

1.3 COMPONENTS OF BIOLOGY LABORATORIES

In most schools and colleges a biology laboratory consists of a main lab, a preparation room, a store and an office. Let us know about them in some detail.

1.3.1 Main Laboratory

As you enter into a main biology lab you see work tables and benches, neatly stacked staining solutions, chemicals, a variety of glassware, instruments and electrical appliances. In schools since there may not be a separate museum, the museum specimens are generally placed in the almirah or shelves and racks fixed to the walls of the main lab or in corridors.

Fig. 1.1: A view of a main biology lab.

Before we list the essential items of a biology lab we should consider the design and organisation of biology labs.

Design and Organisation

In Units 1 of LT-1 course, while considering the design of laboratories, we discussed the need for appropriate space; benching and their surfaces and storage; lighting and ventilation; heating and cooling; services-gas, water and electricity; flooring; access to and from the lab and security. Here we will discuss them in relation to the biology labs.

While visiting a lab it is a good practice to try to get an idea of the space. A main biology lab is quite spacious and its size is comparable to physics and chemistry labs. In an average size lab approximately 15 to 20 students can be accommodated.

Generally biology labs in public institutions are of fixed design with 3 to 4 long benches, each accommodating 4-5 students in a row. The arrangement of benching may vary from lab to lab. The sinks are generally provided at the end of the benches, but fewer in number than found in the chemistry laboratory. The sink may be fitted with a large strainer to remove organic material. Benches are generally made of wood and their surfaces are treated to resist corrosion by acid and alkali spills on them. In some labs bench tops may be of granite or Kota stone which are easy to clean and maintain.

Since quite a few experiments such as dissections, preparations of slides and their microscopic examination are performed while sitting, you will find several stools. There is also a large table for the demonstration of experiments. The other essential items of furniture are : open and closed shelves for keeping chemicals, a built-in cabinet for keeping microscopes, racks for dissecting trays and boards, slide cabinets and provision for under bench storage.

Fig. 1.2: Provision of light in a biology lab.

In a biology lab living plants and animals are studied extensively so arrangements for an optimal light is made. For receiving adequate daylight, very large glass windows are installed in the lab. There is a provision for permanent supplementary artificial light (PSAL) on the walls or ceiling. In addition, there are movable lamps which are an additional source of light when one is working with a dissecting microscope, hand lens or for observing the details of a dissected animal. So several electrical outlets are provided in biology labs.

Do you know what kind of odour is typical of a biology lab?

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To minimise the odour of plant and animal materials and preservatives typical of a biology lab, adequate arrangements for ventilation are made.

You will find that biology labs are equipped with gas, water and electricity. Some labs are provided with LPG cylinders instead of gas pipeline because the use of gas is not so frequent at school or college level. There are several outlets for electricity and water.

For distilled water there may be a distillation apparatus in the lab.

Cleanliness is of paramount importance for biology work. A good design, high quality construction and appropriate use of material on walls, floor, ceiling, benches and other furniture help in the maintenance of cleanliness. But, *we find that these aspects are generally most neglected.* The materials used in public places should be of superior quality and workmanship and lot of emphasis should be given to proper day-to-day maintenance.

Fig. 1.3: Microscopes stored in an almirah.

Instruments

Instruments are essential feature of any science lab. Among instruments balances, pH meter, spectrophotometer, ovens, magnetic stirrers and shakers, chromatography apparatus are commonly found in chemistry and biology labs. The following are specific for a biology lab: magnifying glasses, dissecting microscopes, light microscopes, dissecting kits and trays, microtomes, knife sharpners such as, hones, strops and mechnical and electric blade sharpners, dissection trays, dissection boards, wax dishes, hammers, bone cutters, autoclave, pressure cooker, safety cabinet for inoculation, recording drum, BOD chamber, haemoglobinometer, haemocytometer, manometor and potometers.

Glassware

Common glassware such as test tubes, beakers, flasks, burettes and pipettes, measuring cylinders, dessicators etc. are present in a biology lab. Besides, bell jars, specimen jars, trough, petridishes, microslides, cover slips, cavity blocks, watch glass cuvetters are specially required for biology work.

Specimens

A variety of preserved and well-labelled specimens of plants and animals displayed in the lab is fascinating to watch. You will also find some rare reptiles, birds and mammals preserved as stuffed specimens. In plants, common cash crops, medicinal plants and diseased plants are on display. Also a variety of fruits, seeds, inflorescence, modified parts of plants are kept preserved and properly labelled. There may also be samples of spices, oil seeds, types of wood, varieties of cereals and herbarium specimens.

Fig. 1.4: Display in a biology lab of specimens.

Models

Models of heart, brain, lungs, digestive and reproductive organs are generally seen in a biology lab. In plants models of the anatomy of a stem, leaf and root are displayed.

Bones

There may be a full-size human skeleton and skeletons of some animals. Besides skull and vertebral column, skeleton girdle, limbs are commonly found in racks or cases along one of the walls.

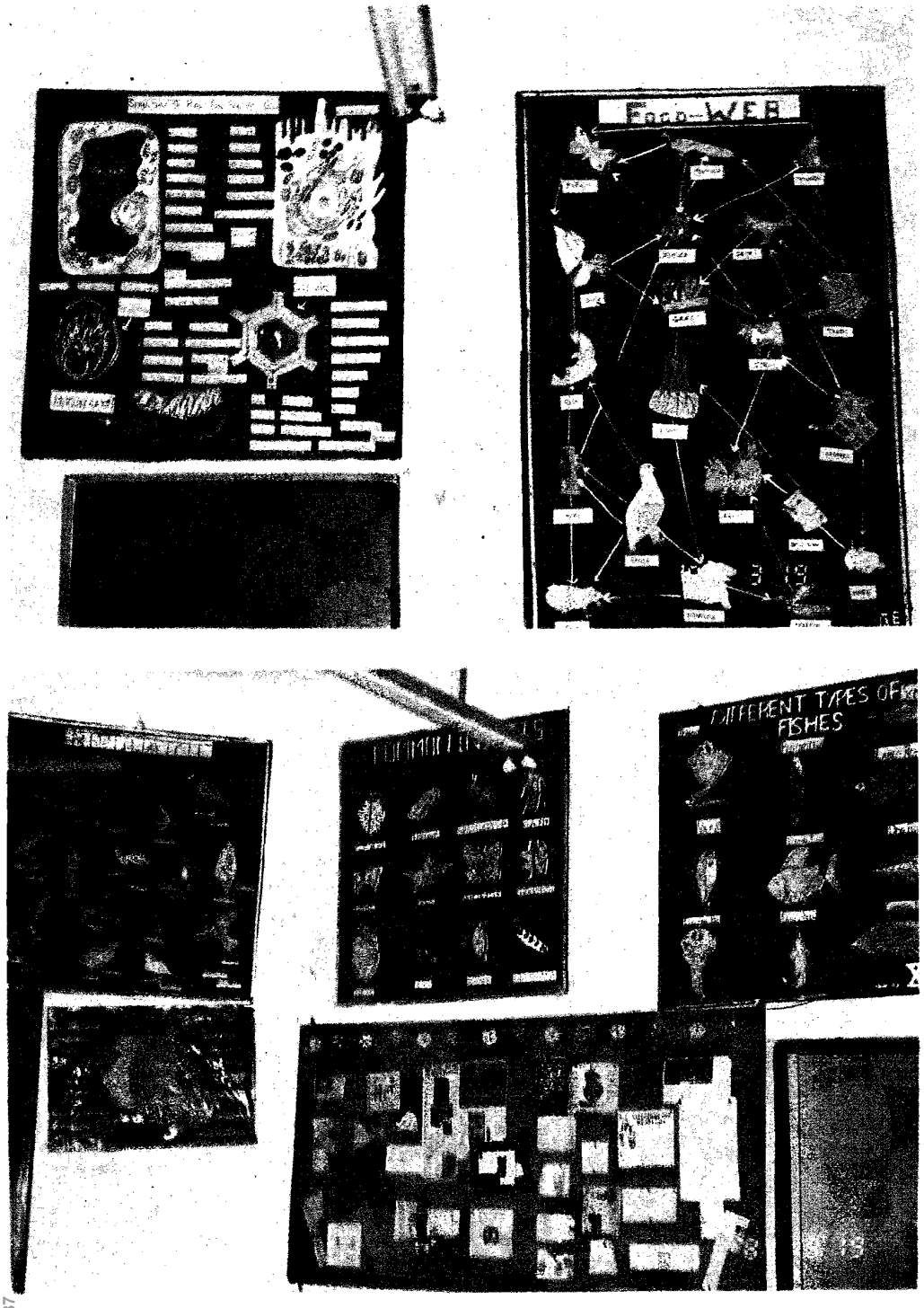

Fig. 1.5: Display in a biology lab of a school.

Display

The activities performed by the students are displayed in lab. Fig. 1.5 shows a few displays in a biology lab of a school.

1.3.2 Preparation Room

There may be a separate preparation room adjacent to the main lab. In this room, slab and sink are provided for wet work. The technician prepares solutions and staining reagents that are used by students in performing experimental work. The technician periodically replenishes the solutions which are used up.

1.3.3 Store Room

As you learnt in unit 4 of LT-1 course in all labs a small room is used for the storage of chemicals, glassware and equipment that is not in use. There are shelves or racks and large glass door almirahs for various kinds of storage. Biology store rooms also have built - in cabinets for storing microscopes and racks for dissecting pans.

1.3.4 Office

There may be a separate office for the lab staff. Like any other office records, stock registers, files, catalogues, acquisition resister, issue register are kept in the office. The records are updated periodically with regard to purchase of various materials of the lab and their issue. The furniture in the office is generally a table, 3-4 chairs, almirah. Among the office equipment there may be a typewriter or even a computer.

1.4 SUMMARY

In this unit you have learnt the following:

- Biology labs in most schools consists of a main lab, a store and an office.
- In colleges there may be more than one main lab, a store, preparation room and additional facilities such as animal house, green house, museum., herbarium etc.
- The main lab is designed keeping in view the specific requirements of biology work. It contains glassware, instruments, specimens, skeletons, bones, charts, models and other display.

1.5 TERMINAL QUESTIONS

1. Describe the features of the biology lab of the school you had studied. What were the outdoor facilities available?

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2. Suppose you are provided with two large rooms for a biology lab in a school. What kind of arrangement would you make to accommodate the necessary features?

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3. What sort of arrangement to receive adequate light are required in a biology lab?

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1.6 ANSWERS

Self-assessment Questions

1. a) Main lab, store, office
   b) Culture room, cold room, dark room, preparation room, microtomy room, museum etc.
   c) i) animal house, green house

ii) biochemistry, physiology or tissue culture

Terminal Questions

1. You may recollect the features keeping in view the one described.
2. Hints:
   Room 1 – for Main Lab. Lt can be used for preparation of solutions, reagent.
   Room 2 for storage and office; and museum and displays.
3. Ref. to section 1.3, Design and Organisation.

UNIT 2 ANCILLARIES OF BIOLOGY LABORATORY

Structure

2.1 INTRODUCTION

In Unit 1 of this course, you have studied about the infrastructure of a biology lab but biology cannot be studied in a closed laboratory only, therefore some other structures become necessary part of a biology lab. As biology is the study of living organisms i.e. plants and animals and plants and animals can be best observed and studied in the open, so field trips and excursions play an important role in the biology lab component. Actual experiences with gardening can be gathered in a botanical garden. Plants and animals are grown and nurtured in green houses and animal houses respectively. Similarly, fishes are housed in aquarium and amphibians and reptiles are grown in a vivarium. Museums (plant and animals) have a display of plant and animal specimens. You will study about these ancillaries of the biology lab i.e. museum, animal house, aquarium, vivarium, botanical garden and greenhouse in this unit.

Objectives

After studying this unit, you should be able to:

- understand the structure of a green house,
- maintain an animal house,
- construct and maintain an aquarium and a vivarium,
- display specimens in museum and herbarium,
- appreciate the importance of a botanical garden, a photographic room and a germinating bed as ancillaries of biology lab.

2.2 GROWING ROOMS AND GREEN HOUSES

Very few plants and animals flourish in an ordinary room for any length of time because of the variation in temperature, humidity and lighting. A satisfactory solution to this problem is the provision of a greenhouse or a growing room.

A green house is a special enclosure made of glass or plastic in which plants are grown and maintained at a specified temperature and humidity. In countries, where freezing temperature are reached in winter, a green house is made with a roof and walls of glass. Plants get adequate light and they can be watered. At the same time heat trapped by the glass enclosure keeps the greenhouse warm. Temperature, however, is also regulated by a specialised system. Greenhouses are permanent structures.

Temperature of a greenhouse is regulated through an automatically controlled heating and ventilation system. A central coal or oil furnace supplies the heat. A peripheral steam heating system is more common. Ventilation is provided a the sides and top. In summer, when the temperature goes up fans and pad cooling are used to lower temperature. Water is circulated through pipes. Cooling pads draw cooled air across a greenhouse. It is more effective when humidity is low.

Instead of glass, plastic films are used to make the wall and roof of glasshous Light absorbing qualities of plastic are similar to those of glass. Rigid plastic or ultraviolet resistant polythene is used. During summer a shade cloth is used to cover the greenhouse.

In an institute where biology is taught, a greenhouse of smaller dimensions ca be constructed to house delicate plants. Plants provide material for botanical studies and students are trained in growing, maintaining and propogating plant under controlled conditions.

Various tools and supplies must be stored in the greenhouse. Shelves and drawers should be available for flowerpots, tools, thermometres and small supplies. Bins should be provided for the storage of soil, sand, fertiliser and peat moss.

SAQ 1

1. How are temperature and humidity maintained in a green house?

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2.3 ANIMAL HOUSE

An animal house is a room in which laboratory animals live, researchers do experiments on the animals and technicians serve the research staff and animals.

Before considering the design any further it is interesting to review the subdivision of the area of the animal house.

Animal room	:	including breeding, stock, reception and quarantine
Plant	:	5%
Corridors	:	15%
Stores	:	food, building, hardware 10%
Wash up area	:	cages, bottles, stacking 10%
Administration	:	offices, toilets and showers 10%

In order to prevent the spread of infection it is important to organise a traffic flow system so that material which may be infected does not come into contact with incoming clean material. Care must be taken to ensure that 'dirty' materials do not mix or meet with 'clean' materials (Fig. 2.1). Dirty bedding and cages will be moved at a different time for cleaning. It is convenient to place the animal room near the 'wash-up area' and store-room, the distance between the animal rooms and laboratories is immaterial. Moving animals a short distance has the same disturbing effect as moving them a longer distance. In general, it is better to separate them fairly widely.

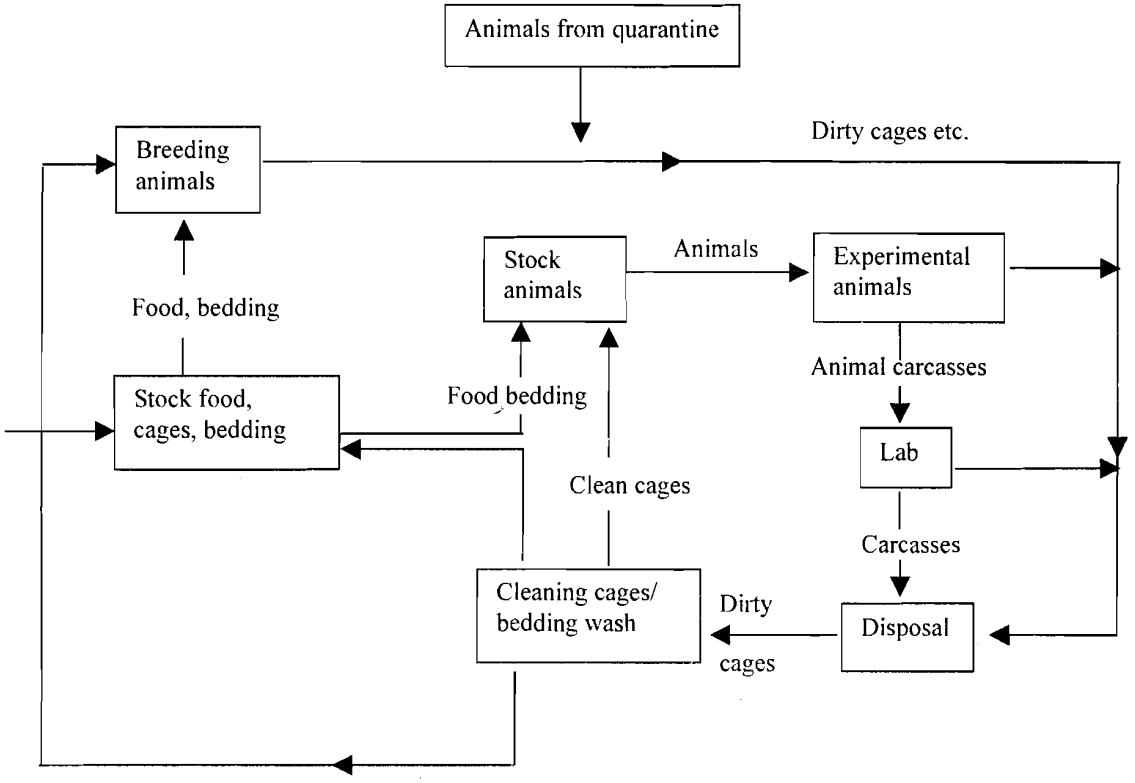

Fig. 2.1: Flow Patterns in an Animal House.

The size of animal rooms should be fairly small, about 50 m^2 is adequate. The idea of small rooms is that any infection can be easily contained and room thoroughly cleaned and sterilised. Adequate provision should be made for washing cages and feeding bottles and for staff washing and changing facilities. The environment of animal houses is quite important for animals are affected by noise, draughts and temperature changes. Ideally, the temperature should not vary by more than ±12°C. The normal range being 18-22°C. The air should be changed frequently, roughly six times per hour.

It should be noted that while the animals in the houses will make attempts to get out, other animals will attempt to get in. The store of food stuffs will be very attractive to rodents, such as rats and field mice. These stores should be protected against such invasion as the rodents will contaminate all that they touch. Animal rooms have to be cleaned from time to time. Floors must be impervious to water, disinfectants, urine, acids, alkali and organic solvents as well as to trolley wheels and reacting feet. Provision of windows is important for natural lighting. Animal houses must have drains to collect dirty water during cleaning but these must not be escape routes for animals. Hot and cold

water and a hose point are important. Good ventilation is important but draughts should be avoided.

2.4 AQUARIUM AND VIVARIUM

An increasing number of biology teachers are collecting and growing aquatic plants and animals from the local area for use in their classes. Some of these specimens must be kept alive for relatively long periods of time. The most economical way to accomplish this is the use of a large aquarium.

An aquarium is a comfortable 'cage' for fishes of selected types where they have enough oxygen, light, correct temperature, the right quantity of water, sufficient and correct type of food.

Fishes are usually kept in glass aquaria. However, an aquarium may be constructed from any material which is water proof – not poisonous to fish, not easily corroded by water. An aquarium for this purpose should have a capacity of 30-50 gallons. Bottoms and ends may be of soapstone, the sides are of plate glass, braced with nonrusting tie rods and sealed with waterproof cement. It should be equipped with noncorrosive drain fittings with overflow and trap and a water supply pipe with a shut-off valve and pantry cock. Use of a separating mesh screen in the 50 gallon size converts it into a dual aquarium, one side of which can be used for water plants such as *Elodea* and *Vallisneria* and the other side for fish, snails and other water animals. Special safeguards are necessary to prevent corrosion of aquarium parts.

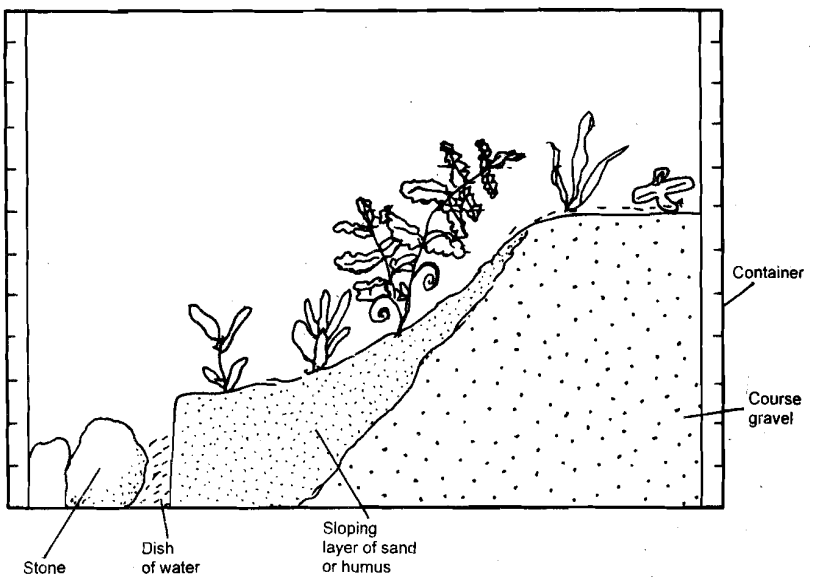

Fig. 2.2: Diagram to show set-up of a vivarium.

For aeration, a small electric pump may be used to force air through a tube to the bottom of the aquarium sending up a spray of small bubbles. Careful

preparation and planting are necessary for success in maintaining animals. Before fishes are added, the tank should be prepared and plants planted in the tank should be washed with coarse sand and water, and rinsed several times. The tank should be filled with water up to 2/3 and the water allowed to stand for a day to mature.

As a technician you have to maintain it daily. Daily maintenance involves feeding the fish, keeping the water level constant, removing dead plants or animals, removing overgrown plants and excess snails.

It is not necessary to change the water in the tank except if fouled or if special care requires it. Yellowish or greenish water is 'conditioned' aquarium water and is good so long as the pH range is between 6.8 to 7.2.

Vivarium: A vivarium is any container that can provide a 'pool' which with a 'beach' surrounding is comfortable to house most kinds of amphibians, and some reptiles. The purpose of keeping aquarium/vivarium in school/college is to study ecology i.e. interrelationship between flora and fauna. Suitable vivarium can be constructed from an old aquarium or large glass jar. A natural habitat is prepared by planting a 'beach' around the pool using layers of moss. Living food is best for amphibians e.g. fresh liver dangled in front of them on a string (Fig. 2.2). Dead animals and uneaten food should be removed within an hour to avoid fouling the tank.

SAQ 2

1. What is the difference between an aquarium and a vivarium?

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2.5 MUSEUM AND HERBARIUM

Museum is one of the most interesting and informative components of a Biology lab. Museum has a display of mounted specimens of representative local plants and animals collected by students and laboratory staff, supplemented by other specimens procured from supply companies. The appearance of such a display can be improved if the specimens are mounted on glass plate, in standard specimen bottles with black tops, rather than in fruit jars. Sketches, photographs or models of the smaller microscopic organisms add to the effectiveness of the display.

Fig. 2.3: Photograph of Museum showing animal specimens.

A human skeleton, an important component of the museum is generally posted near the door. Skulls of various animals and articulated bones that show the entire skeleton of the organism are displayed and non-articulated bones are kept in wooden cases. Some of the museums also have stuffed animals such as owls, rabbits, hedgehogs, squirrels, bats, and a variety of snakes and at times special ones like dolphins (Fig. 2.3).

Sometimes small aquaria also form part of the museum. Variety of fishes and other aquatic life forms in an aquarium attract visitors.

Insects which are collected by students, teachers and laboratory technicians are displayed in insect boxes. Napthelene balls are put in insect boxes to save the insects from ants or any type of infection.

Similarly plant specimens e.g. miniature lichens are displayed on the trunk of small trees. Plants of economic importance e.g. edible mushrooms, rosewood etc. are often included. Cereals that form the staple diet are also displayed e.g. immediately harvested crops, grains with husk and dehusked grains of wheat are displayed.

Murals showing the development of life on earth and charts showing the relationships between various groups and with man provide an interesting supplement to such a museum display. The wall-space above the tackboard can be used to display these murals and charts.

It is very important for the lab technician to take care of specimens and maintain them in proper condition. A laboratory technician should try to make the display as interesting as possible.

Herbarium is a collection of plant specimens. You will study in detail about a herbarium in Unit 5.

SAQ 3

1. What is the difference between museum and herbarium?

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2.6 OTHER ANCILLARIES OF A BIOLOGY LAB

In some large institutions, due to more facilities available, in addition to a museum, aquarium, greenhouse and animal house, a few other ancillaries are also present e.g. darkroom, botanical garden, germinating bed etc. You will study about these ancillaries in the following subsections.

2.6.1 Botanical Garden

Since biology instruction is effective to the extent that it influences what people do, how they live and what they enjoy, actual experiences with gardening. Such experiences may well include planning for flower growing as well as vegetable gardening should be considered. A well-maintained garden where plants are classified and grown with care and where plants may even be multiplied for the purposes of observation and research is called a botanical

garden. A garden full of plants, both flowering and non-flowering is indeed a treat to see. But a botanical garden is not for entertainment alone but is associated with Botany teaching and research.

In our country the main national botanical gardens are in Lucknow and Calcutta. The one in Lucknow is called the National Botanical Garden and has plants from Uttar Pradesh including the hilly regions of Garhwal. The Indian Botanical Garden is in Shibpur near Calcutta. It has a more than hundred year old Banyan tree.

A school/college botanical garden is developed on a much smaller scale. The plants grown in it are mostly the ones which form the study material for botany. A patch of ground within the school premises where ample sunlight comes is the ideal place where a botanical garden may be developed. An ideal situation is when (i) new plants are added from time to time (ii) plants are labelled with labels carrying the botanical names as well as common names. (iii) A catalogue has to be prepared giving a number and concise description.

2.6.2 Photographic darkroom

In many high schools and colleges, photographic work is included in one or more science courses. An area that can be totally darkened, with a work table, sink and running water is necessary. In addition to the photographic work, there is need for such an area for experiments with light. A room that can be darkened well is essential to microprojection in biology classes. The darkroom is equipped for students to work in small groups with a microprojector. This room provides storage space as well. The nature of the items to be stored in this room is determined by the use of the room.

2.6.3 Experimental Farms

An increasing number of schools and colleges are acquiring tracts of land so that students and teachers can have direct experience with continuation practices and with outdoor living. The availability of a school forest and farm can aid in focusing attention on local plants and animals and can establish life-long interests in many students. On such a tract of land a nature trail can be laid out.

2.6.4 School/College Ground

A school or college ground itself can become a place for studying biology. Trees that are representative of those found locally should be preserved. Shrubbery should be selected and placed so that birds are encouraged to come there. Bird houses, bird baths and winter feeding stations should be planned. If the grounds are large enough, a nature trail may be laid out.

2.6.5 Germinating bed

In many institutions germinating beds which have been made locally are used in the biology rooms or in the greenhouse. The box should be zinc-lined with a strainer and dripcock, at the center. Part of the box should be made of plate glass for observing the root growth of seedlings. The table on which the tray is mounted should have 4 rubber tired casters so that the unit can be moved to different positions in the room.

2.7 SUMMARY

After studying this unit, you have learnt that:

- A green house is a special enclosure in which plants are grown and maintained at specific temperature and humidity. A greenhouse is made up of glass or plastic. A greenhouse is a permanent structure.
- An animal house is a room in which laboratory animals are kept. Good ventilation is very important in an animal house. An animal house should be protected from rodents as food stuffs in an animal house attract rats. Infection should not spread in an animal house.
- An aquarium is a cage for fishes where they have sufficient oxygen, light, correct temperature, enough water and food. A vivarium is a container to house amphibians and reptiles. Daily maintenance of an aquarium and a vivarium is very important.
- A museum displays plants and animal specimens. Human skeleton, stuffed animals, insects, skeletes, and models of smaller organisms make the display more effective.
- Botanical garden, germinating bed and dark room are other ancillaries of a biology lab which increase the effectiveness of biology instructions.

2.8 TERMINAL QUESTIONS

1. What is the importance of an aquarium/a vivarium in a biology lab?

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2. What precaution should be taken while maintaining an animal house?

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2.9 ANSWERS

Self-assessment Questions

1. In an aquarium fishes are housed in a container in which sufficient oxygen, light, correct temperature, enough water and food are provided whereas a vivarium is a container to house amphibians and reptiles.
2. A museum is a room in which local plant and animal specimens are displayed. Sketches, photographs or models of smaller organisms, human skeleton, insects increase the effectiveness of display. Herbarium is a collection of plant specimens.
3. The temperature of greenhouse is regulated through an automatically controlled heating and ventilation system. Water is circulated through pipes. Cooling pads draw cooled air across a greenhouse.

Terminal Questions

1. Aquariums and vivariums in schools and colleges are built to study ecology i.e. interrelationship between flora and fauna.
2. Daily maintenance of an aquarium and a vivarium involves feeding the fishes, amphibians and reptiles, keeping the water level constant, removing dead plants or animals, and overgrown plants and excess snails.

UNIT 3 EQUIPMENT USED IN BIOLOGY LABORATORIES

Structure

3.1 INTRODUCTION

Biology owes much to the allied sciences of chemistry and physics for the development of new tools and techniques. In this unit you will study about the equipment which is necessary for a biology lab. You should keep in mind that mastery of simple apparatus is a prerequisite for work with more advanced equipment. As a biology laboratory assistant you must know about various types of equipment, its uses and working. Some equipment, which are mostly used in chemistry and physics, has merely been referred to and you will study them in detail in respective courses.

Objectives

After studying this unit you will be able to:

- list and describe the glassware and equipment used in a biology lab.
- list all the equipment for heating and weighing used in a biology lab and describe its uses.
- appreciate the importance of sterilization in biological techniques, describe various types of sterilization equipment, their working and use.
- describe a microbiological safety cabinet, its working and use.
- describe a centrifuge and explain its use.
- describe various types of microtomes, their working and use.
- list the microtome knives and describe its working.
- state the use of a colorimeter.
- define pH and state the application of pH meter in experiments.
- describe the parts of distillation units.

3.2 IDENTIFICATION OF APPARATUS

When you work in the lab you should know the names of various apparatus, their working, uses and maintenance. In this unit we are going to divide the types of apparatus into two categories.

(1) Glassware
(2) Equipment

Glassware

Scientific equipment made of glass are generally known as glassware. We will try to give some information about glassware so you are acquainted with them well.

1. Beaker

Beakers are important pieces of glassware used in a biological lab. They are made up of Corning or Pyrex glass. Sometimes they are made up of plastic. Beakers are of different capactiy from 10 ml – 100 ml – 1000 ml – 10000 ml. The beakers that are used should be sound. If they are chipped or cracked they should be discarded.

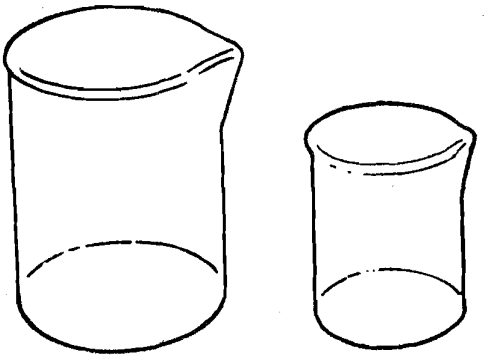


Fig. 3.1: Beaker.

2. Bell jars

The open type needs a ground – glass stopper which should be highly greased.

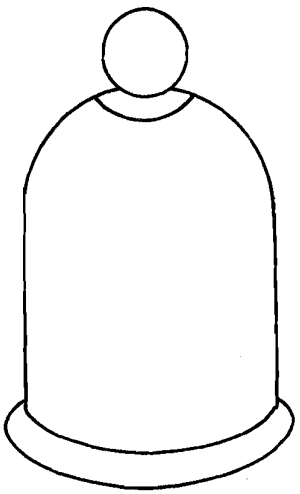


Fig. 3.2: Bell jars.

3. Desiccators

Desiccators are used to remove the last trace of moisture from apparatus or substances and to keep them dry. Objects are dried by placing them on the wire rack over a desiccant – a substance that readily absorbs water. The most common desiccant is anhydrous calcium chloride but others are also used. The ground-glass rims of the lid and the base should be lightly greased and the lid moved smoothly about to spread the grease, to give a tight seal. When placing something hot in a desiccator, beware of pressure building up inside because it can blow the lid off (and onto the floor). Either leave the lid ajar or open the tap until the object has cooled. When removing the lid, slide it across the base.

Caution: Do not try to lift it off, many back injuries have been caused in this way.

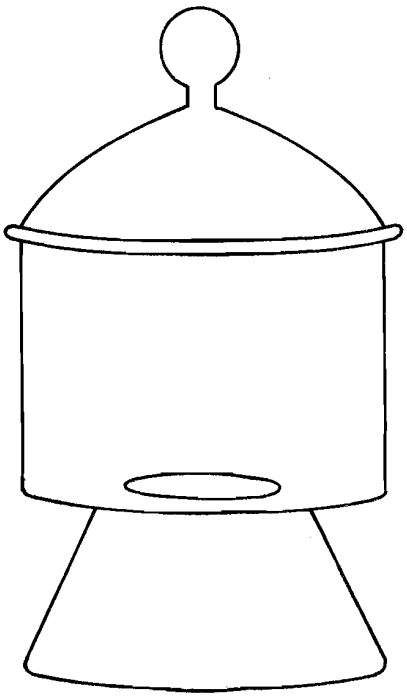


Fig. 3.3: Desiccator with lid.

4. Watch glass

Smaller concave glasses which are used to keep materials are known as watch glasses.

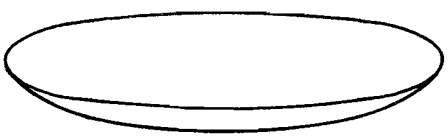


Fig. 3.4: Watch glass.

5. Test tubes

Glass tubes which are used for testing materials and cultures are known as test tubes. Test tubes with a diameter of 16 mm are often preferred because a thumb fits over the end quite easily. Never boil a liquid in a test tube. Instead

use boiling tube. Some common sizes and capacities of test tubes and culture tubes are mentioned below:

Test tube-size 15.5 cm x 2.5 cm
12.5 cm x 2.5 cm

Culture tube (with or without a rim):
Size 15 mm x 125 mm
15 mm x 150 mm
18 mm x 150 mm
25 mm x 100 mm
25 mm x 150 mm
25 mm x 200 mm

Culture tube with screw cap and rubber liner:
Size 15 mm x 75 mm
15 mm x 125 mm
25 mm x 100 mm

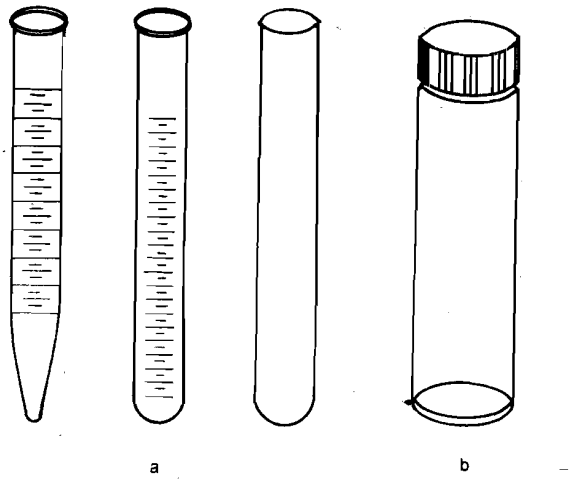


Fig. 3.5: (a) Test tube. (b) Culture tube with screw cap and rubber liver.

6. Petri dish

This is also used for culture in microbiology. Petri dishes made of glass and can be reused after proper sterilization, but disposable petri dishes once used should not be reused as they cannot be adequately sterilized. Each dish is 2 cm in depth. Its diameter ranges from 5 cm to 20 cm.

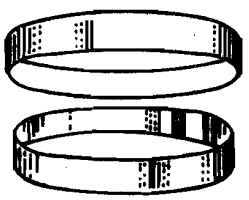


Fig. 3.6: A pair of Petri dish.

7. Conical flask

Conical-shaped beakers are used in gravimeteric analysis or preparation of chemical compounds. This along with the beaker and test-tube is a corner-stone of practical science.

8. Flat-Bottomed flask

This type of flask should not be heated or subjected to pressure changes as the glass is under stress around the flat bottom.

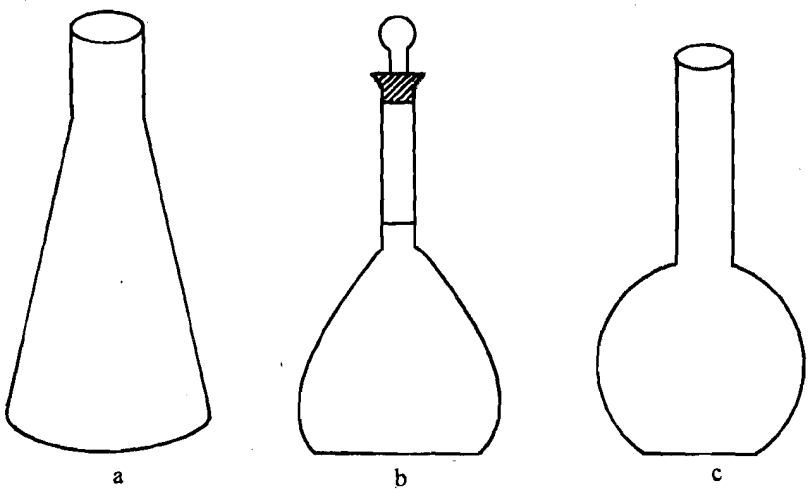


Fig. 3.7: (a) Conical flask (b) Measuring flask (c) Flat bottom flask.

9. Round-bottomed flask

This type of flask should be used whenever it, or its contents, are exposed to strong heat or variations in pressure.

10. Distillation flask

As the name implies these flasks are used for distillation. These are often used with other pieces used in distillation. You will study them in detail in the chemistry course LT-3.

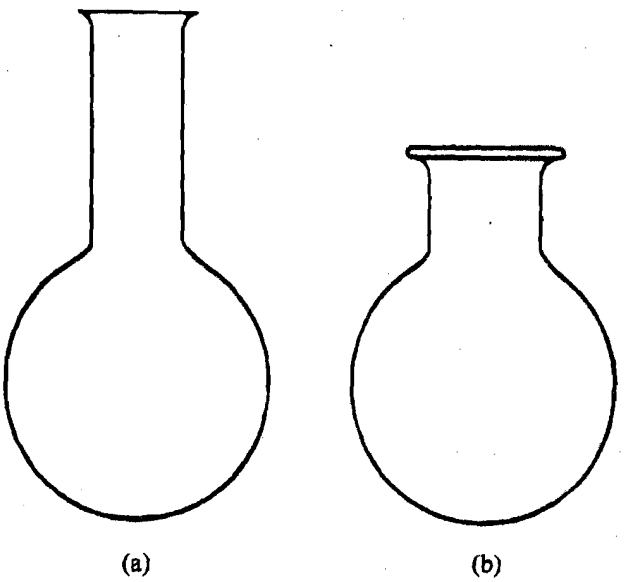


Fig. 3.8: Round bottom flask.

11. Pestle and mortar

The container is the mortar. When used, the pestle should be grasped firmly by the handle and moved in a circular grinding motion in the mortar.

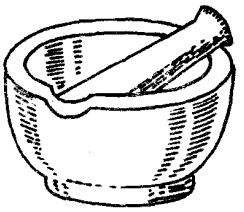


Fig. 3.9: Pestle and mortar.

12. Measuring cylinder

Measuring cylinders are available in capacities ranging from 10 ml to 2 litre and are used to dispense the volumes of solutions, with a lesser degree of accuracy.

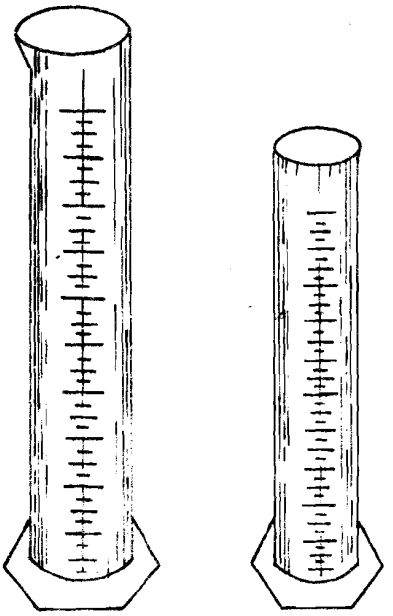


Fig. 3.10: Measuring cylinders.

13. Volumetric flasks

Such flasks are designed with a pear-shaped bulb, a long and narrow neck with graduated mark and a stopper. The volumetric flasks are containers for specific volume of solutions at one specific temperature i.e., 5°C or 20°C. They are available in the capacities of 10 ml to 5 litres (see Fig. 3.7 c). You will study more about them in the chemistry course LT-3.

14. Jars

Jars are especially designed to preserve plant and animal materials in various types of fixatives and store them in the museum for a longer period. Rare specimens, mounted on glass sheets, are also kept in the jars for future reference. The jars are also commonly called specimen jars or museum jars and are vary much in their size and shape. Two chief types are common in use.

15. Rectangular jars

These are provided with glass sheet covers or lids which are fixed to the mouth with the help of grease for making the jars airtight. Rectangular jars are available in these sizes:

15 cm x 10 cm x 5 cm to 30 cm x 17 cm x 9 cm

16. Round jars

The round jars either have bakelite screw caps or are provided with knobbed glass stoppers. These vary in their size from 15.5 cm x 5 cm to 38 cm x 15.5 cm.

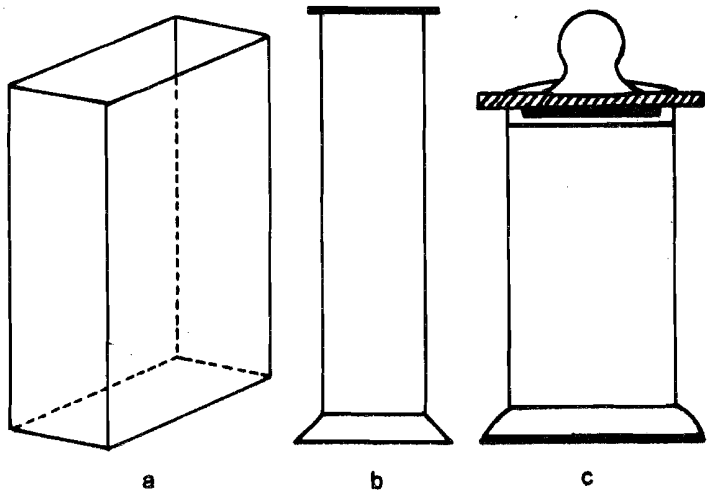


Fig. 3.11: (a) Specimen jars. (b) Specimen jar with lid. (c) Specimen jar with round and knobbed stopper.

17. Troughs

The glass trough is circular in shape varying in diameter from 20 cm to 30.5 cm and chiefly serves as wash basin for cleaning the glassware. Various types of glassware are dipped in chemicals filled in a trough. After the required period of dipping the glassware is washed thoroughly in tap water.

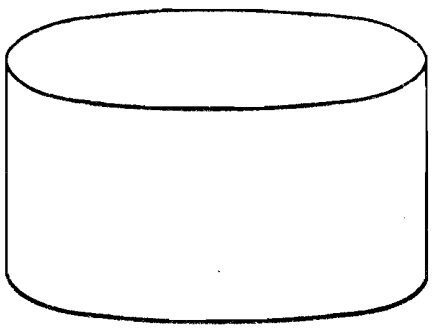


Fig. 3.12: Trough.

18. Micro Slides

Micro-slides are manufactured from the best selected glass sheet. These are optically flat and are used in microscopic work. Generally two types of slides are available in the market. Some of them have ground-polished edges and others with cut edges (unground and unpolished). The standard size is 75 mm x 25 mm. But the thickness may be 1 mm or 1.4 mm.

In addition to the above ordinary slides, micro concavity slides or just cavity slide are also used in the laboratories each slide may have one or two or three cavities present on one surface. Microorganisms are kept in these cavities. Sometimes pollen is also germinated in cavity slides. Generally two types of micro-concavity slides are available with manufactures.

1. Size - 75 mm x 26 mm
   Thickness - 3 mm
   Cavity diameter - 16 mm x 1.75 mm deep
2. Size - 75 mm x 25 mm
   Thickness - 1.5 mm
   Cavity - One, two or three.

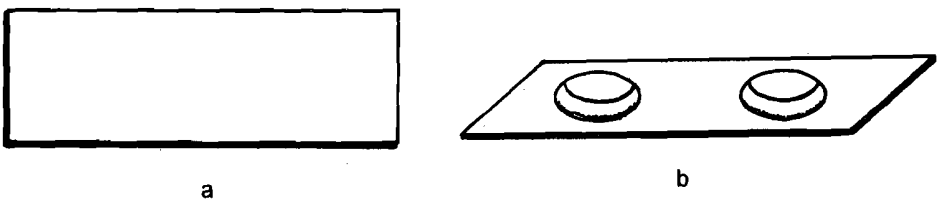
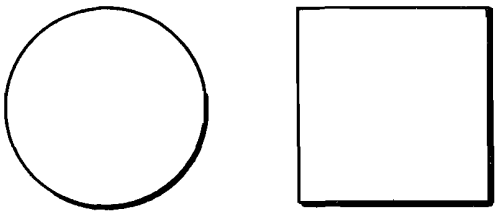


Fig. 3.13: a) Micro slide, b) Micro slide with cavity.

19. Cover Slips (Cover glasses)

Cover slips are optically flat and usually free from waviness. These are manufactured from fine quality very thin glass-sheet and may be circular, rectangular or square in outline. Cover slips are a must for microscopic studies of plants and animals. These are used for mounting the materials in a suitable medium taken on a slide. Cover slips are available in different sizes:

Circular: 18 mm x 22 mm
Rectangular: 22 mm x 25 mm
22 mm x 30 mm
22 mm x 40 mm
22 mm x 50 mm
22 mm x 60 mm
Rectangular special:
24 mm x 30 mm
25 mm x 60 mm
Square: 18 mm x 18 mm
22mm x 22 mm



a b

Fig. 3.14: Cover slips. (a) Round cover slips. (b) Square cover slips.

20. Cavity block

Cavity blocks are solid rectangular blocks of glass with thick walls with a cavity inside. This block is used for staining material safely in it. The blocks are strong and can be held firmly. They are generally of small size.

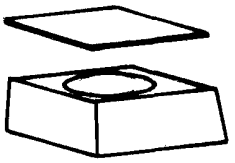


Fig. 3.15: Cavity block.

21. Vessels designed for Staining

The vessels originally designed for staining are known as Coplin jars and are vertically standing glass vessels which have ridges down the insides to accommodate slids placed in them lengthwise.

For the production of many slides at a time, a further vessel may be used called a staining trough. This is a rectangular glass box which is fitted with ridges to accommodate slides lying lengthwise, with the fluid just covering the slide's width.

It is, however, more likely that you will be using vessels of a non-specific kind in which you will only be able to deal with one or two slides at a time.

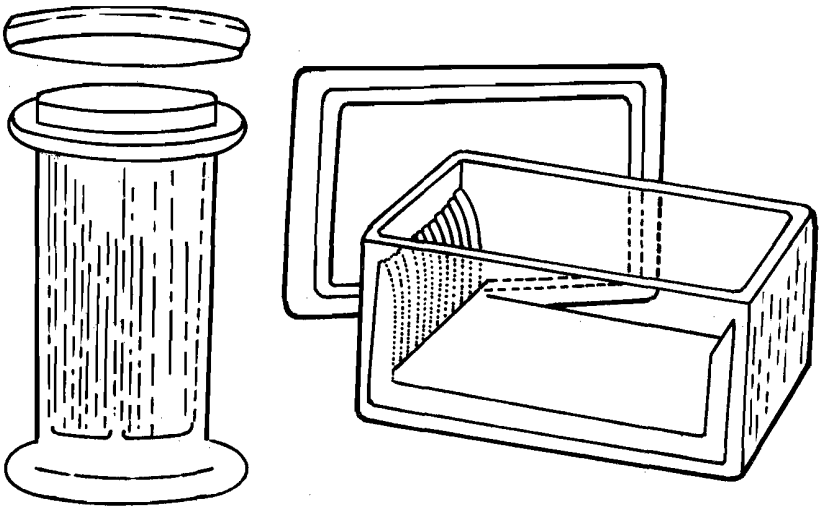


Fig. 3.16: Coplin jars and staining trough.

22. Staining bottles

Stains are kept in staining bottles, which can be with a lid or with a dropping stopper. The bottles with the droppers are more useful since a small quantity of the stain can be dropped directly on the material. The stain can also be taken out with droppers.

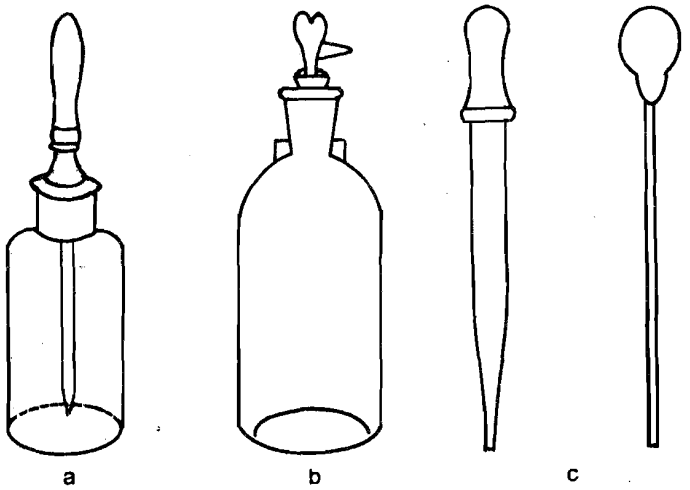
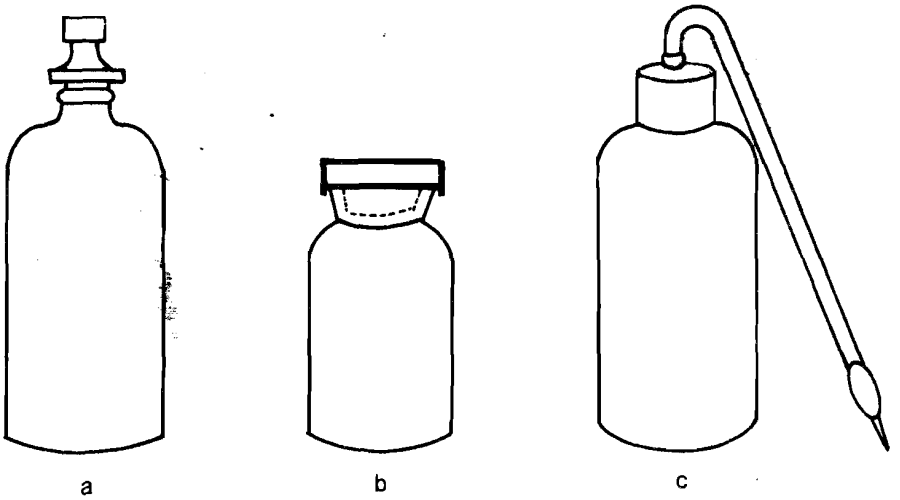


Fig. 3.17: (a) Bottles with dropper. (b) Dropping bottles. (c) Droppers.

23. Washing bottles

Staining rack is incomplete without washing bottle. The stained materials are washed with the help of washing bottle. The weak acid and alkali solutions are kept in washing bottle.



3.18: Types of washing bottles.

Cleaning of Glassware

Though glassware break so easily even then they are generally used in labs. Glass is a unique material and relatively inert and transparent. It can withstand a certain amount of heat and can be easily cleaned. The borosilicate glass can be heated upto about 500°C. The glassware must be thoroughly cleaned before using. Several chemicals, in the form of solutions, are employed for cleaning glassware.

Cleaning of glass

30 g sodium or potassium dichromate is dissolved in 100 ml water. It is made up to a litre by adding concentrated sulphuric acid. The mixture is simultaneously cooled while adding sulphuric acid because much heat is produced during the process (The acid must always be added to water gradually). This mixture should always be prepared in a pyrex glass vessel because ordinary glassware may break when heat is produced. The glassware to be cleaned is first soaked in a freshly prepared acid cleaner for sometime. Later the cleaner is drained from the glassware. Then the glassware are washed for at least 10-12 times with tap water and then rinsed twice with distilled water. Finally the cleaned glassware is kept for drying.

Caution: Handling of acids and caustic solutions should be done with utmost care. If the skin gets splashed with acid or caustic, it should be immediately washed with sufficient amount of water and then neutralised with two percent solution of neutralizer. In case of injury due to acid 20% of sodium carbonate solution is applied to the affected part. When injury is due to caustic solutions, spillage is neutralized with 20% acetic acid solutions.

Commercially manufactured cleanser can also be used in laboratories.

SAQ 1

Write whether the following statements are true (T) or false (F).

1. To seal a desiccator grease the rims of the lid. ☐
2. The most common desiccant is anhydrous calcium chloride. ☐
3. When placing a hot material inside a desiccator the lid is closed immediately. ☐
4. Watch glasses are used for keeping small material. ☐
5. Test tubes are used for boiling material. ☐
6. Petri dishes are used in microbial culture. ☐
7. Flat-bottomed flasks should be used for strong heating. ☐
8. Jars are especially designed to preserve plant or animal material. ☐
9. Sometimes pollen grains are germinated in cavity slides. ☐
10. Coplin jars are used for staining slides. ☐

3.3 EQUIPMENT FOR HEATING

The following heating equipment is used in some experiments in a biology lab.

1. Spirit lamp
2. Bunsen burner
3. Water bath
4. Oil bath
5. Sand bath
6. Hot plate
7. Heating mantle
8. Heating block
9. Immersion heater

The most common equipment in a biology lab is a spirit lamp. The heating of slides, solution and stain is done on a spirit lamp. This lamp is very handy and easy to use.

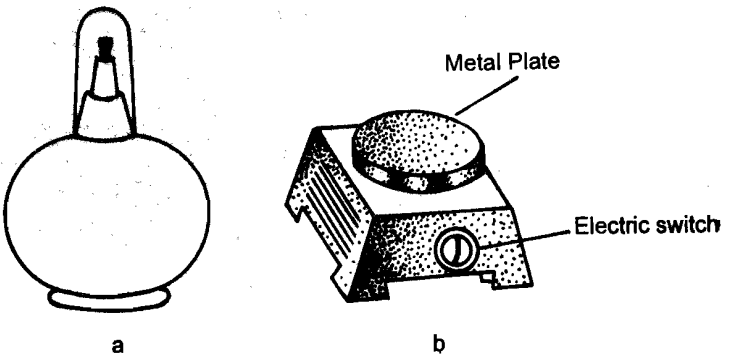


Fig. 3.19: (a) Spirit lamp. (b) Hot plate.

The instruments listed above from 2-9 will be studied in LT-3, Unit 1 – Introducing scientific apparatus section 1.3 – Apparatus for heating in chemistry course.

3.4 EQUIPMENT FOR WEIGHING

Weighing is an important process in scientific work. Some experiments need rough weighing, for a general purpose and some need weighing very accurately. Thus the balances are of many types such as:

1. Pan balance
2. Mechanical balance
3. Mechanical analytical balance
4. Electronic balance

Pan Balance

The pan balance is used when you have to weigh upto ½ kg. or 1 kg. For example, for making a medium or broth you have to weigh of 500 gm shredded agar, or 500 gm of glucose etc.

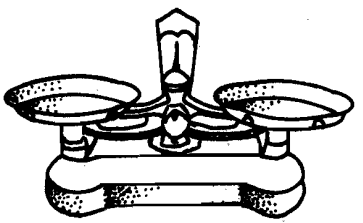


Fig. 3.20: Pan Balance.

Mechanical Balance

This is a simple balance which is handled mechanically and has two pans. This balance weighs upto 1 kg.

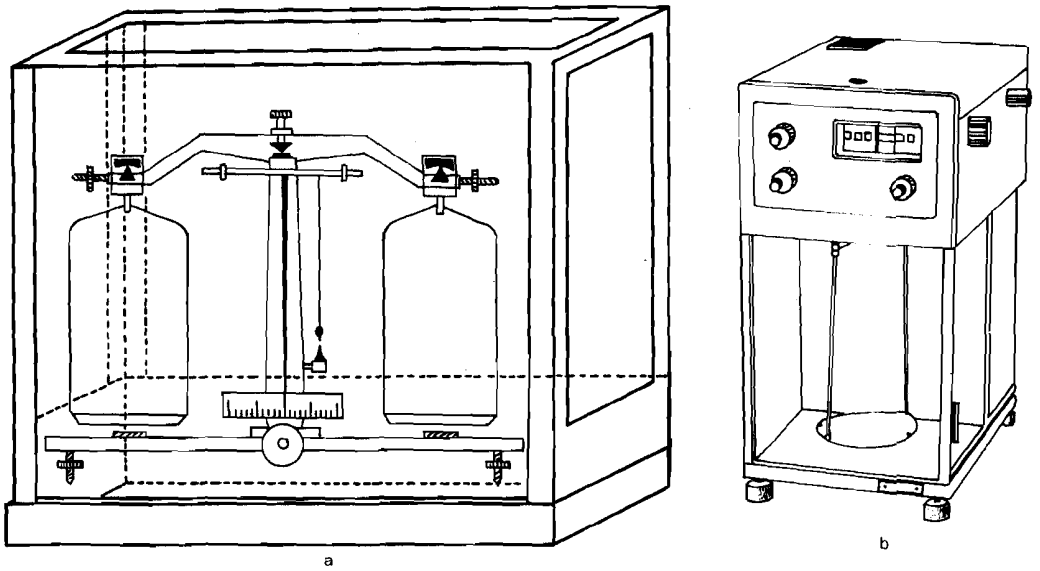


Fig. 3.21: (a) Mechanical balance. (b) Electronic balance.

You will study the other two Mechanical analytical balance and Electronic balance in Unit 2 of chemistry course LT-3 – Measurement and measuring devices in section 2.6 in detail. In this section you will also study their installation, care and uses.

3.5 EQUIPMENT FOR STERILIZATION

Sterilization means, making any material or object free from organisms. Maximum care should be taken in sterilizing the media, glassware and other accessories for scientific work.

The following methods are used for sterilization by heat.

1. Sterilization by heat
   A. Wet heat sterilization
   B. Dry heat sterilization
2. Sterilization by filtration
3. Sterilization by chemical action
4. Sterilization by ultra-violet rays (radiations)

1. A. Wet heat sterilization

The principle of wet heat sterilization is mainly based on the holding periods at a particular range of temperature for warming up and cooling down. At high temperature the activity of cytoplasm ceases thus killing the organisms. All the instruments such as inoculation needles, scalpels, culture tubes, slides, Petri dishes, syringes, etc., are kept in a container filled with distilled water. These are allowed to boil for at least 15 minutes so that all the articles become free from organisms. Sometimes bacterial spores remain alive and resist boiling. It is essential to boil the water for several hours to remove the bacterial spores. The boiled apparatus should be taken out with the aid of sterile forceps. If the articles are not to be used immediately these should be stored in a sterile container.

A device for sterilization by steam in a steamer was suggested by Koch. It is done in two ways: single exposure and intermittent exposure.

Under single exposure the unsterile materials are exposed to steam at 100°C for 40-70 minutes. This much of exposure is sufficient to kill a majority of organisms except thermophylic bacteria which normally grow above 50°C.

During intermittent exposure the unsterile material are exposed to steam for three successive days. The temperature on each day is maintained at 100°C for a duration of 20-30 minutes. The basic principle underlying this device is simple. On the first day of steaming, vegetative parts of organisms are destroyed but not the spores. Before the second day, the remaining, live spores will germinate to form vegetative parts and on the next day they are killed in steaming. Further live spores may germinate which will be killed during the third steaming.

The above two methods are not suitable for sterilizing glassware but are most effective for substances like gelatine. A most suitable method of sterilization by increased pressure has been devised recently. It is well-known that at normal atmospheric pressure water boils and produces steam at 100°C. (The boiling point of water being 100°C at the sea level). With the increase of pressure the water boils at a higher temperature. Thus the temperature of steam is increased. In other words the temperature at which the water boils rises with the increase of pressure. This is the principle adopted in an autoclave to sterilize the materials. The apparatus utilized in sterilization under increased pressure is known as an *autoclave* similar to a large *pressure cooker* in which sterilization can also be done. It should be remembered that the temperature achieved at a certain pressure depends upon the amount of air mixed with the steam. Only pure steam will produce maximum temperature at a particular pressure.

Pressure cooker

A pressure cooker of 12 litres capacity should be used for sterilization of glassware, instruments and media. The principle of a pressure cooker is to cook or sterilize materials at 122.2°C (1.055 kg per cm^2). The water boils at a higher temperature with the increase in pressure. That higher temperature and faster sterilization is achieved by utilising pressure was known since long but it was not known how to control the necessary pressure for safe domestic use. It has now been achieved with the invention of the pressure cooker. Different types of pressure cookers are available in the market e.g., Hawkins, Prestige, Cookwell, etc. The capacity of each ranges from 1.5 to12 liters.

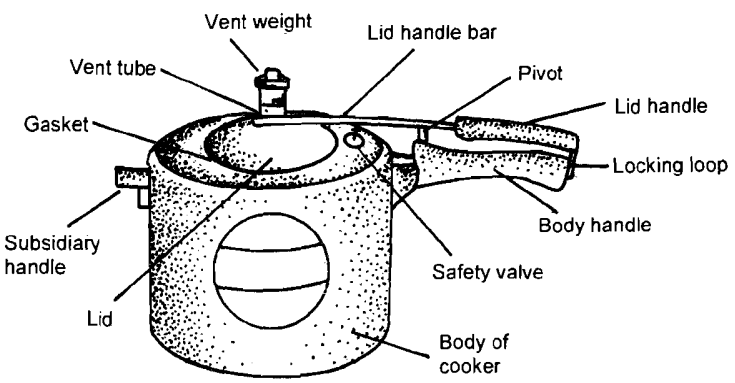


Fig. 3.22: Pressure cooker.

These days sterilization is very conveniently done in laboratories with the aid of an autoclave. These are of different types such as: (1) *Simple autoclave* (2) *Steam-jacketed autoclave* and (3) *Automatic autoclave.*

1. Simple autoclave

The body of a simple autoclave is made up of gun metal. It is cylindrical in appearance and closed at one end by a hinged door, also made up of gun metal. A gasket seal is provided between the door and cylinder. It can withstand high temperature. The gun metal cylinder of an autoclave may be horizontal in position varying in capacity (Fig. 3.23).

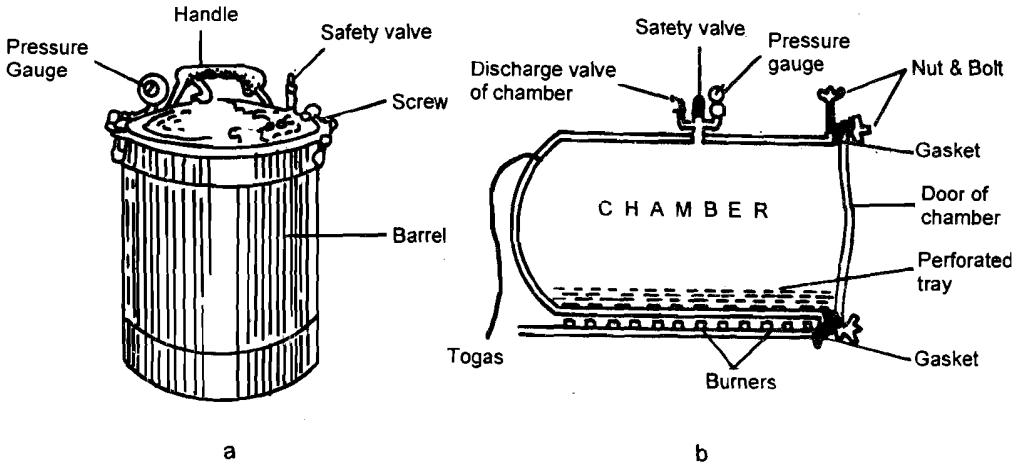


Fig. 3.23: (a) Vertical autoclave. (b) Horizontal autoclave.

A perforated metal tray is provided within the barrel which is used for keeping those articles which are to be sterilized. The water present below the perforated tray is boiled by gas, stove or electric heater to produce the steam. A pressure gauge is also provided which tells the pressure in pounds (lb)/cm^2 above atmospheric pressure. There is an outlet for steam operated by a tap. There is a safety valve which is regulated beforehand for any required and safe pressure. Generally the safe pressure is regarded at 10°C above the pressure needed for general autoclaving.

2. Steam-jacketed autoclave

This is a modified form of simple autoclave. In a simple autoclave much of the heat is wasted from the surface of the barrel. To check this, a steam-jacket is provided around the barrel in large autoclaves (Fig. 3.24).

The steam is filled in this jacket which is at the same temperature and pressure as that of steam inside the barrel. It is operated by steam produced outside the autoclave. A condensation trap is fitted to steam lines for collecting condensed steam. Due to this arrangement water-logging does not occur in the steam and cotton wool plugs get prevented from being soaked, thus cutting down the chances of contamination by bacteria.

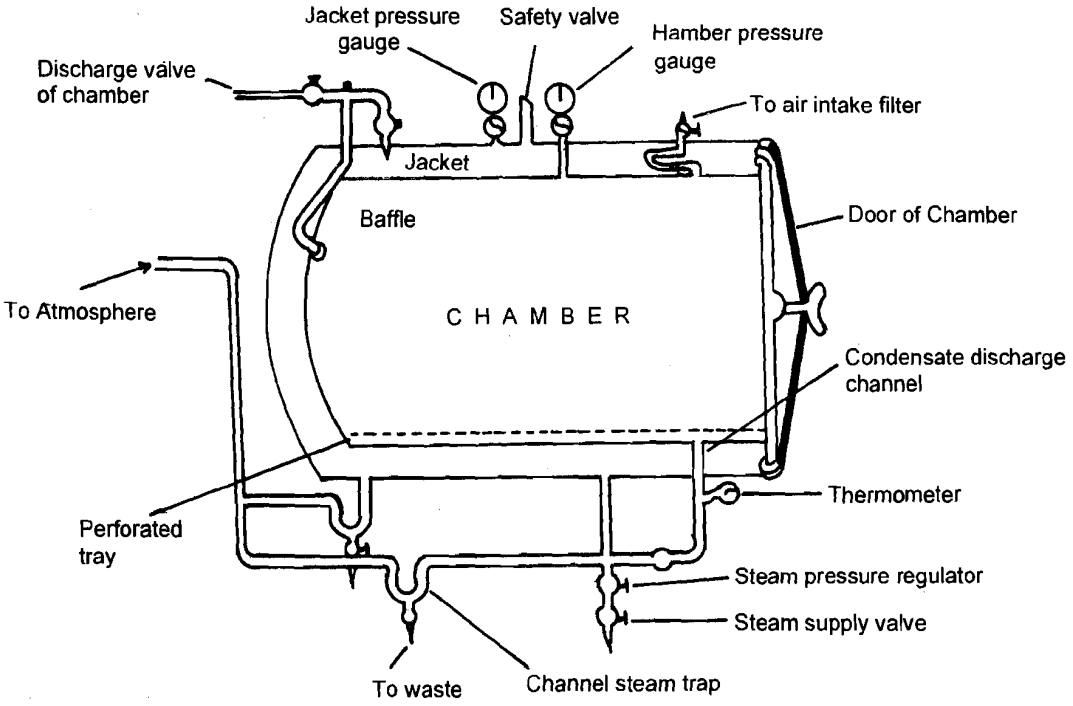


Fig. 3.24: Steam-jacketed autoclave.

1. B. Dry heat sterilization

Dry heat sterilization is done by flaming the articles or keeping some of them inside the oven. The inoculating needle, points of forceps, scalpels and other articles are dipped in absolute alcohol and flamed with burner or spirit lamp to make them sterile. The slides are wiped with cotton wool soaked in absolute alcohol and passed into the flame. The neck and mouth of specimen tubes, flasks and culture tubes are also passed through a flame till they become sterilized. The above process of sterilizing the article with a flame is called *flaming*.

Another method of dry heat sterilization is to keep thoroughly washed and dried glassware, such as petri dishes, beakers, flasks, specimen tubes, etc. packed within a box provided with a lid, inside a thermostatically controlled electric oven. The specimen tubes, flasks and bottles should be plugged with cotton wool before keeping them in an oven for sterilization.

Hot Air Oven

A wider application of dry heat is the hot-air oven in which electrically heated air is blown over the articles to be sterilized. The heater is thermostatically controlled so that the oven can be set to maintain the required temperature. A good sterilizing temperature is 160°C. This temperature should be maintained for a period between 1 and 2 hours, depending on the load. Once again you can draw a parallel with cooking in an electric oven. Some containers, particularly plastic ones, are rapidly destroyed in an oven. In addition, food is altered, e.g., it dries up, and may even be destroyed if cooked for too long. Thus, culture media are not sterilized by dry heat. However, it is a very useful method for

bulk sterilization of glassware, which is often required in large quantities in a laboratory.

The sterilizing cycle of an oven should include:

(a) The time required for the oven and its load to heat up.
(b) The time for which the load must be held at its sterilizing temperature, i.e., the holding period.
(c) The time allowed for the load to cool down, to prevent glass cracking because of exposure to rapid temperature changes.

Finally, a word about packaging the articles prior to sterilization. This is important so that after sterilization they may be handled and stored without risk of re-contamination. Glassware must be securely capped but if no caps are available, aluminium foil may act as a convenient cover. Similarly, many articles may be wrapped in foil which transmits the heat without itself being destroyed.

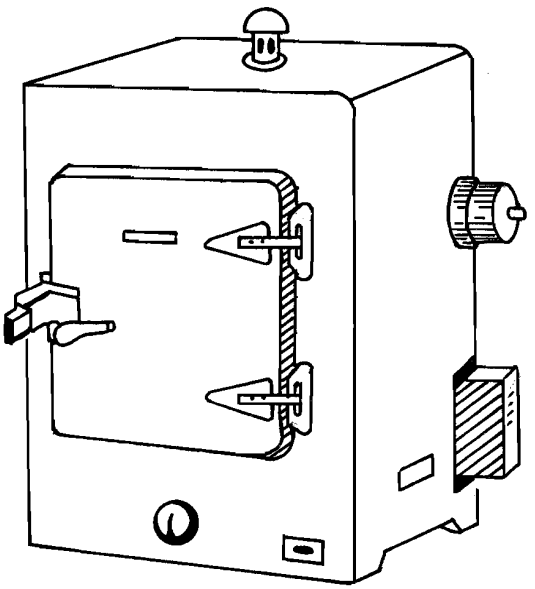


Fig. 3.25: Electric oven.

2. Sterilization by filtration

Sterilization by filtration is employed for the liquids contaminated with bacteria. Such liquid is filtered through a bacteria-stopping filter. These filters do not allow the bacteria to pass out and the filtrate becomes sterile. This filtrate should always be stored in a container which has been made sterile before hand. Several types of bacteria-stopping filters are available with suppliers. These filters are also used for the separation of a particular bacteria or virus present in some extract which may be used for cultivation in the laboratory.

3. Sterilization by chemical action

It is a quick method of sterilizing instruments, glass apparatus or any other article used in culture technique. Various types of chemicals and antiseptics serve as good sterilizers. A brief account of these are as follows:

1. 95% alcohol is used for dipping scalpels and inoculation needles and later the alcohol is flamed.
2. 0.1% mercuric chloride is used for disinfecting herbarium specimens from microbial attack.
3. 0.5-1% aqueous solution of calcium hypochlorite is utilized to make the spores of bryophytes and seeds sterile, thus allowing them to germinate to give rise to a pure culture.
4. 3% solution of lysol or cresol has proved to be a strong antiseptic for sterilizing petri dishes, instruments or any other apparatus. These are dipped in this solution to make them sterile.
5. The surface of a inoculation table is wiped with cotton soaked in lysol or cresol solution to make it sterile before use.

4. Sterilization by ultra-violet rays

Sterilization by ultra-violet rays is usually done with a ultra-violet lamp. The rays travel in a straight line and are lethal to living organisms. It is also utilised for sterilizing culture rooms and tables. The materials to be sterilized are taken-out into special glass tubes which do not absorb ultra-violet rays and are exposed for an hour. Ordinary glass is not used because it absorbs ultra-violet rays and material kept in an ordinary glass container remains unsterile.

SAQ 2

Write the names of instruments which are used in wet sterilization in a biology lab.

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3.6 MICROBIOLOGICAL SAFETY CABINET

Laminar flow also known as inoculation chamber, is used for inoculation purpose.

In larger laboratories, a microbiological safety cabinet may be used for inoculation. Depending on the type of cabinet, air is either filtered and passed over the work surface to prevent contamination of cultures, or air is drawn upwards and passed through a filter to eliminate organisms released during handling techniques.

Figure 3.26 is of a modern safety cabinet designed to protect the operator and the environment from exposure to pathogenic micro-organisms.

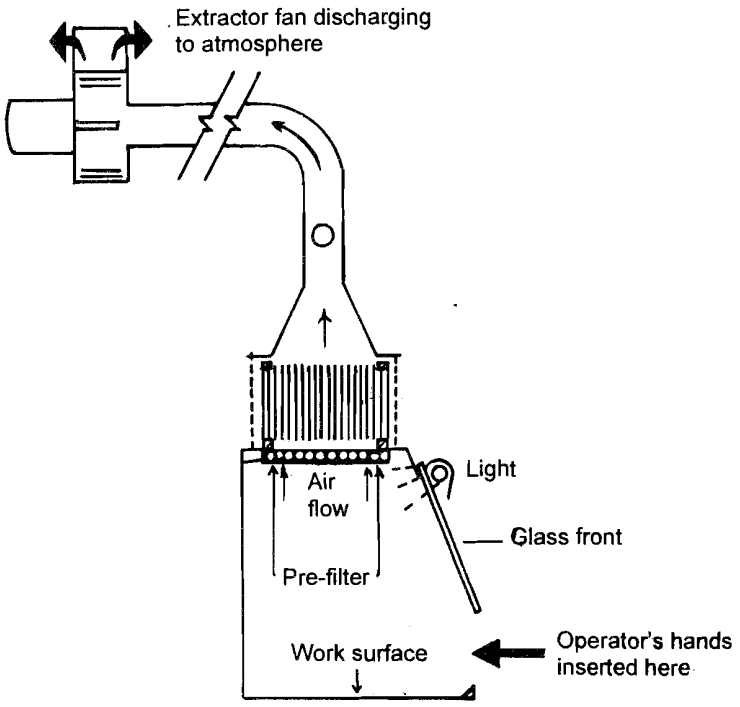

Fig. 3.26: Class I safety cabinet.

Micro-organisms are handled within the cabinet with air being sucked through the gap in the cabinet front where the operator's hands are. The air is sucked upwards through a pre-filter and a main filter which traps any airborne micro-organisms. The extract fan is housed above the filters and exhausts air to the atmosphere. This type of cabinet, designed to protect personnel but not prevent contamination of handled cultures, is termed Class I.

In contrast, Fig. 3.27 illustrates a Class II cabinet in which prime consideration is given to keeping contamination of material being handled to a minimum, whilst still affording some protection to the operator.

In a Class II cabinet air is passed downwards over the work surface and then passed upwards to be exhausted outside the room. The prefilter and main filter are situated below the work surface so that the contaminated air is sterilized prior to being discharged into the atmosphere.

SAQ 3

A Class I microbiological safety cabinet ..... (tick the correct completing statement).

1) Protects the operator from the cultures he/she is handling.
2) Is designed to prevent contamination of the cultures being handled.
3) Is designed for neither (1) nor (2).

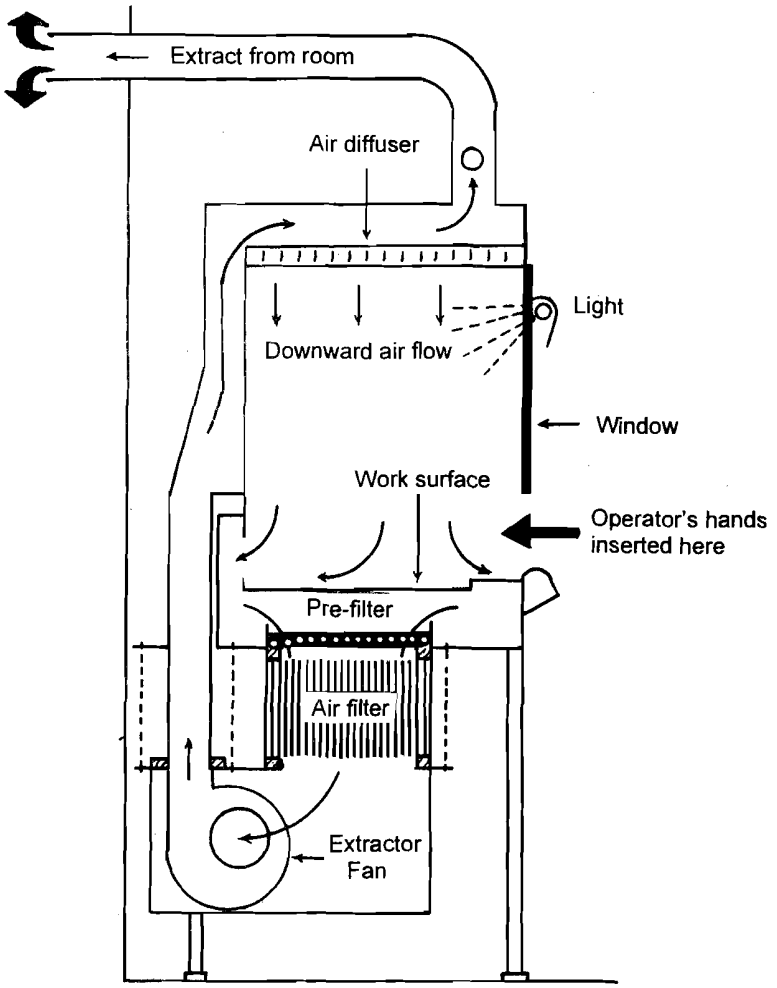

Fig. 3.27: Class II safety cabinet.

3.7 CENTRIFUGES

This is an important equipment found in a biology lab.

Centrifuges are widely used in the lab for the concentration or separation of suspensions (including differential separation) and the breaking of emulsions. The earliest kinds of centrifuges were rather like domestic cream separators – hand cranked devices with limited performance.

Once electric motors were coupled to centrifuges, the designed became superior and today there is a wide range of centrifuges available with RCFs on smaller instruments of up to 2,500 g and on the largest instruments up to 100,000 g.

The latter class of instrument, which typically runs at speeds of 60,000 rev/min, are usually referred to as ultra-centrifuges. They are expensive and you would usually find these in only advanced analytical labs. They are outside the scope of this unit.

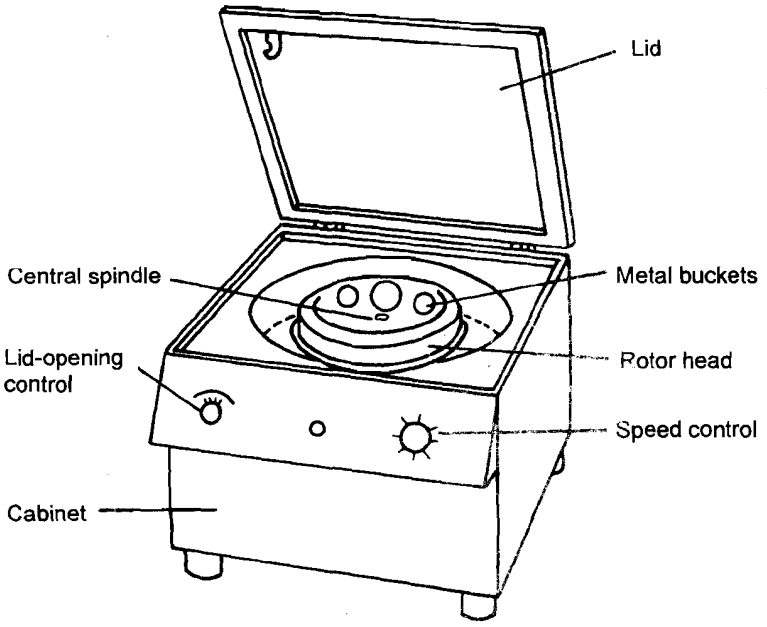

Fig. 3.28: Bench top centrifuge.

SAQ 4

When you are next in your lab, check to see if there are any centrifuges there. If you can obtain access to one it will help you to understand the description that follows. In any case, use any centrifuge you find to answer this questionnaire.

1) Make and model of centrifuge:

2) Approximate maximum speed:

3) Type of rotor head:

4) Approximate RCF:

You are more likely to encounter bench-top centrifuges which have a speed of upto 5,000 rev/min, developing RCFs of around 3,000 g. An example of this kind of centrifuge is illustrated in Fig. 3.28.

3.8 MICROTOMES

You will also come across microtomes in a biology lab. The mechanical cutting of plant/animal materials are done by the aid of microtomes and the process is commonly known as microtomy.

Microtomes can be divided into four basic groups:

1) Rocking
2) Rotary
3) Sledge (sliding)
4) Freezing

All microtomes, however, have a few things in common; all have a means of holding a block, the chuck; all have a knife past which the chuck can be moved in order to cut the block, and all have a mechanism by which the chuck can be advanced micron by micron, or in some cases by sub-micron steps, towards the

blade. The blade in all types is angled with respect to the chuck, and that angle may be varied.

1. Rocking Microtomes

The commonest rocking microtome is called a Cambridge Rocker. Figure (3.29) is a basic diagram. The mechanism of the microtome is mounted on a heavy cast iron base which helps to reduce vibrations by providing an inertial 'sink' and thus keeping all the fixed points truly fixed.

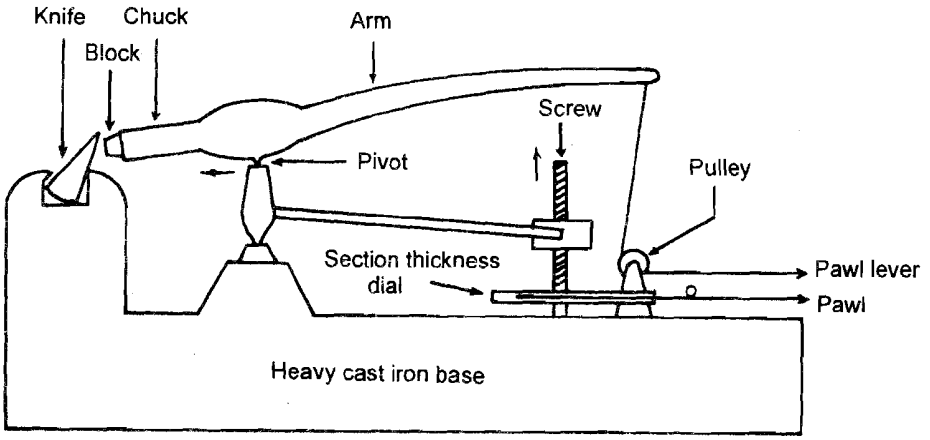

Fig. 3.29: The Cambridge rocking microtome.

The block for sectioning is fixed to the chuck which is rocked up and down about the *pivot* by the action of the *pawl lever* on the *arm*. With each rocking movement the chuck passes the *knife* which is only a little heavier than a cut-throat razor, and which has its cutting edge upward and a slide from the block on the downward stroke. On the upward stroke the *pawl* itself is engaged and moves the chuck forward by an amount which can be varied between two and twenty microns in two micron steps, by means of the *screw*. The downward stroke is neutral.

There are other rocking microtomes, but all such instruments suffer from the same disadvantage, namely that the section is necessarily curved since the chuck moves in an arc. For many purposes this is unimportant but it would not do for an embryological specimen, for example, where the juxtaposition of groups of developing cells might be critical, or for making serial sections of animals to ascertain the position and extent of the internal organs. A rocking microtome is generally best for cutting sections thicker than six microns.

2. Rotary Microtomes

Rotary microtomes are larger and heavier instruments, even more heavily damped to prevent vibrations and are usually capable of making thinner sections than the rocking variety. They are much more expensive. The pass, made past the much heavier knife by the chuck, is straight and relatively fast and is succeeded by a return upward stroke during which the chuck is advanced. A basic diagram of the machine can be seen in Fig. 3.30, which shows the flywheel used to keep sections flowing from the knife by smoothing out the action. Rotary microtomes enable sections of 5 microns thickness to be cut.

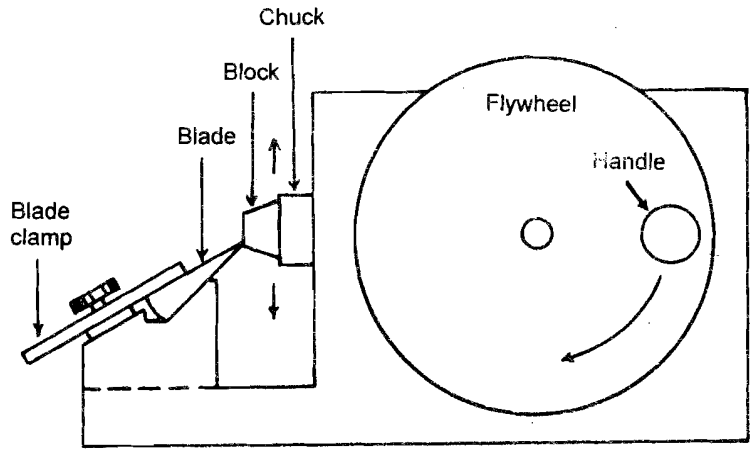

Fig. 3.30: The Rotary Microtome.

3. Sledge Microtomes

The sledge microtome is a device which may weigh as much as fifty kilograms, and consists of a heavy base into which are machined flat tracks on which the chuck sledge slides. The chuck holds the block with the surface to be cut uppermost. The chuck slides past a very heavy blade which is held horizontally on mountings which are bolted to, or drawn from, the base. Fig. 3.31 shows the basic diagram but can convey little of the weight of the machine. Each section is cut by a movement of the sledge past the blade and the return stroke advances the chuck. Various devices may be attached to sledge microtomes among which are chuck stages for the sectioning of small entire animals. Sledge microtomes are damped extremely well and may be used to cut sections down to two or three microns.

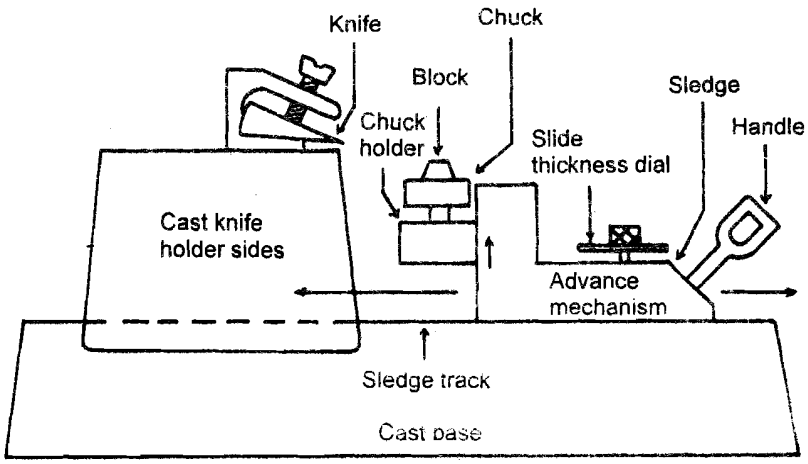

Fig. 3.31: A sledge microtome.

4. The Freezing Microtome

The only other type of microtome of interest here is the freezing microtome. This device usually has a wedge blade and the instrument is unusual as the blade is moved past the fixed chuck rather than the other way around. Fresh tissue to be sectioned is placed on the hollow chuck, and carbon dioxide gas passed rapidly through the chuck under pressure from a cylinder. The expanding CO_2 is sprayed on the tissue from above, and on the blade to cool it.

The cold blade is passed through the frozen tissue and the single sections removed with a cooled section lifter or forceps to be floated in water. Such microtomes are sometimes mounted in chilled cabinets to make working with them easier. It is now possible to cut frozen sections using freezer aerosols instead of CO_2 and freezing chucks are made which are electrically cooled by means of Peltier cells.

Microtomes of all kinds should be cleaned of wax after use, possibly by using a little xylene or less harmful solvent, and any parts liable to rust should be wiped with a little thin oil. The action should be examined and lubricated where necessary, and any soiled paintwork cleaned. The cover should always be on if the machine is not in use, but should be removed regularly for examination during periods of prolonged idleness.

SAQ 5

Fill in the blank.

1. For .... sections cutting from rocking microtomes is not advisable.
2. A rocking microtome generally cuts section thicker than ....
3. A rotatory microtome can cut the sections of .... thickness.
4. In freezing microtome ..... gas is sprayed on the tissues.
5. All the microtomes should be cleaned with ..... or with some less harmful .....

SAQ 6

To which part of a microtome is the wax block attached for sectioning?

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3.9 MICROTOME KNIVES

There are three basic cross sectional shapes of knives, wedge, plano-concave and double concave, all of which are shown in Fig 3.32.

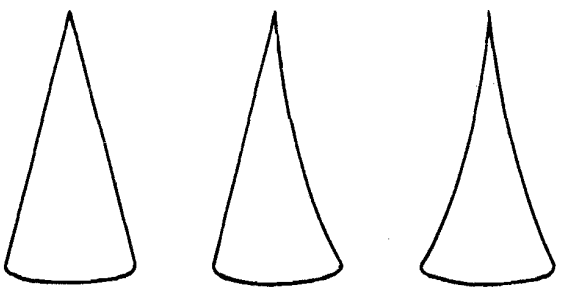

Fig. 3.32: Microtome knives.

The plane wedge knife is generally, though not exclusively used for cutting frozen sections, and plano-concave knives are often used for sectioning soft

materials such as celloidin embedded tissues. Botanical sectioning razors are generally plano-convex.

The best knives for the cutting of paraffin blocks are the double concave type, which should be of a heavy pattern cross-section to prevent vibration, i.e. the taper should be short and the base wide.

Blades are designed to undertake particular jobs, but microtomists are essentially experimentalists at heart and often use them for other purposes than those for which they were intended. Thus anything that we say about the uses of blades may be contradicted by someone else's experience. Nevertheless, there are certain things about microtome blades which are unalterable: for instance, a blunt one won't cut! Blades should be treated not only as dangerous but as delicate. The weighty hunk of stainless steel which is a microtome blade has been made with the precision of a Swiss watch and for section cutting its edge should look perfect when viewed under a microscope at about fifty times. This is how they come from the manufacturer! The job of a microtomist is to keep it that way.

In use, a blade gradually becomes blunted and may even develop a saw edge if left too long.

3.10 THE COLORIMETER

This instrument measures the density of the colour of a coloured solution. For example, it can be used to estimate the concentration of a compound solution, e.g., the intensity of the blue colour when iodine is added to starch, or the amount of haemoglobin in samples of blood.

A simple colorimeter is illustrated in Fig 3.33. When the coloured solution is placed in the instrument, light passes through the solution onto a photo-sensitive element. The amount of light passing through is registered on a meter scale. If the colour is dense, relatively little light passes through onto the photo-sensitive element. On the other hand if the colour is weak, more light passes through. The readings on the meter scale vary accordingly.

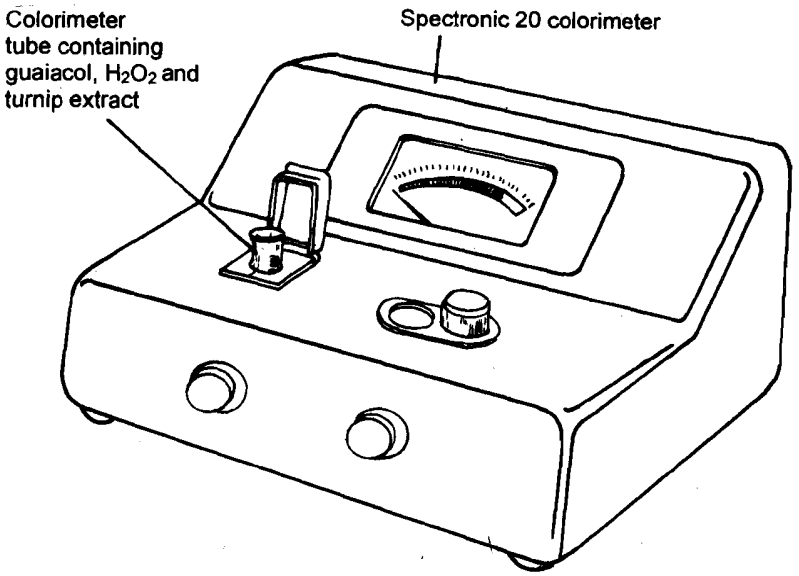

Fig. 3.33: The colorimeter.

3.11 pH METER

A pH meter is an electronic voltmeter of requisite sensitivity having a scale calibrated directly in pH units. The scale normally extends from 0-14 pH units. A pointing needle moves across the graduated scale and the pH of the solution can be read directly on scale. The pH meter is used to determine the pH of various solutions used in various biochemical investigations. pH meter is dealt in great detail in unit 4 of Block I of Chemistry course LT-3.

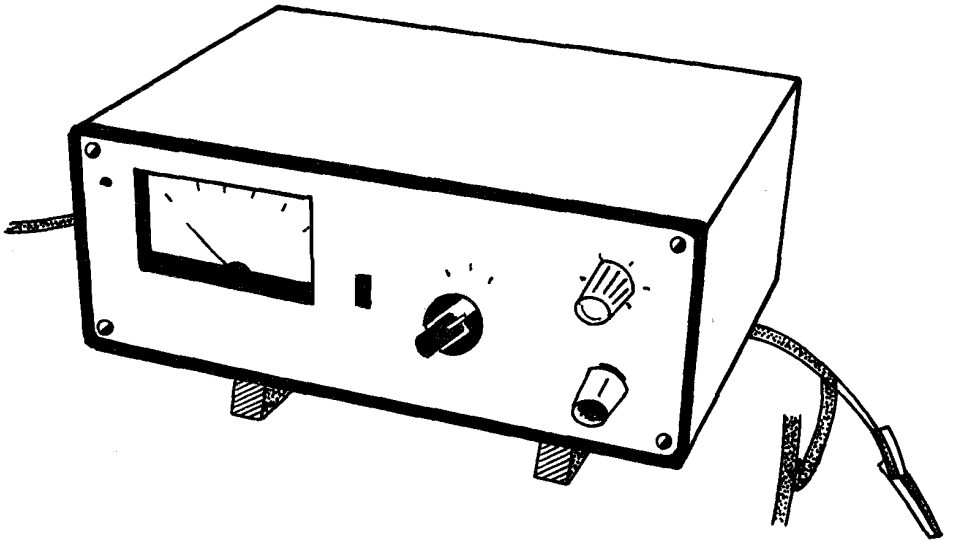

Fig. 3.34: pH meter.

3.12 EQUIPMENT FOR DISTILLATION

Distilled water is an important requirement in the laboratory. All impurities have been removed from distilled water. The detail unit on distillation is given in chemistry course LT-3, Unit 10.

The distilling unit, which is commonly used in a Biology lab consists of the following parts.

i) **Distilling Flask:** The size of flask varies depending on requirement. It is filled with water and heated over flame or hot plate.

ii) **Leibig condenser:** It consists of an inner glass tube surrounded by a glass jacket through which water is circulated. The steam, passing through an inner tube, condenses due to the cooling effect of cold water flowing in the glass jacket.

iii) **Adapter:** It is used to facilitate delivery of distillate into the receiver.

iv) **Receiver:** It is a simple conical flask, where distillate is collected. It is attached to an adaptor. All the connections are made through corks. Certain precautions are taken before the start of the distillation process:

Distilled water is used for preparing several reagents.

(1) All cork connections should fit tightly.
(2) The apparatus should be held firmly by the help of clamps.
(3) The condenser should be full of water.
(4) The distillation flask should be kept over the wiregauze before heating.

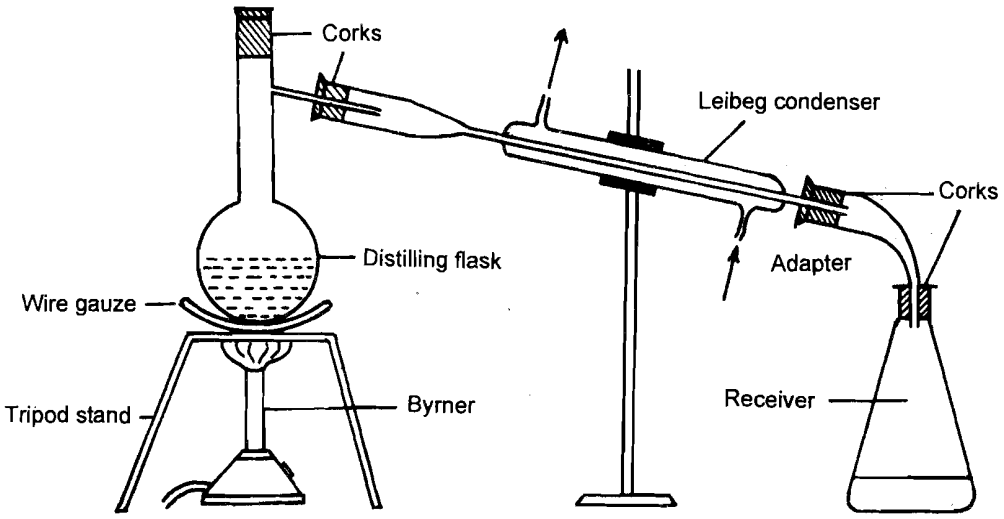

Fig. 3.35: A distillation unit.

3.13 EQUIPMENTS FOR MICROSCOPY

Various types of microscopes are used in studying the various structure and activities inside a cell. These are:

i) Simple microscope
ii) Compound microscope
iii) Phase contrast microscope
iv) Electron microscope
v) Scanning electron microscope (SEM)

Details of these microscopes will be given in Unit 6 of this course.

3.14 INCUBATOR

Incubator is an appliance which maintains the desired temperature inside it. An incubator is a box or container with insulated walls and a door fitted with a latch to close the door firmly. There is a hole in the center of its roof for insertion of a thermometer to read the temperature of the inside chamber. Its base contains a heating unit heated by electricity. On the front of the base on one side is a knob which can switch – on and switch – off the instrument. On the back is fitted a thermostat to regulate the desired temperature. In the centre of the front or besides the knob a bulb is fitted to indicate whether the instrument is off or on. The internal chamber is provided with one or more shelves.

Uses: The incubator is used for the following:

i) To keep section cutting material embedded in paraffin wax (at 50°C - 55°C).
ii) To keep cuttings at a given temperature.
iii) To incubate eggs.

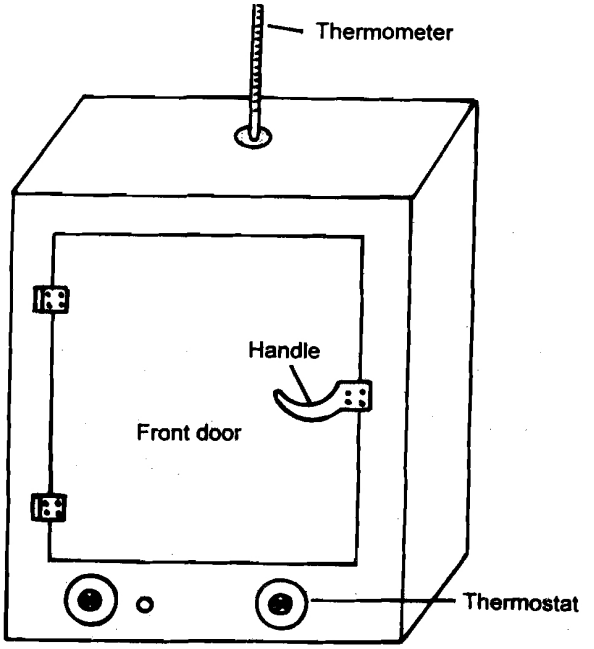

Fig. 3.36: Incubator.

3.15 SUMMARY

In this unit you have studied about:

- Several types of glassware and their uses.
- Several types of equipment which are used in a biological lab.
- Various types of heating apparatus.
- Various balances with their appropriate uses.
- Simple autoclave, pressure cooker, steam-jacketed autoclave, their working and the precautions which should taken when they are used.
- Microbiological safety cabinet used in microbiological inoculation and to protect the operator and the environment from exposure to pathogenic microorganisms.
- Centrifuges used in separating the cell organells or separation of suspensions.
- Microtomes used for cutting biological material for microscopic examination. There are four basic groups:
  (i) Rocking
  (ii) Rotary
  (iii) Sledge
  (iv) Freezing
- Microtome knives and their functions.
- Colorimeters used to find out the amount of substance dissolved in a solution.
- pH, which can directly measured on a pH meter.
- Different types of microscopes used in a biology lab.
- Incubator, a chamber with a thermostat which is used for incubating eggs and for wax embedding of section-cutting material.
- Structure of distillation unit and how distilled water is obtained.

3.16 TERMINAL QUESTIONS

1. List all the glassware which you come across while visiting a biology lab.

2. List various techniques of sterilization and describe any one in detail.

3. What are the drawbacks in inoculation without safety cabinets?

4. List the types of microtomes and write a note on their disadvantages.

5. What is the final product we get from a distillation unit?

6. Mention the use of a thermostat in an incubator.

7. List the different parts of a distillation unit.

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8. Mention the use of an incubator.

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3.17 ANSWERS

Self-assessment Questions

1. 1 – T, 2 – T, 3 – F, 4 – T, 5 – F, 6 – T, 7 – F, 8 – T, 9 – T, 10 – T
2. i) inoculation needles,
   ii) scalpels,
   iii) culture tube,
   iv) slides,
   v) Petri dishes etc.
3. 1, 2,
4. This answer will vary for every student.
5. i) embryological specimens
   ii) six micron
   iii) five micron
   iv) CO_2
   v) xylene or solvent
6. The chuck

Terminal Questions

1. List all the glassware and equipment found in a biology lab.
2. i) Wet heat sterilization -- in Pressure cooker or Autoclaves
   ii) Dry heat sterilization – Hot air oven
   iii) Sterilization by chemicals or untra violet rays
   iv) Dry heat sterilization – by flaming or Hot air oven
   v) Sterilization by ultra – violet rays.
3. See section 3.6. Microbiological safety cabinet.

4. See section 3.8 Microtomes
5. Distilled water in which all the impurities have been removed
6. Thermostat regulates the temperature
7. See section 3.12 equipment for Distillation.
8. Incubator is used to
   i) keep section-cutting material embedded in paraffin wax (at $50^{o} - 55^{o}C$).
   ii) keep cutting at a given temperature
   iii) to incubate eggs.

UNIT 4 COLLECTION AND PRESERVATION OF BIOLOGICAL SPECIMENS

Structure

4.1 INTRODUCTION

Every biology laboratory should have a collection of a variety of good plant and animal specimens. This is to facilitate the carrying out of experiments, observations and demonstrations. The earth is populated by great numbers of different plants and animals from which the collection may be made. In order to make the collection, proper methods and equipment are used. Once these specimens are collected it is important to know how to identify, kill and preserve them. Many biology laboratories have a museum for specimens. A herbarium is a collection of plant specimens within a museum. Within the museum the specimens are grouped according to their taxonomic characteristics.

Objectives

At the end of this Unit, you should be able to:

- list the characteristics of the main phyla of the plant and animal kingdoms broadly,
- describe correct techniques to collect plant and animal specimens,
- group plant and animal specimens,
- know correct techniques of preservation of plant and animal specimens.

4.2 DISTINGUISHING FEATURES OF MAIN PHYLA IN THE ANIMAL AND PLANT KINGDOMS

Living organisms are grouped according to their evolutionary closeness. The science of classification is known as **taxonomy**. In classification the organisms are grouped into units called **taxa**. The largest taxon is a **Kingdom** which is subdivided into **Phyla**. Each phylum is made up of **Classes**. Each class has several **Orders**. Each order is made up of several genera (singular genus). Each genus has many **Species**.

A species is the basic unit of classification. It is a group of organisms that can interbreed to produce viable offspring.

The main phyla in the animal kingdom and their distinguishing features are as follows:

1. **PORIFERA** (Sponges)
   - the body has pores hence the name of the phylum Porifera. The pores link interconnected chambers in the organism to the outside.
   - sessile.
   - skeleton made up of calcium, silica or horny spicules.
   - all are marine.
     Examples: *Leucosolenia, Sycon*

2. **COELENTERATA**
   - sea-like body cavity which also serves as a gut cavity and is called **enteron.**
   - radial symmetry.
   - the body wall has two layers of cells i.e., they are diploplastic, the outer layer is called ectoderm and the inner is called endoderm.
   - they have stinging cells called nematocysts.
     Examples: *Hydra, Obelia,* Portuguese man of war, Coral, Sea anemone.

3. **PLATYHELMINTHES** (flatworms)
   - the body is flattened dorso-ventrally
   - the body has only one opening – the mouth but no anus
   - branched alimentary canal
   - they use special cells called flame cells for excretion and osmoregulation
     Examples: flukes, tapeworms

4. **NEMATHELMINTHES/ASCHELMINTHES (Round Worms)**
   - rounded in cross-section
   - they have a narrow body pointed at both ends
   - they have a thick elastic cuticle cover
   - they are all parasites
     Examples: round worms, hookworms, filaria worms, threadworms

5. **ANNELIDA (segmented worms)**
   - the body is made up of a serial repetition of segments that are formed in the embryo stage. This is called metameric segmentation.
   - they are triploblastic i.e., the body has three layers i.e. ectoderm, endoderm and a middle mesoderm. The mesoderm secretes a fluid called coelom and therefore the organisms are said to be coelomate.
     Examples: Earthworms, leeches.

6. **MOLLUSCA (soft bodied)**
   - the **ventral** side of the body has a soft muscular **foot** hence the name of the phylum.
   - On the dorsal side is the **visceral hump** containing the main digestive organs.
   - the visceral hump is generally (but not always) protected by a **shell.**
   - most have a tongue like structure used for feeding called **radula.**
     Examples: snails, octopus,squids, slugs.

7. ARTHROPODA (joint-legged animals)
- they have an exoskeleton made up of cuticle.
- they shed the exoskeleton periodically – a process called moulting or ecdysis.
- they have jointed appendages (projections from the body)
  Examples: crustaceans, millipedes, centipedes, arachnids, insects.

8. ECHINODERMATA (Spiny skinned animals)
- the skin has plate-like structures called ossicles and spines made up of calcium.
- they are exclusively marine.
- they have bilateral symmetry as larvae but radial symmetry as adults.
- they have a water-vascular system with tube feet for locomotion.
  Examples: sea urchins, brittle stars, sea cucumbers and sea star.

9. CHORDATA
- they all have a strengthening rod known as **notochord**
- they have a dorsal hollow **nerve chord**
- they have muscle blocks called **myotomes**
- a post anal tail and perivisceral clefts.
  Examples: Tunicates, Lancelets, Jawless fishes, Jawed fishes, Amphibians, Birds, Reptiles, Mammals.

Distinguishing Features of main Phyla/Divisions in the Plant Kingdom:

1. THALLOPHYTA
- The plant body is a thallus i.e. not differentiated into root, stem and leaf.
- They have a wide variety of bodyforms and sizes, ranging from microscopic unicellular organisms to large sea weeds.
  Examples: Green algae (chlorophyta) e.g. *spirogyra* – blue-green algae (cyanophyta) – Brown algae (phaeophyta) – Red algae (Rhodophyta)

2. BRYOPHYTA
- They show clear – cut alternation of generation
- Haploid gamete producing gametophyte alternates with diploid spore
- Gametophyte is the more prominent phase in the life cycle.
- Gametophyte is anchored to ground by filaments called rhizoids.
- Sporophyte is parasitic on gametophyte.
  Example: Mosses and Lichens

3. PTERIDOPHYTA
- These show prominent **sporophyte** in the life cycle.
- Sporophyte has roots, stems and leaves with vascular tissues.
- Sporophyte is independent of gametophyte. They have more differentiation of tissues.
  Example: Ferns and horsetails.

4. SPERMATOPHYTA
- Seed bearing plants.
- They have separate male and female spores (Pollen grains and embryosac respectively)
- Embryosac is enclosed in **ovule** which, after fertilization, develops into **seed**.

They have complex **vascular tissues** in roots, stems and leaves. Examples: Gymnosperms and Angiosperms.

Gymnosperms are 'naked seed-bearing', not dependent on free water for fertilization e.g. cycads and conifers. Angiosperms are flowering, seed-bearing, depend on pollinating agents, do not depend on free water for fertilization e.g. monocots and dicots.

SAQ 1

i) State the phylum to which each of the following organisms belong to:
   a) *Hydra*
   b) Man
   c) Moss
   d) Maize

4.3 SOURCES OF SPECIMENS

Specimens for the biology laboratory can be collected from a variety of sources. The sources are usually the places where the organisms live (habitats). The habitats may be in water (aquatic) or on land (terrestrial). Aquatic habitats may be ponds, rivers, lakes and oceans. Terrestrial habitats may be on land, in soil or on other plants and animals.

The methods used to collect specimens depend on:

a) Type of specimen being collected
b) Habitat of specimen

Plant Specimens

Source: Lower plant specimens like algae are obtained from stagnant or very slow moving waters.

These are collected by using nets or even picking by hand. There are special nets (plankton nets) for collecting the unicellular specimens. Higher plants are collected from land in the various areas where they grow.

In collecting Angiosperms, it is important to note that:
- The specimens must be collected during dry weather.
- As much of a plant as can help in identifying it should be collected.
- The specimens must not be sterile. Usually, a fertile shoot with flowers, fruits and leaves will help in identifying a plant.

Animal Specimens

These are also collected from various areas they live in. In this subsection we shall deal with methods of collecting invertebrates. Invertebrates can be collected using the following methods:

1. Sweeping methods by using special nets such as sweep nets and mist net.
2. Hand picking.
3. Trapping – using special traps such as light traps, pheromone trap, yellow can and pitfall traps.
4. Using insecticides – In cases where specimens are not required to live.

You will study in detail about methods of collection of animals and plant specimens in Exp. 4 and 5 of LT-2 course.

4.4 IDENTIFICATION OF SPECIMENS AND USE OF KEYS

There are various systems of classifying organisms. The information you have been given so far is based on the natural system of classification. This is the most commonly used system. Another system is the artificial system of classification which uses non-biological criteria for grouping such as alphabetical order of names and economic importance.

In classification there must always be a method that can be used to determine the name of a particular organism. This is done by constructing an identification key. A key is essentially a printed information – retrieval system into which one puts information regarding a specimen-in-hand and from which one gets an identification of the specimen to whatever level the key is designed to reach.

In the absence of the originally identified specimen of the species utilised for comparisons, the published description becomes the only tool. In lower groups with many species, it is the most tedious task to compare specimens with hundreds or thousands of published description. This task can be solved if the keys to the main group are available. The main purpose of the key is to facilitate identification. It is a tabular device designed for rapid identification.

When you visit your study centre, your counsellor will demonstrate to you how to use such an identification key.

4.5 PRESERVATION OF SPECIMENS

Every biology laboratory should have a collection of specimens. Since it is not possible to keep these specimens in live condition it becomes necessary to preserve these specimens and then store them in a life-like form. This is done using special techniques.

4.5.1 Preservation of Plant Specimens

The most common method of preserving plant specimens is by first drying them and then mounting them.

In **drying,** the specimen is placed between folds of absorbent paper e.g. newspapers. Care must be taken to display the parts of the specimen as closely as possible to their natural position. This can be done by placing a weight over the absorbent paper or by using a plant press where available. During drying the papers must be changed daily to prevent growth of moulds. The drying process can be speeded up by placing near a source of heating e.g. a charcoal burner. Quick drying of the specimen helps to preserve the green colour of the plant.

In **mounting** the dried specimen is attached to manila paper using either glue or strips of gum paper. On the manila paper a label must be attached giving

name of specimen, location and date of collection. The mounted specimens should be placed in folders and stored according to taxonomic groups.

ACTIVITY 1

Read and make notes on the drying of plant specimen.
When you visit your study centre next, your tutor will demonstrate you the process of drying and mounting plant specimens.

4.5.2 Humane Killing and Preservation of Animal Specimens

Humane killing is painless killing of animals i.e. the animal is made to pass quickly and quietly into an unconscious state and death follows before consciousness is regained. Humane killing is called euthanasia.

Chemical methods may use chloroform or ether. This is done in airtight containers like desiccators and care must be taken that the animal does not come into contact with the chemical.

Some of the specimens of killed animals may need to be preserved for future use. This is done a) using chemicals called Preservatives e.g. Formalin (5%, 8% or 10%) and 70% alcohol b) by drying the specimens and then mounting them in storage boxes. This is common with insect specimens. You will study in detail about preservation of insect specimens in Exp. 4 of LT-02 course.

SAQ 2

Which chemicals are commonly used as preservatives?

.....

.....

ACTIVITY 2

At this stage collect and preserve at least three different animal and three different plant specimens. Present the preserved specimens to your tutor when you visit the study centre next time.

SAQ 3

Define Humane Killing.

.....

.....

.....

.....

4.5.3 Arrangement in Museum and Herbarium

You would recall from Unit 2 of this course that a museum is a display of a collection of plant and animal specimens. A museum consists of mounted specimens of representative local plants and animals collected by students, supplemented by other specimens procured from supply companies sketches. Photographs or models of the smaller microscopic organisms add to the effectiveness of the display.

A herbarium is a collection of pressed and dried plants arranged according to some valid system of classification and available for reference. The herbarium – a repository for plant collections is a research, training and service institution that functions as a reference centre, documentation facility and data store house. Collections consist of specimens that are samples of populations and taxa from nature, experimental garden or laboratory. The main steps in the collection of specimens for a herbarium are pressing of plants, mounting of specimens and identification of specimens. For collection of plants, you can plan a field trip. Before planning a field trip, you should obtain necessary permission from the authorities concerned to visit a forest, national park or a sanctuary and you should always keep a copy of all essential documents in your computer. You will study details of a herbarium in Unit 5 of this course.

You should try to visit a museum nearest to you to see the variety of specimens and their organisation.

4.6 SUMMARY

Let us summarise what all you have studied in this unit.

- The main phyla in animal kingdom are Porifera, Coelentrata, Platyhelminthes, Nemathelminthes, Annelida, Mollusca, Arthropoda, Echinodermata and Chordata. The main phyla in plant kingdom are Thallophyta, Bryophyta, Pteridophyta, Gymnosperms and Angiosperms.
- The methods used to collect specimens depend on the type of specimen and habitat of specimens.
- After collection, animals and plant specimens are preserved, mounted and identified.
- A museum is a display case consisting of mounted specimens of local plants and animals. Herbarium is a collection of pressed and dried plants arranged according to some system of classification.

4.7 TERMINAL QUESTIONS

1. State two distinguishing features between a) Coelenterata and Platyhelminthes
   b) Bryophyta and Pteridophyta.

   .....

   .....

   .....

   .....

   .....

   .....

2. Why is it necessary to preserve plant specimens?

   .....

   .....

   .....

   .....

4.8 ANSWERS

Self-assessment Questions

1. a) Coelenterata
   b) Chordata
   c) Bryophyta
   d) Spermatophyta
2. Formalin (5%, 8%, 10%) and Alcohol (70%).
3. Painless killing of an animal.

Terminal Questions

1. a)

Coelenterata	**Platyhelminthes**
Diploblastic	Triploblastic
Radial Symmetry	Bilateral symmetry

   b)

Bryophyta	**Pteridophyta**
has Rhizoids	has roots
gametophyte dominant	sporophyte dominant

2. a) Because it is not possible to keep all plant specimens in the laboratory alive.
   b) Some of the plant specimens are not easily available in the locality.
   c) Preserved specimens occupy less space in the laboratory.
   d) Preserved specimens form a reference collection.

UNIT 5 HERBARIUM TECHNIQUES

Structure

5.1 INTRODUCTION

Herbarium is a collection of dry, pressed and preserved specimens of plants arranged systematically for the purpose of reference and identification. The herbarium may also be regarded as a museum with a wide representation of plant species from various geographical regions.

The modern herbarium is more or less a great filing system and can furnish data in any field of plant research. In this unit you will study about various techniques involved in the making of a herbarium because as a lab technician you will have to collect, preserve and store plants.

Objectives

After studying this unit you will be able to learn about:

- various equipment, which are required for plant collection,
- pressing material to be used,
- plant parts which are to be pressed,
- keeping of wet plant material,
- drying, mounting and labelling of plant specimens,
- storing of the herbarium sheets.

We will describe in detail all the techniques used in preservation of plants for their taxonomic studies.

5.2 COLLECTION PROCESS

As a plant collector you will need the following equipment:

i) a gardener's knife
ii) a plant press or vasculum
iii) blotting papers to dry plants
iv) trowel to dig and uproot the plants
v) collecting and mounting sheets

vi) gum tape, labels, waterproof ink and pen
vii) other equipment used in collection are collecting pick, secateur, vasculum, hatchet, spade, etc.

Before coming to actual collection you must know about vasculum and plant press.

Vasculum: Vasculum is made up of a metal cylinder with a sliding door usually worn on a strap over the collector's shoulder into which plant specimen are placed (Fig. 5.1). Polythene bags and paper bags are also used for putting fruits, seeds and small specimens after collection.

Plant Press: Plant press is an indispensable tool for pressing fresh plant specimens to subsequently dry them and mount permanently. The plant press is of two types as shown in figure 5.1.

The collection of plants can be done by three methods. The most common method is to press the individual plant as soon as you see a particular plant. This you can do by pressing in the field. You can press the plant in blotting paper or newspaper in a field press.

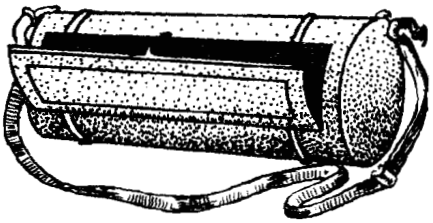
At times specimens can be kept in vasculum. But before keeping plants in vasculum you must line the vasculum with wet blotting paper or newsprint to avoid wilting of the specimen collected. You should not overcrowd the vasculum, it is always safer to fill the vasculum partly. You can keep plant specimens in good condition for about twelve hours by keeping the vasculum in a cool place. The specimens should be pressed immediately after this period.

The second method is to collect plants in polythene bags or rucksack (a bag strapped to shoulders) and pressed as soon as there is an opportunity for it. You can adopt this method for the plants of tropical rain forests and chiefly in rainy season when there is too much humidity.

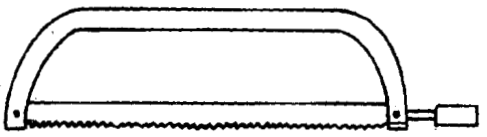
Things to remember:

i) You should always collect specimen which is in flowering and fruiting stage.
ii) Herbs are collected complete with their underground parts.
iii) At least five specimens of plants, preferably in different stages of flowering and fruiting should be collected.
iv) You must visit the locality several times during the year.

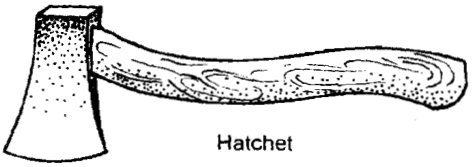
You should select the blotting paper that absorbs a given volume of water in the shortest period. You can also use newspapers. The standard size of a folded blotter should be 30x45 cm. The blotting paper should be changed after every 24, 48 and 72 hours. You can reuse it after drying it. In case of fleshy plant parts and during rainy season, remove blotters at short intervals.



Vasculum

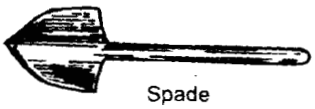


Hack saw



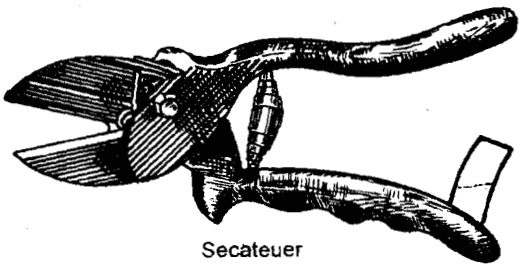
Hatchet

Collecting pick

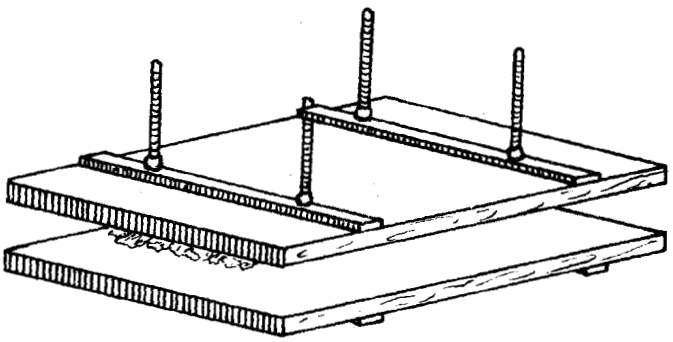


Spade

Trowel



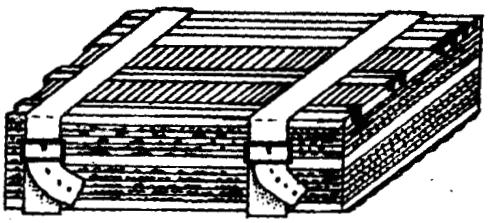
Secateuer



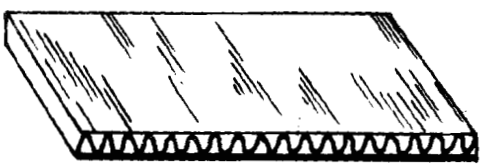
Plant press of wooden planks

Knives

Knives



Perforated plant press



A corrugate or ventilator

Aluminium plant label

CPLT-02/62

Fig. 5.1: Various equipment used in collection of plants.

SAQ 1

Write T for true and F for false for the following statements.

1) Newspapers can be used in place of blotting papers. ☐

2) You can keep as many plants as you like in the vasculum. ☐

3) Polythene bags or rucksack are used for plant collection in a tropical rain forest or in rainy season. ☐

4) The standard size of a folded blotter should be 30 x 45 cm. ☐

5.3 PRESSING OF PLANTS

Arranging plant specimens with pressing paper is most important step in the preparation of herbarium and needs careful attention. Before pressing the plant specimen the following points should be taken care of:

1. Underground parts like root, rhizome, etc., must be included in herbs.
2. Make it sure that specimens are in flowering or fruiting stage. Sterile specimens are generally useless.
3. Ensure that specimens are free from insect infestation, fungal infection and any pathological symptoms.
4. Fresh specimens are found all-over land, thus their parts can easily be arranged within the pressing paper. The art of plant pressing comes from experience. However, some suggestions are:

    1) Pressing is most effective when the maximum surface of pressing paper is covered with the plant specimen to be pressed.
    2) As a general rule a single specimen should be pressed in a folded pressing paper. In case of a large-foliaged specimen, it may be cut into two or more pieces and each piece is arranged and pressed into two or more folded pressing papers.
    3) In case of small herbaceous plants, many specimens may be pressed in one pressing paper.
    4) Herbaceous specimens, longer than 40 cm. may be folded in toto into a V-shape, N-shape or M-shape and then pressed. The points of folds should be moderately macerated before bending brittle stems to avoid breaking them.
    5) Pruning of specimens is essential to avoid overlapping of plant parts. Care should be taken to leave the basal portion of pruned parts (pedicel, petiole, etc) attached to the plant to indicate their natural position.
    6) A few leaves should be arranged with their lower surfaces facing upward.
    7) When the leaf is large and pinnate compound, it is necessary to excise all except one leaf. If the remaining leaf is still too large, leaflets of one side may be removed leaving the terminal leaflet, if present. Even one complete leaf may be split lengthwise and half of a leaf pressed.
    8) A large palmately compound leaf is usually split in half (lengthwise) and one half is discarded while pressing.
    9) Exceptionally tall herbs are pressed in three parts i.e., one from the base, another from the middle and a third from the top indicating their position.

10) At times it is not possible to accommodate more than a leaf and an inflorescence within a herbarium sheet. In such cases, the arrangement of leaves is noted down. Entire petiole and the portion of stem, from which it is produced, may be included.
11) A few gamopetalous flowers should be pressed separately from the specimens. Some of the flowers should be split open and then pressed to expose their essential organs and the nature of the thalamus.
12) Roots and underground parts should be washed thoroughly before pressing to remove soil particles.
13) Fleshy and bulky organs should be sliced open to facilitate quick drying. Paper padding or cotton batting around such organs is helpful for drying.
14) To avoid decoloration and moulding, blotters should be changed frequently depending upon the nature of the specimen and humidity.

Members of Iridaceae, Commelinaceae, etc. produce flowers with deliquescent corolla or perianth (quickly becoming semiliquid). Such corolla or perianth often stick to pressing paper or become so thin that they cannot be removed easily. When such a specimen is pressed, a layer of absorbent cleaning tissue is put over and beneath the flower before pressing. This tissue is peeled off after the specimen becomes dry.

SAQ 2

Suppose you collect a plant 60 cm long. Show with the help of diagrams how you will press this plant.

5.4 KEEPING WET MATERIAL

You may find it difficult to press the fleshy and non-fleshy plants during the rainy season. Two techniques have been found useful in such cases.

Schultes (1947) has favoured the following technique. Specimens are pressed between blotters for about 24 hours. Later these are taken out of the plant press and dipped in a solution containing 2 parts of 40% formaldehyde and 3 parts of water. After a few seconds of dipping, specimens are kept again in the pressing papers. All the sheets containing specimens are piled one above the other. No blotters and corrugates are put in between them. After applying pressure for a few hours, the entire bundle is sealed inside cellophane, or a cloth bag with several layers of thick rubber. Later, specimens are removed and dried at convenience.

The plant specimens may be kept in a wet condition for a month or so. If the drying process is delayed somehow, they may be taken out after a month and dipped again in commercial formaldehyde-water solution. Rubber gloves should be used when working with this solution to prevent cracking of skin on hands. The specimens made by this method are of inferior quality as they lose their natural colour and beauty.

Fosberg (1947) had proposed an improvement over this method. Schultes' solution was replaced with one part of formaldehyde and two parts of 70% alcohol. The solution was applied to specimens with a 5 cm flat brush instead of dipping. The solution penetrated inside the specimens and there was found to be a better control from decomposition.

Hodge (1947) observed that substitution of 40%-50% alcohol as preservative was more satisfactory. Moore (1950) advocated the use of hydroxyquinoline sulphate as a preservative.

It has been found that 15g mercuric chloride ($HgCl_2$) mixed with one litre of 70% alcohol serves as a good preservative. The specimens are dipped in this solution for a few seconds and then placed in pressing papers. The only disadvantage in this method is that the solution leaves a blackish mark on pressing paper, which, of course, cannot be avoided. Specimens treated with this solution are neither spoiled nor decomposed. Even these are well protected from insects.

5.5 DRYING OF SPECIMEN

Drying of plant specimens without artificial heat

It is almost followed universally and supposed to be most satisfactory. Plants are pressed in pressing papers between blotters. Corrugates are not used in this method. Plant press is locked for 24 hours. This is called the 'sweating period'. After the sweating period is over, the press is unlocked, blotters are removed and pressing papers are turned back. Now the specimens are rearranged as the situation demands. At this time most of the plant parts become flaccid. They lose their natural spring and turgidity and now it becomes easier to arrange the branches, leaves, flower buds, flowers and their parts in the desired position. Much attention is to be paid at this stage. A careful arrangement of plant parts will result in nicely arranged specimens after drying when plant parts become stiff and are fixed in a desired manner. After rearranging, folded pressing paper is kept in between two fresh blotters. All the specimens are provided with fresh blotters in this manner and are locked again in the plant press for the next 24 or 36 hours. A third change follows and specimens are locked up tightly for another 48 or 72 hours. Most of the specimens dry in a week's time. Fleshy and succulent materials may require a much longer period to dry completely.

There are certain disadvantages in this method of drying which are mentioned here:

1. A much longer time is needed for drying the specimens. It may be a week's time or sometimes even more than that when plants are fleshy and succulent.
2. Blotters are to be changed at least 3 or 4 times during the entire process and wet ones are to be dried in the sun before using them again.

3. A large number of blotters are needed in comparison to drying the specimens with artificial heat.
4. Few specimens can be processed per collector as compared to drying with artificial heat.
5. Specimens dried without artificial heat are liable to be infected by fungi because much time is taken up in complete drying.
6. Larvae present within the flowers eat away some of the important parts.
7. The labour cost of drying per specimen is exceedingly high in comparison to drying with artificial heat.

5.6 MOUNTING OF SPECIMEN

Dried specimens are mounted on herbarium sheets of standard size 29 x 42 cm. The herbarium sheet must be of long lasting and durable paper and heavy enough to support the specimen. A hundred percent rag paper has been found to be the best but it is quite expensive. A paper of lower rag content is mostly used since it is cheaper as well as easily available. It is not advisable to use paper of much lower rag content because while lifting, it bends with the weight of the mounted specimen and the specimen is liable to be broken (as it is brittle after drying). Thick drawing sheets may also be used as herbarium sheets. Curators of most herbaria use hundred percent rag paper for mounting the specimens.

There are several ways of mounting. Generally a paste or glue is used to fix the specimen to mounting sheet. It is applied to the lower surface of a specimen with a brush and the specimen is properly placed on a mounting sheet. Sometimes the glue or paste is spread in a thin layer over a glass plate of size 36 x 51 cm. The specimen is lifted with the help of a forceps from pressing paper and is placed over the glued surface of glass plate with the lower flat surface in contact of the glue. It is then lifted and placed over the mounting sheet. The pressing paper (of unprinted newsprint) is placed over it, gently pressed, taken off and is rejected. It removes the excess of glue or paste which comes outside the lower glued surface of the specimen. The glue is to be reapplied on the glass plate after two or three specimens have been brought into contact with glued-glass surface. It is better to scrap off the glue from the glass plate before reapplying it as broken parts of dried specimen sometimes get stuck to the paste. After the specimen has been mounted, white gummed cloth strips (holland cloth) are also used to fix the specimen on the mounting sheet. While using these strips, care should be taken that strips do not hide important parts of the specimen.

SAQ 3

Write T for true and F for false for the following statements in the given brackets.

1. It has been found that 100% $HgCl_2$ is a good preservative against insects. ☐
2. The locking of a plant in plant press for 24 hours is known as sweating period. ☐
3. Specimens dried without artificial heat are liable to be infected by fungi because much time is taken in complete drying. ☐
4. Larvae present within the flowers eat away some of the important parts. ☐

SAQ 4

Fill in the blanks with appropriate figures or word(s).

1. The standard size of herbarium sheets on which dried specimens are mounted is .....
2. Curators of most of herbaria use ..... per cent rag paper for mounting the specimen.
3. Specimens are mounted on sheet with the help of ..... or .....
4. One should not use paper of much ..... rag content because while lifting it will bend and specimen will be broken.
5. The ..... on the sheet provides some important data of about the specimen collected.
6. The entries in labels must be in legible handwriting using .... black ink.

5.7 LABELLING OF SPECIMEN

After mounting the specimen, herbarium sheet is given a final touch by pasting a label at its lower right hand corner. The label is of 11 x 7 cm size but its size may vary by 1.2 cm on either side. Some herbaria prefer to get the label printed on sheets. The label provides some important data of specimen which cannot be gathered by simply studying the herbarium material. A label must provide the following information:

Flora of ..... No .....
Family .....
Botanical name.....
Local name .....
Habit .....
Flower colour .....
Habitat.....Locality..... Altitude.....
Ecological notes .....
.....
.....
Date of collection .....
Collector .....

It is advisable to type the data on the label. Entries on the label may be made in legible handwriting using waterproof black ink.

5.8 STORING OF HERBARIUM SHEETS

After proper drying, the herbarium sheets are ready in all respects to be stored. You can use wooden or steel almirahs which are generally dust proof. Now the specimens are arranged according to the Bentham & Hooker or Engler and Prantl classification.

Each species is kept in a folder which is lighter in weight and smaller in dimension than the genus cover. Many species (kept in separate species cover) of a genus are kept together in a genus cover. The genus cover usually has the name of genus written or printed at the lower left hand corner. When the cover is restricted to specimens of a single species, the genus initial and the name of

species are written at the lower right hand corner. It is customary to arrange the genera and species in alphabetical manner under each family. In large herbaria the specimens are also segregated by the name of the continent to which these are indigenous.

Preservation of Specimens

In all the herbaria great attention is paid towards the preservation of herbarium specimens because there is every likelihood of their being attacked by several insects. Some of the most common insects which damage the specimens are herbarium beetle, drugstore beetle and booklouse. All these insects complete their life cycle within the dried specimens, eating away the plant parts, herbarium sheets and badly damaging several specimens kept one above the other. The herbarium beetle is the worst of all and completes its life history within two or three months. To overcome insect-damage combined use of repellents and insecticides has proved to be most effective.

Repellents are chemicals which prevent attack of specimens by insects. These chemicals simply drive them back, when trying to advance towards herbarium specimens but are neither lethal nor toxic to them.

Two chief repellents are naphtha flakes (naphthalene compounds) and paradichlorobenzene (PDB). Merril (1948) has reported that two parts of naphthalene compound mixed with one part of paradichlorobenzene brings the most effective result. Two to three ounces of this compound may be kept in the pigeonhole of a herbarium almirah. The bags containing this mixture are refilled once a year.

Insecticides are fatal to the insects. These kill the insects either by coming into contact or by being digested by them. Some of these are as follows:

1. Cyanide gas
2. Paradichlorobenzene (PDB)
3. Carbon disulphide gas
4. A mixture of 3 parts ethylene dichloride and 1 part carbon tetrachloride.
5. Dichloro diphenyl trichloroethane (DDT)
6. Mercuric chloride.

1. Cyanide gas is extremely poisonous and is a more effective insecticide. The specimens are kept inside an air-tight chamber or room and are fumigated with this gas. Cyanide gas is exclusively used at Kew (Ballard, 1938).
2. Paradichlorobenzene crystals vaporise at 43°C to 60°C. The specimens are kept in a air-tight chamber for 3 to 4 days and paradichlorobenzene is allowed to vaporise in that chamber killing all sorts of insects injurious to herbarium specimens. It is more commonly used as a repellent than as an insecticide in most of the herbaria.
3. Carbon disulphide is a highly inflammable and volatile liquid. It is converted into gas at 10°C to 15.5°C. This liquid should be kept at a distance from the flame as it easily catches fire. Specimens are fumigated with this gas in air-tight chambers taking all precautions that it does not catch fire. Two to three days of fumigation is sufficient to kill all the insects and their eggs which are harmful to specimens.
4. A mixture of 3 parts (by volume) ethylene dichloride and 1 part of carbon tetrachloride has been found to be a most effective insecticide. The mixture is volatile at room temperature and does not catch fire. The specimens are fumigated in the air-tight chamber.

5. Dichloro diphenyl trichloroethane (DDT) is available in powder form. Howard (1947) has suggested dusting of dried specimens after taking them out from the plant press. It is used either in 100% or 25% to 50% strength and has been found effective in killing herbarium insects. It is not a permanent insecticide. Specimens need frequent dusting by DDT after an interval of 1 or 2 years.
6. Mercuric chloride is a very strong poison for all sorts of herbarium insects. A saturated solution is prepared in 95% of alcohol at room temperature. One part of this stock solution is mixed with a part of alcohol and dried specimens are dipped in it. After dipping, specimens are kept in blotters for 24 hours and then mounted on herbarium sheets. Some botanists advocate the use of 2% solution of mercuric chloride in a mixture prepared by adding 1 part of petroleum ether to 2 parts of 95% alcohol. Specimens are dipped in it and dried for mounting.

While dipping specimens, penetration of liquid in fleshy and thick parts of plants is not appreciable. The application of this liquid remains more or less superficial and thus the insects and larvae, which have already entered deep in the plant tissue, are not killed. Being a stomach poison this does not kill them until they have already eaten some parts of the plants along with this poison. It is not a permanent poison and loses its potency after a few years. The application of mercuric chloride is not prescribed for already mounted specimens because it dissolves some of the pigments of plant material and leaves a black residue on herbarium sheets.

An other way of killing insects has been suggested by O'Neill (1938). It has been found that a temperature of 60°C to 77°C is enough to destroy all the insects, their eggs and larvae in 4 to 5 hours. This much of heating neither burns nor makes the specimens more brittle. Steel herbarium almirahs are fitted with thermostatically controlled electric heating units which keep a constant temperature of 60°C to 77°C within the compartments.

5.9 HERBARIUM ETHICS

Though ethics is not central to this topic we would like to mention a few points in this regard:

Every herbarium should provide information on working days and hours, filing arrangements, loan procedures, availability of microscopes, use of library collections, etc. A visitor should be aware of these regulations beforehand.

Even otherwise, one should always practice the following rules while working in a herbarium:

1. Specimens are fragile; the sheets on which they are mounted should be kept flat.
2. Folders and sheets should be lifted one at a time.
3. While carrying the specimens from the cabinet to the working table, a supporting sheet should be kept below.
4. Heavy books or elbows should not be placed on the sheets.
5. Any broken part should be kept in a small packet in the same folder.
6. Materials for dissection should be taken only sparingly and with permission.
7. Damaged specimens should be kept aside for repair.

5.10 SUMMARY

In this unit you have studied that:

- A plant collector needs equipment such as a gardener's knife, plant press or vasculum, blotting papers or newspapers, trowel, mounting sheets, gum or glue tapes, labels, waterproof ink and pen, collecting pick, secateur, hatchet spade etc.
- Blotting papers are a very important part of plant drying. They should be changed every 24, 48 and 72 hours.
- Before pressing the plants some important points should be taken care of during selection of plant specimens.
- The drying of specimens is of utmost importance and is done without artificial heat for natural and better drying.
- Labelling is a very important task for the collector because it provides a lot of information about the plant specimens.
- Storing is one of the most important tasks to be performed because without proper storing all the hard labor involved in collecting, pressing, drying, mounting, labelling is lost forever.
- Herbarium ethics should be followed for the preservation and conservation of the specimens.

5.11 TERMINAL QUESTIONS

1. Write down important things one must remember while collecting plants for making a herbarium.

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2. Write any ten points that you think should be kept in mind before pressing the plants.

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3. Describe the Schultes method (1947) of keeping wet material.

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4. Describe the disadvantages of drying plant material without artificial heat.

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5. Take a plant of your choice and fill in the information given below.

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Flora of ..... No .....
Family.....
Botanical name.....
Local name .....
Habit .....
Flower colour .....
Habitat.....Locality..... Altitude.....
Ecological notes .....
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Date of collection.....
Collector .....

6. List various insecticides used as preservatives for plant specimens and describe any one in detail.

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7. Write down the important points which should be kept in mind before storing dried herbarium sheets.

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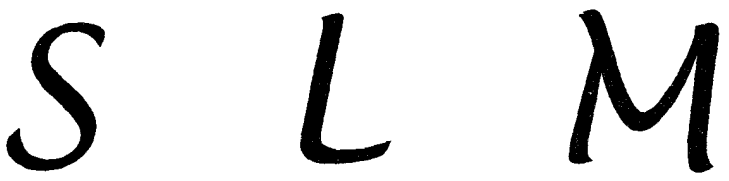
5.12 ANSWERS

Self-assessment Questions

1) 1 – T, 2 – F, 3 – T, 4 – T
2) You can fold a plant in given the shapes.



3) 1 – F, 2 – T, 3 – T, 4 – T,
4) 1) 29 x 42 cm, 2) 100%, 3) paste or glue, 4) lower, 5) label, 6) water proof

Terminal Questions

1. Refer to section 5.2.
2. Refer to section 5.3.
3. Refer to section 5.4.
4. See section 5.5. Drying of plant specimens.
5. The answer will vary from person to person. You can get the help of your counsellor for this question.
6. See section 5.8. Storing of Herbarium.
7. The points which should be remembered:
   i) Specimens should be stored either in steel or in wooden almirahs which must be dustproof.
   ii) Specimens should be arranged according to the Bentham & Hooker or Engler or Prantl systems of classification.

UNIT 6 TYPES OF MICROSCOPES

Structure

6.1 INTRODUCTION

A microscope is a versatile instrument, that by its various modes of operation, provides valuable information of the objects viewed by it. Its applications in biological sciences are immense. Therefore, it is a very common instrument of the biolabs. Its value lies in:

a) giving a magnified image of the object that can be identified and also drawn or photographed;
b) revealing the fine internal and external details of a structure or of small organisms;
c) taking measurements of the size and shape of the magnified objects; and
d) taking various physical measurements in the analysis.

The unaided human eye can see objects as small as 0.1 mm (100 μm) diameter, e.g., an amoeba cell or a mammalian ovum in good lighting conditions preferably side lighting against a dark background. Of course, one cannot discriminate any details within these minute objects. Also one cannot observe smaller objects such as the normal cells of plants, animals and micro-organisms. For these reasons a variety of microscopical methods are used in biology, medicine, veterinary medicine, agriculture, genetics, microbiology, forensic science and other areas of study. Microscopical techniques enable us to magnify objects upto 1000 times (also written as 1000x) and thereby observe organisms as small as 0.5 μm. At these magnifications, the microscopes are operating at the limits of their *resolving power* which is governed by the wavelength of light and the quality of their lenses. By using short wavelength, high voltage electron beams, both the magnifying and the resolving powers can be increased many times, e.g., up to 20,000x as in the electron microscopes.

In this unit, you will study about a variety of microscopes – from light to electron microscope. We begin the unit by discussing the commonly used terms and units related to microscopy. Then we go on to the compound microscope, phase contrast, polarizing and fluorescence microscopes, followed by the electron microscope.

Objectives

After studying this unit you should be able to:

- define the common terms and units used in microscopy;
- identify the main components of a compound microscope;
- trace the path of light in a compound microscope;
- describe the basic principles involved and the components of phase contrast, polarizing, and fluorescence microscopes;
- describe the basic principles involved in the electron microscopy.

6.2 RELEVANT TERMS AND UNITS

Before proceeding for the study of various types of microscopes you should be familiar with the meanings of the common terms used in microscopy. They relate to the quality of the image and to the features of the microscope used in obtaining a high quality image. Several of the terms are used in other definitions too, so you will need to work right through all the five terms explained in this section to achieve a clear understanding. In case, you are not familiar with a microscope, we advise you to first observe the Figs 6.2 and 6.3 before beginning your study of this section.

Magnification

Magnification (M) is the theoretical ratio between the apparent size of an object when it is viewed directly (without the aid of an optical system) and the apparent size when it is viewed under a microscope.

$$M = \frac{\text{Size of image}}{\text{Size of object}}$$

Some microscopes, especially the traditional type, have an adjustable tube length. For these instruments, the following formula gives an estimate of total magnification:

$$\text{Magnification} = \frac{\text{Magnification of eyepiece}}{\text{Focal length of objective}} \times \text{Mechanical tube length}$$

Most modern instruments have a fixed tube length of 160 mm. In this case, magnifications are determined by the manufacturer and the total magnification is more easily calculated by:

$$\text{Magnification of eyepiece} \times \text{Magnification of objective}$$

Magnification can therefore be changed by one or more of the following:

1) by rotating the turret to bring a different objective into position,
2) by changing the eyepiece and in some instruments by altering the draw tube,
3) or in some binocular microscopes it may be necessary to change the objective lenses.

A total of eighteen different magnifications can be obtained with many microscopes by having three eyepieces (for example, with magnifications of

7x, 10x and 15x) and six objectives (for example, with magnifications of 3.7x, 8x, 10x, 20x, 40x and 90x oil immersion). These can be conveniently expressed on paper in the form of a grid.

SAQ 1

a) Below we've set out a magnification grid for the range obtainable using a common student microscope. The magnifications of the eyepieces and objectives are shown and we've filled in three of the magnifications obtainable as examples. All you have to do is complete the grid and then compare your grid with the one given at the end of the unit.

Magnification Grid for a Student Microscope.

Eyepiece	Objective					
	3.7x	8x	10x	20x	40x	90x
7x		56				
10x	37.0					
15x				300		

b) Using the microscope which you obtained, just as for the question given above, draw up a magnification grid. Make a neat job of it as the grid will be useful to you in later work. Don't worry if your microscope may have fewer objectives than the six listed above.

Tube Length

Two dimensions are used – one for *optical* tube length and another for *mechanical* tube length. The latter has the more important practical value in instruments with adjustable draw tubes and we will only be considering this dimension.

Mechanical tube length is defined as the distance between the top of the eyepiece lens and the base of the objective (see Fig. 6.6). The majority of modern lenses are designed to give the best results at a tube length of 160 mm, and this length is normally fixed at the time of manufacture in most modern instruments. If the tube length is adjustable, it is normal practice to pre-set it to 160 mm. Then, if greater magnification is required, the eyepiece tube can be withdrawn, or a screw motion is best for this purpose.

Numerical Aperture

Numerical aperture (NA) is a very useful and important value as it is a measure of the resolving power, clarity, definition and brightness of the objective lens. The higher the NA for a given magnification, the better is the lens and the better the image it can produce.

The NA is the ratio of the diameter of the lower objective lens to its focal length. Figure 6.1 shows a cone of light entering an objective lens from a point on a slide.

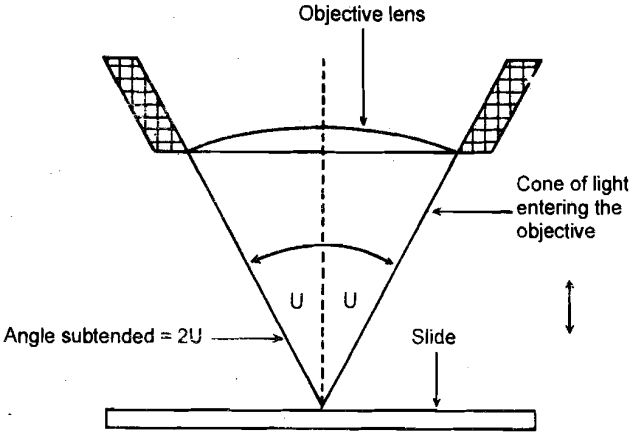


Fig. 6.1: Numerical Aperture.

If the lens had a greater diameter for the same focal length, then obviously more light could enter it giving a brighter image. The ratio is determined at the time of manufacture and is engraved on every lens. Mathematically, this ratio is defined as follows:

$$\text{Numerical aperture} = \text{Refractive index} \times \sin U$$
$$\text{or} \quad NA = n \sin U$$

For air, n = 1 and for immersion oil, n = 1.5

The NA will increase as:

1) The objective lens is made wider, and
2) The objective moves closer to the slide (angle U increases, see Fig. 6.1)

In practical terms it is not usual to use magnification greater than 1000x NA, i.e., there is no point in using an objective with a low NA and high power (20x) eyepiece.

SAQ 2

Look again at the microscope you used in the earlier question. Then in the grid below note the NA of each objective lens and the magnification power of each one. Use this information to answer the next SAQ.

Lens	*NA*	*Magnification*

Briefly describe the relationship between the NA and the magnification power of objective lenses?

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Resolution

In introduction, i.e., Section 6.1 we mentioned the term 'resolving power', this is an indicator of the quality of the optical system of a microscope. It is a measure of the ability of the lens system to produce a high quality image in which the fine detail of the object being magnified is distinctly made visible to the observer.

The resolving power of a microscope is related to the resolution of which its lenses are capable. Resolution (R) is a technical term defined as follows:

$$R = \frac{\lambda}{2n \sin U}$$

where R = resolution in μm,
n = refractive index of the medium between the coverglass and the objective lens (for air, n = 1 for immersion oil, n = 1.5),
U relates to the cone of light entering the lens [see Numerical Aperture (NA) discussed earlier], and
λ = wavelength of light used.

What this rather complicated looking formula indicates is the ability of a lens to discriminate between two small objects on a slide which are close together. Poorer quality lenses, with low NA will tend to show the two small objects as a single entity. A better quality lens of the same magnifying power but with higher NA may be able to reveal that the two objects on the slide are in fact separate.

A cheaper way to improve the resolution of a microscope is to use light of short wavelength. For this reason, a blue or green filter is often used in the light path.

Resolving power, thus depends mainly on two factors:

1) The quality (freedom from aberration) and the numerical apertures of the lenses, and
2) The quality of the illumination.

Working Distance

The working distance is the distance between the object on the slide and the lower side of the objective lens when the microscope is in focus. You will see from Table 6.1 that this value approximates to the focal length of the low power lenses, and at high powers the working distance is much less than the focal length. It is important to use a coverglass of correct thickness. Some high power lenses with high NA have extremely short working distances and very thin cover-glasses must be used. For example, BS (British Standard) coverglass no. 1½ is only 0.16-0.19 mm thick.

Table 6.1: Technical Data for a Typical Microscope's Objectives.

Focal length (mm)	NA	Initial magnification	Working distance (mm)
45	0.80	2.7	57.5
36	0.11	3	37.5
24	0.22	6	12.125
16	0.18	10	16.5
16	0.28	10	5.5
4	0.70	42	0.59
4	0.85	45	0.223
1.5	1.30	105	--

SAQ 4

Identify which of the following three definitions correspond to:

a) Working distance
b) Mechanical tube length

1) **The ratio of the diameter of the lower objective lens to its focal length.**

2) **The distance between the top of the eyepiece lens and the base of the objectives.**

3) **The distance between the object on the slide and the objective lens when the microscope is in focus.**

You should have one statement 'left over'. What does this define?

.....

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SAQ 5

What is meant by resolution? (Tick the correct answer)

1) The variation in focal depth possible with an optical system. ☐

2) The capacity of a lens to discriminate between two points separated by a very small distance. ☐

3) The ability of an optical system to be focused by adjustment of the lens tube. ☐

SAQ 6

How would you define magnification?

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SAQ 7

Why is the NA of a lens so important?

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Now check your answers with the ones given at the end of the unit before continuing. It is important that you have a clear understanding of these definitions. Do read through the relevant portions again if you're still unsure about these terms.

SI UNITS

The most commonly used unit is the *micrometer* (μm) which is one millionth (10^{-6}) of a meter, i.e., one thousandth part (10^{-3}) of a millimeter. The common skin bacterium *Staphylococcus* is about 1 μm in diameter and if stained, it can be seen as a dark spot at 900x; red blood cells of mammals are about 7 μm in diameter; and most animal cells lie in the range 10-15 μm. Plant cells are generally larger and are about 30-80 μm.

High quality optical microscopes and electron microscopes have high resolution and can distinguish among very small objects inside the cells. It may then be appropriate to use the *nanometer* unit (nm) to express their sizes.

$1\ nm = 10^{-9}$ meter, i.e., 1000 nm = 1 μm

Very slender bacterial spirillae may be 0.3 μm, or 300 nm in diameter. Nerve axons or dendrons may also be about this diameter.

SAQ 8

Define a micrometer (μm).

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SAQ 9

A nanometer (nm) is: (tick the correct answer)

1) 10^{-4} meter
2) 10^{-12} meter
3) 10^{-9} meter
4) 10^{-10} meter

6.3 THE COMPOUND MICROSCOPE

A compound microscope consists basically of two parts: the stand, and the optical system.

The stand of a microscope is simply a framework on which the component parts of the optical system are carried. Many different types of stand can be made, but all must meet the following fundamental design criteria:

1) The optical 'units' in use must be held in such a relation to one another that the optical axis passes through the centre of each unit. In other words, there is a strict requirement for alignment.
2) At the same time, there must be a means of accurately adjusting the positions of the units so that they can be focused.
3) The object to be viewed has to be held on some kind of platform or other working area which is maintained at 90° to the optical axis, even though it may be permitted movement in other directions.

The stand of a 'traditional' type of microscope (Fig. 6.2) consists of a base or 'foot' (usually open and of horseshoe shape) to which is hinged a curved

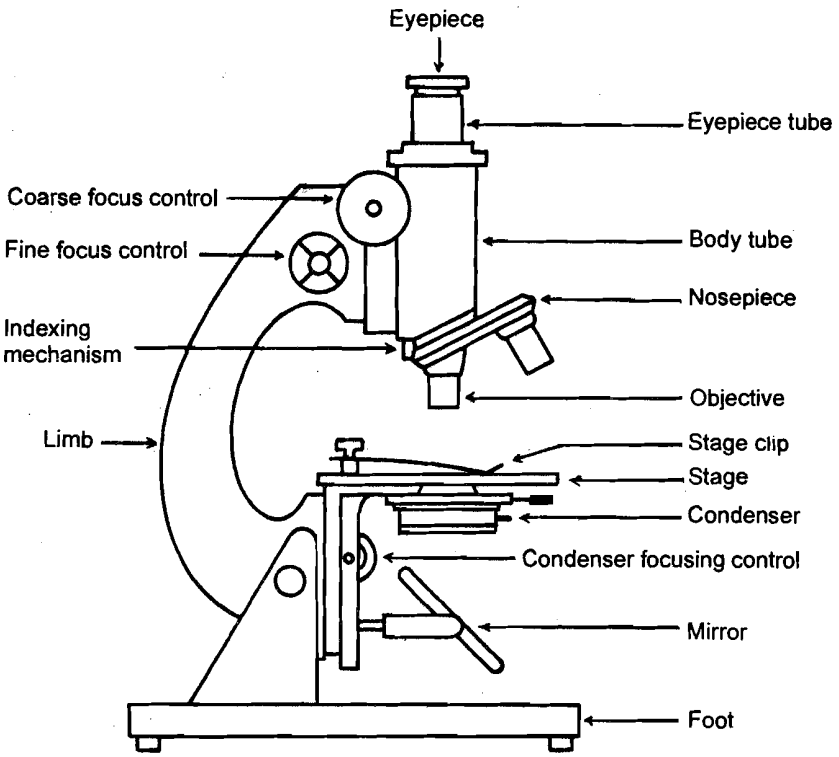

Fig. 6.2: A 'traditional' microscope.

'limb'. The limb carries the optical components and the focusing mechanism. The lower end of the body tube carries the mounting for the objectives, usually a rotating nosepiece; at the upper end is the eyepiece, usually in an upright tube. The base should be heavy, and should have sufficient spread to provide stability. Because of the hinging of the limb and the base, the limb and body tube can be inclined, but this also alters the angle of the stage.

There are focusing adjustments to raise or lower the body in relation to the stage. In simpler instruments, there is only a single, coarse adjustment, but more advanced instruments also have a fine adjustment. Below the stage there is a mounting for a condenser and its supplementary equipment called a substage.

There may also be some mechanism provided for altering the distance between condenser and stage so that an image of the light source can be focused. At the bottom of the limb is mounted a mirror or a built-in illuminator.

In the more 'modern' type of stand (Fig. 6.3) a curved or angled limb is fixed in position relative to the base, so that the stage is permanently horizontal.

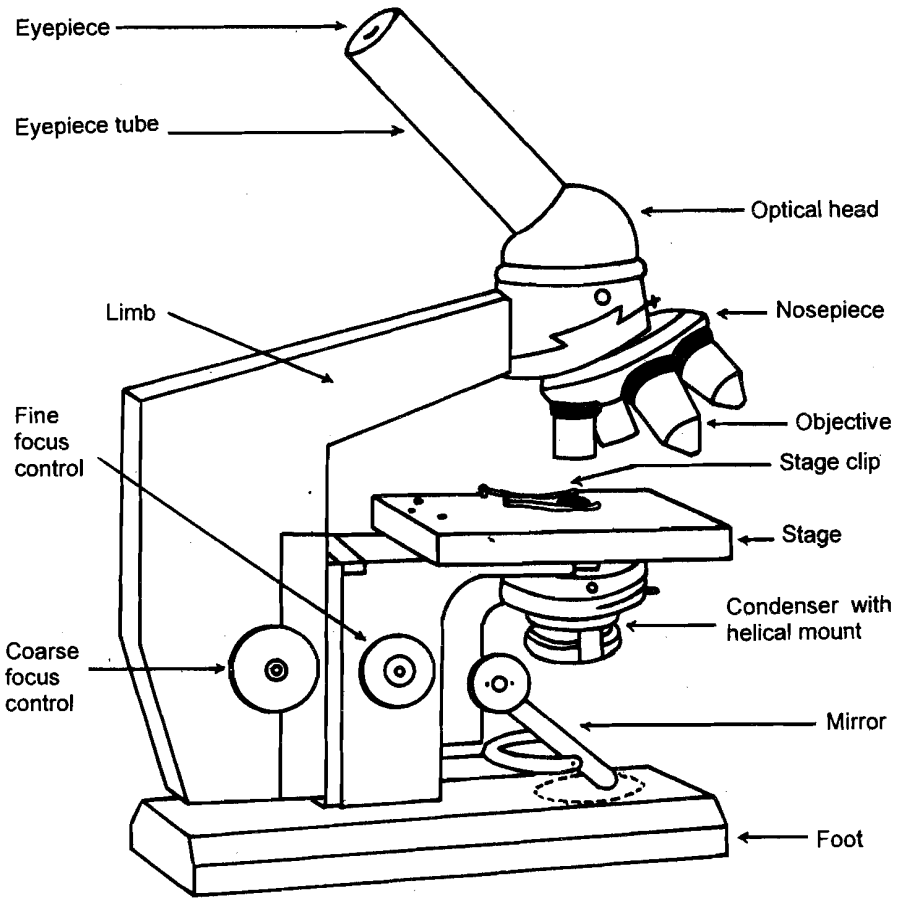

Fig. 6.3: A modern microscope stand with an inclined limb and stage focusing.

The upper part of the body tube is inclined towards the user, the light path being altered by the use of prisms in the optical head. The base of the microscope can be open or closed and may carry a mirror or a built-in illuminator. If there is built-in illumination, it is usually a low voltage type, so the base may also contain a suitable transformer.

In this type of microscope, focusing is usually achieved by moving the stage relative to the limb. This has an advantage that the controls are low down and are more convenient to handle than those on a traditional stand, where they are usually mounted high on the limb. This distinction between the two types of stand is not completely clear-cut – some instruments with open bases and upright heads employ stage focusing.

The advantages and disadvantages of these two main types of stands are fairly obvious. Most observers find it more comfortable to view with the body tube at an angle to the vertical. This can be accomplished on the traditional upright type of stand only by angling the stage. Many might feel that this is a considerable disadvantage when working with fresh preparations; however, this should not be the case as long as the angle of inclination is not too great. With angled head instruments the viewing height is fixed, so the smaller models may be uncomfortable for tall users.

There is little doubt that the more traditional type of instrument is usually easier to maintain and to service. For the do-it-yourself enthusiast it is also more versatile, in that simple microprojection and photo-micrography are easier to arrange with an instrument that has an open base and upright end.

In addition to the two main types of stand, there are specialised variants. For example, there is the inverted microscope which has the light source and specimen mounted above the objective. Another variant is the compact instrument. There are also stereomicroscopes in which pairs of objectives and eyepieces are used to produce a 3D effect and give 'depth' to the image.

ACTIVITY 1

With the permission of your supervisor/counsellor, obtain a microscope from your workplace/laboratory and with the microscope besides you, read the preceding text again. This time, as you read through the text, use your microscope to identify the parts shown in Figures 6.2 and 6.3 and the functions of the controls. If you can obtain a stereo-microscope, you will find it worthwhile examining its construction and controls too. Don't put the ordinary microscope away yet. You'll need it again during your study of this unit.

Don't worry if a microscope is not available at your workplace. You will be handling a microscope in your practical session.

SAQ 10

Write down two disadvantages of the 'traditional' microscope with a vertical tube.

i) .....

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ii) .....

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SAQ 11

The 'modern' microscope with inclined tube has some disadvantages. Write down two of them.

i) .....

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ii) .....

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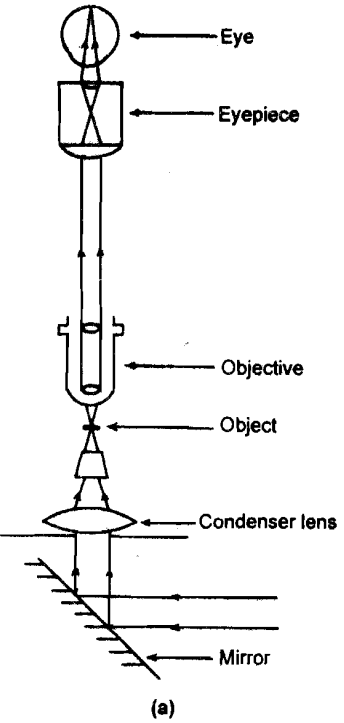
ACTIVITY 2

Briefly describe about the microscope components numbered as 1, 2, 7, 8/9/10, 18 and 21 in Fig. 6.4. Your description should include each component's name and its function. The entire description should not be more than 100 words in length.

Component	Function
(1)	
(2)	
(7)	
(8/9/10)	
(18)	
(21)	



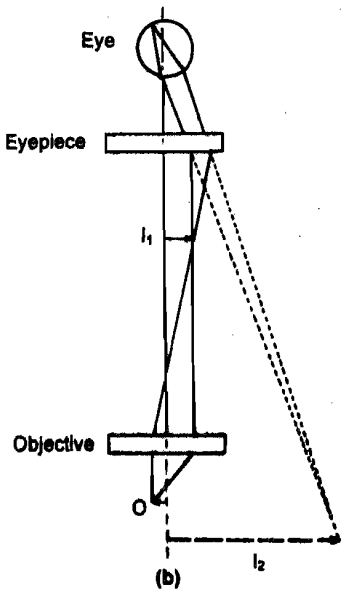


Fig. 6.5: The optical system of compound microscope. a) The light path. b) Image formation. O – object, I_1 – Primary image, I_2 – Final image.

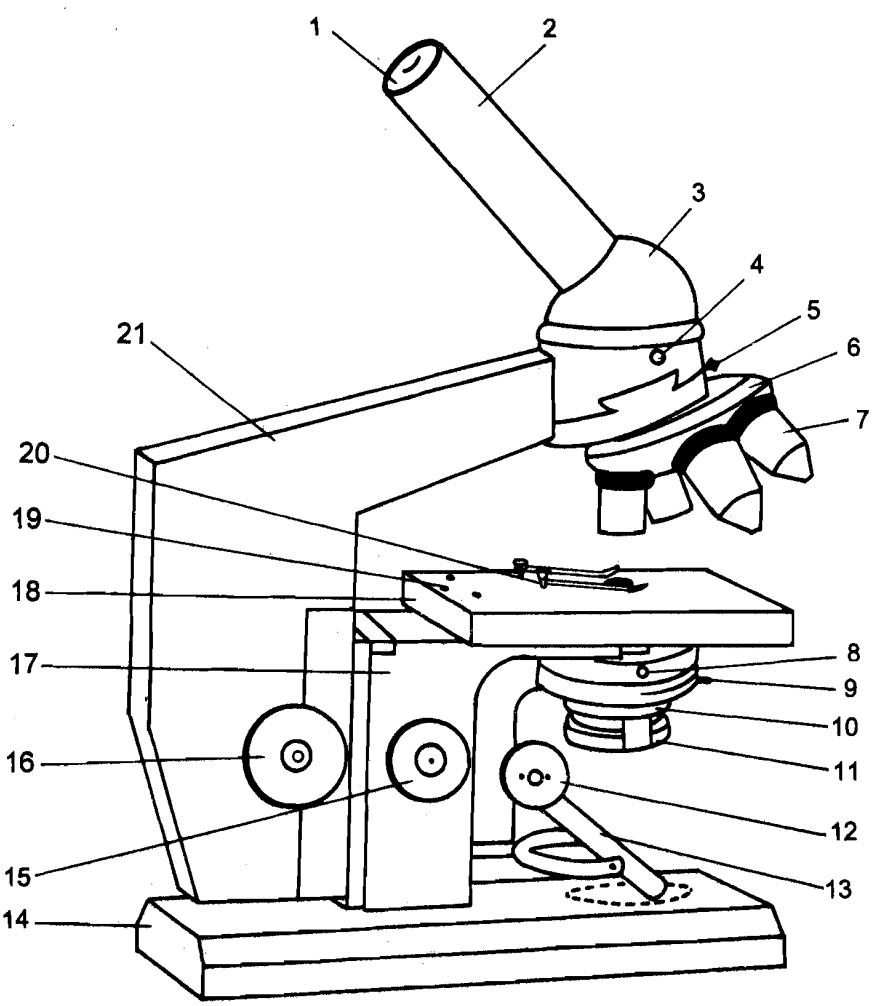


Fig. 6.4: Diagram for Activity 2.

Coming to the compound microscope's optical system the objective lens system forms an enlarged, real, inverted image of the object; this image is further magnified by the eyepiece to form a virtual image (see Fig. 6.5). This magnified virtual image seen through the eyepiece may appear to be anywhere between infinity and the viewer's near point, depending on the focusing of the microscope and the adjustment of the eye.

In order that this system of lenses may be properly illuminated, many microscopes have a third optical unit fitted between the light source and the objective – the condenser lens system.

Now look at Fig. 6.6 which shows the optical path in an actual microscope.

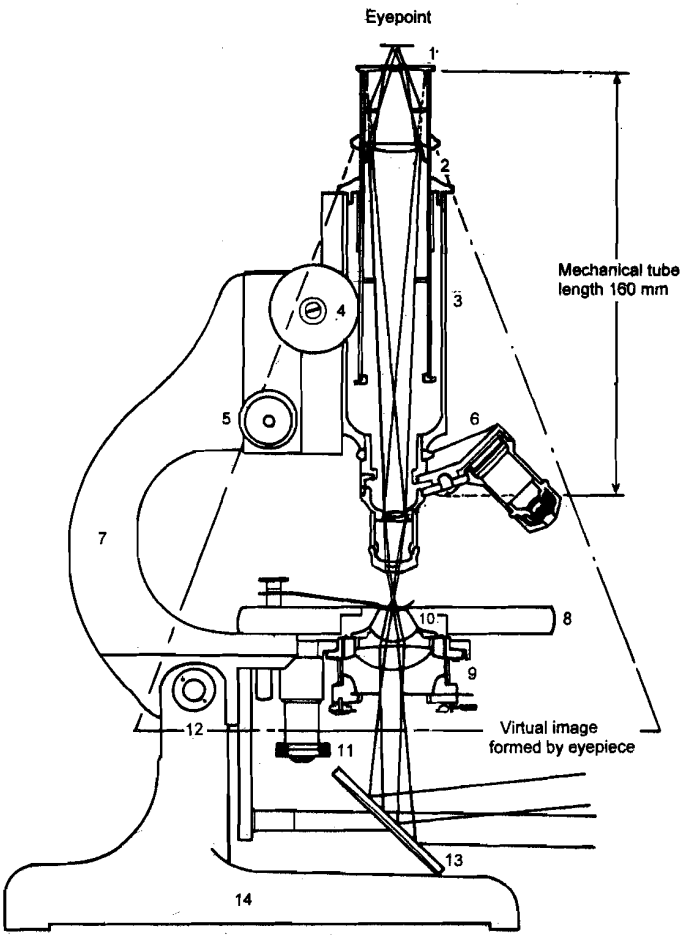


Fig. 6.6: Optical path in a compound microscope (Illustration courtesy: Prior Instruments).

Key:

1. **Eyepiece**
2. **Draw tube**
3. **Body tube**
4. **Coarse focusing adjustment heads**
5. **Fine focusing adjustment heads**
6. **Dustproof revolving nosepiece**
7. **Limb**
8. **Stage**
9. **Substage fitting**
10. **Condenser**
11. **Substage focusing adjustment**
12. **Axis of inclination**
13. **Mirror**
14. **Horseshoe foot**

You can see in Fig. 6.6 that a beam of light from an external light source (or substage illuminator) is focused by the condenser onto the slide on the stage. Light passing through the slide is magnified by the objective lens. The image so formed is then re-magnified by the eyepiece.

SAQ 12

Which of the following are true for the image formed by the objective lens of a microscope? (Tick the correct answers)

☐ 1) enlarged
☐ 2) diminished
☐ 3) upright
☐ 4) inverted

SAQ 13

The movements of the slide appear reversed when viewed through the eyepiece. Why is it so?

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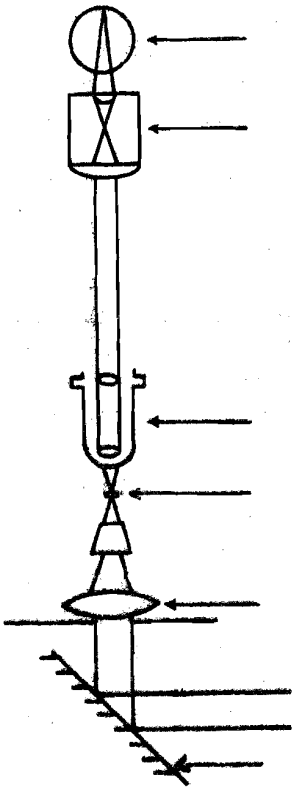
SAQ 14

Look at the following optical diagram of the compound microscope and see if you can name the features indicated. Also insert arrowheads to show the direction of the light beam.



6.4 THE PHASE CONTRAST MICROSCOPE

Phase contrast microscope is a modification of light microscope that enables one to observe living or unstained preparations. This is made possible by the two special devices fitted in the microscope – an annular disc (Fig. 6.7) placed beneath the condenser and an objective fitted with a phase-plate (Fig. 6.8). Microscopes fitted with built-in contrast equipment are also available. The above two devices, convert minute phase changes in transmitted radiations (due to differences in refractive indices of cell organelles) into perceptible shades of grey so that the organelles in the living cells become visible with fair contrast.

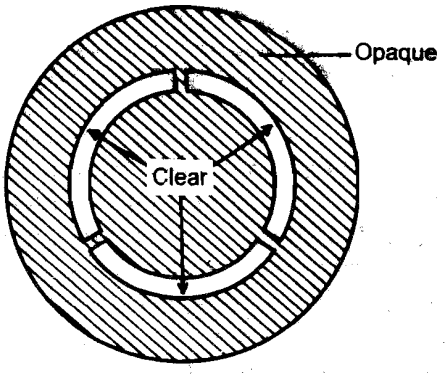


Fig. 6.7: Substage annulus. It is made up of a glass plate which is blacked out except for an annulus which is left clear through which the light rays can pass. Sometimes it is made up of opaque material, and a ring-shaped area cut from it, with three bridges left to prevent the centre from falling out.

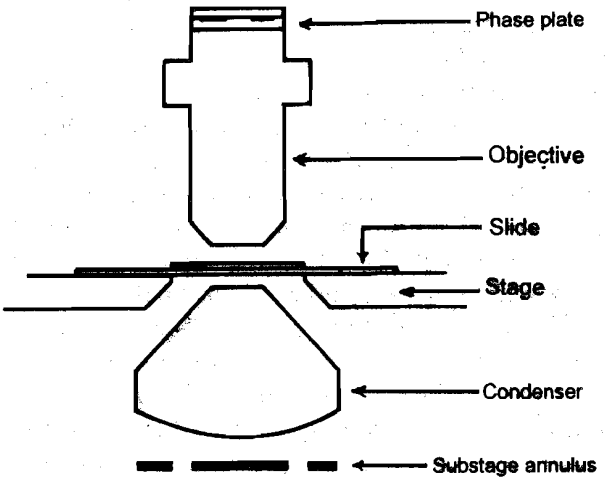


Fig. 6.8: Phase Contrast Components in position.

Advantages and Uses of Phase Contrast

The phase system comes in handy in the study of living material, for which it was originally developed. It is particularly useful when that material is in water, which has a low refractive index. When a drop of pond water is viewed by means of a normal microscope which is properly set up, many objects can be seen, but when the same field is viewed by means of phase contrast, much more can be seen. Separate internal areas of a section, or whole animals or plants which differ only minutely in refractive index are easily differentiated, as are the internal differences of single cells. Phase contrast imparts no colour itself, but often a green filter is used which lends colour to the field and improves contrast. It must be stressed that phase contrast is really only effective with objects which have little or no colour of their own. Stained preparations do not really benefit from this technique. However, it is beneficial for cleared and mounted but unstained, permanent preparations.

Now try the following SAQs.

SAQ 15

What are the key components of phase contrast microscope?

.....

SAQ 16

What is the principal reason for employing the phase contrast system?

.....

.....

6.5 THE POLARIZING MICROSCOPE

Polarization of Light

This microscope involves the principle of polarization of light. Polarized light consists of waves which are oriented in one plane only (see Fig. 6.9). Imagine a beam of light having waves all radiating from the same point in a random way, some horizontal, some upright and some in intermediate positions. If they were polarized they would all be in the same plane. Looking at the ripples on a pond you can see that they are polarized: they all go up and down, and they are all in the same plane. Comparing it to a light source the radiations would not only be horizontal in the way ripples on a pond are, but are vertical and at all angles from the vertical to the horizontal. In Fig. 6.9 you can see an assortment of waves which are not polarized, but in several planes.

Light is not normally polarized, simply because it radiates in all directions from a source. So to make it polar it has to be passed through a *polarizing filter*. The filter material is dark, like the lenses of sunglasses, and is generically known as *polaroid*. It only allows light of one orientation to pass between them. All other orientations of light are absorbed by the polaroid.

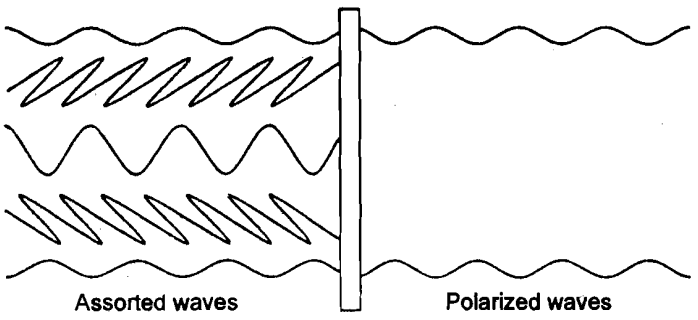


Fig. 6.9: Diagram showing how a polarizing filter works.

If you take two sheets of polaroid and, holding one still, you rotate the other in front of it, you will see the light which is coming through the polaroids dim and brighten at certain positions. When the light is at its brightest, the polaroids are in the *same* orientation. When it is at its dimmest the polaroids are crossed. With good polaroids it is possible to extinguish the light from a fairly powerful tungsten source by holding them in the crossed position. Now, if between these two polars set in the crossed position, a crystal that has the capacity to rotate light is placed, this turned light can pass through the second polar. Changed

position of the crystal can rotate more light. The more the light is rotated, the more of it can pass through the second polaroid and therefore the brighter that part of the image appears. A range of colours proportional to the rotation of the light by the specimen is exhibited through the second polaroid. Materials like crystals that rotate polarized light are known as *anisotropic* and the ones that don't rotate polarized light are known as *isotropic*.

Further, in the example in Fig. 6.10, a coloured image of the specimen could be seen through the second polaroid. If you now rotated the second polaroid slowly, the coloured image could be extinguished. This is because the second polarizer is crossed with the new orientation of the light. In consequence, the area surrounding the specimen becomes lighter due to the partial uncrossing of the polaroids. The specimen now appears as a dark object on a lighter ground.

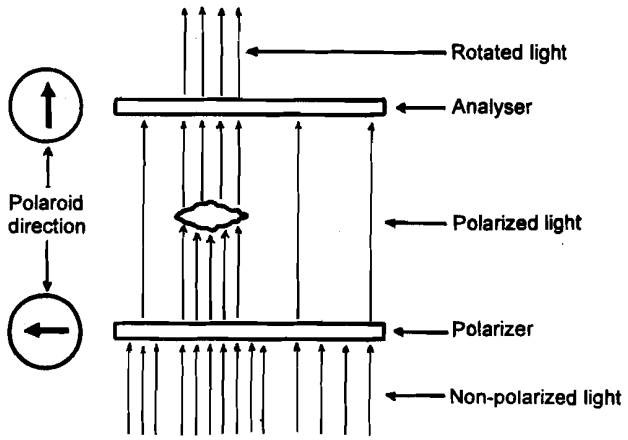


Fig. 6.10: Crossed Polarizers and an Object.

When such an instrument is used in this way, the fixed polaroid is known as the *polarizer*, and the movable one as the *analyser*. These are shown in Figs. 6.10 and 6.11. The movable polaroid may be fitted with a vernier, or protractor with which the change in rotation of the specimen may be noted.

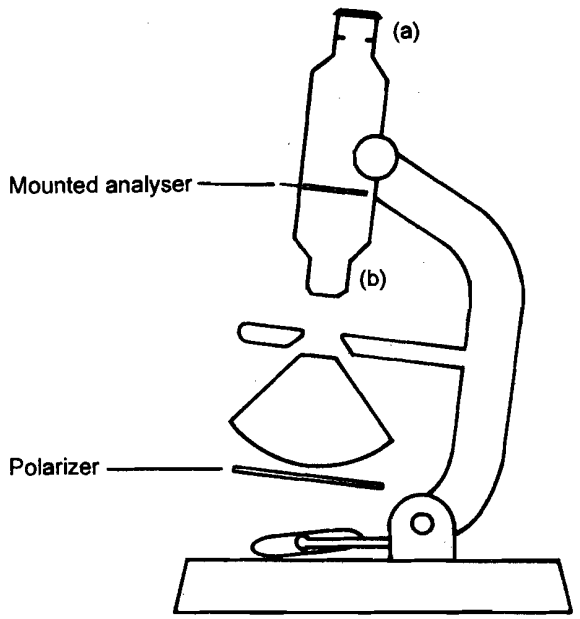


Fig. 6.11: A Polarizing microscope.

Any microscope may be used as a polarizing instrument simply by the addition of two pieces of polaroids (see Fig. 6.11). One is introduced beneath the substage condenser and the other is placed above the specimen stage either at the bottom of the body tube or in the field stop of the eyepiece. One of the two pieces is rotated until the field is at its darkest and the object in question is placed upon the specimen stage. Low powers are generally sufficient since polarizing objects are often quite large.

The polarizing microscope is used to distinguish the isotropic substance from anisotropic ones. As described above, this method is also used for studying the details of anisotropic materials, by inducing varied colours in them, without actually dyeing or staining them.

ACTIVITY 3

View a slide containing some crystals, and read these notes. You can take a slide of leaf of *Ficus elastica* (Indian rubber plant)* containing crystalline masses known as cystoliths in its leaf. View these crystals at a magnification of x400. Various colour bands may be seen in the crystals due to the different angles of the facets presented to the polarized light giving the various interference colours. You can see that the lignified (xylem) tissue of the section is also shown up by the polarizing conditions. This material simply rotates the light, giving a bright image.

Now try the following SAQs.

SAQ 17

What is special about polarized light?

.....

.....

SAQ 18

Where are polaroids placed in a microscope to turn it into a polarizing instrument?

.....

SAQ 19

Which of the two polaroids is called the analyser?

.....

SAQ 20

What kind of materials give no reaction to polarized light?

.....

.....

* cystoliths are also found in many members of Acanthaceae, Moraceae, Urticaceae, Ulmaceae, and Cannabinaceae.

What distinguishes an isotropic substance from an anisotropic one?

.....

.....

6.6 THE FLUORESCENCE MICROSCOPE

Before we begin with a discussion on the fluorescence microscope, let us briefly understand what is meant by **fluorescence**. This would enable you to understand better how this microcope works.

Fluorescent substances, when irradiated with short waves, i.e., high energy electromagnetic waves of near ultraviolet and ultraviolet (UV) light, emit further electromagnetic radiations which are within the visible spectrum of light. Thus they seem to 'glow in the dark' under ultraviolet light, and to light up under other high energy illuminations (see Fig. 6.12). This property is known as **autofluorescence**, or **primary fluorescence**. Non-fluorescent substances produce no such reaction and remain indifferent to ultraviolet or other high energy radiations. If a non-fluorescent substance can be impregnated with a material which exhibits primary fluorescence, it can be made to fluoresce artificially. This phenomenon is known as **secondary fluorescence**. The fluorescent material which is used to produce the secondary fluorescence is then known as a **fluorochrome**. These properties are used in microscopy both to differentiate one substance from others within a given specimen, e.g., chromosomes within a dividing cell, and to make whole objects more visible, e.g., bacteria.

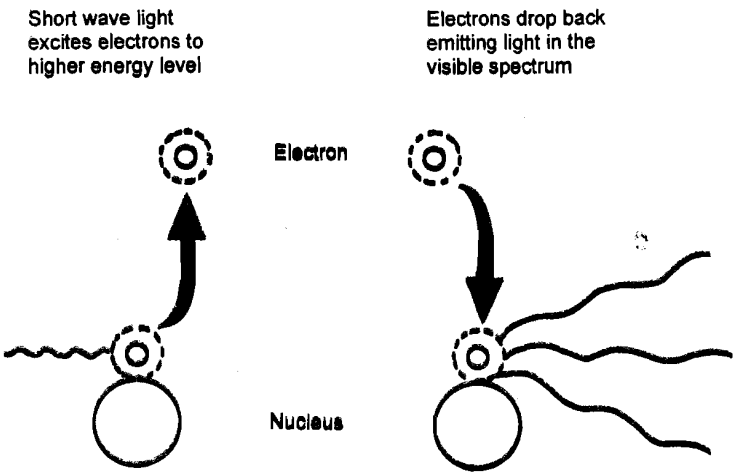


Fig. 6.12: Diagrammatic representation of the Fluorescence Effect.

Ultraviolet light (UV), the primary requirement for fluorescence microscopy, is a high energy radiation and may be produced from many sources. Mostly, a high pressure mercury vapour lamp is used as UV source for microscopy and can be tailored to give the wavelengths required in ultraviolet and near ultraviolet light by careful design and by the use of suitable filters in the emission output.

In practical microscopes, the UV has to be transmitted by lenses to the specimen, and glass is notorious for the absorption of ultraviolet. Therefore,

the lenses used to transmit the UV are not made of simple glass but of fluorspar, a non-absorber of UV, or more likely nowadays of a specially formulated glass which firstly does not absorb UV, and secondly just as importantly, does not itself fluoresce.

Though it is perfectly possible to do so fluorescent specimens are not often viewed in brightfield conditions but usually in darkground conditions, since it is the secondary emissions of the specimen itself which are being studied and these might get subdued by the visible light.

UV sources emit light over the whole visible spectrum and into the infrared region so filters must be used to eliminate the unwanted parts of the emission. Having done its work of exciting the specimen, any excess or escaping UV itself becomes an unwanted emission, largely because of the damage it can inflict upon the eyes of the microscopist! (UV destroys the visual pigment of the retina causing temporary blindness and in severe cases, permanent blindness). Despite the fact that glass absorbs UV quite well, it does not necessarily absorb all the UV, and also some glasses are autofluorescent. For these reasons absorbing filters are placed in the microscope in order to remove unwanted UV.

The fluorescence effect is not confined to UV but is associated with short wavelength high energy light, i.e., light from the blue end of the spectrum as well as UV (which is just off the end of the spectrum). Excitation may be by violet, blue or green light as well as UV. The fluorescence light from the specimen will always be of a longer wavelength (and therefore of lower energy) than that of the exciting light, since some energy is lost in the transformation, i.e., in the change from one wavelength to another by the fluorescence phenomenon. Thus UV, violet or blue exciting light might give green fluorescence, but green exciting light cannot, though it might give any lower colour, e.g., yellow or orange. UV, of course, may give any lower colour of fluorescence.

Exciting light filters (also known as source filters) will allow either UV, violet, blue or green light from the light source into the microscope. Since the fluorescence from the specimen is of a longer wavelength and of a lower energy, the exciting light must be filtered out so that the fluorescent image is not swamped. To this end, the excitation suppression filters are exactly matched to absorb the exciting light without suppressing any of the fluorescence light, i.e., they are opaque to exciting light and transparent to all other lower energy light.

Fluorescence microscopes often have a built-in UV suppression filter between the objective and the eyepiece. This is to ensure that in the event of careless setting up UV will never be transmitted to the observer. However, you should NEVER RELY ON THIS.

Now try the following SAQs.

SAQ 22

What is a fluorochrome?

.....

.....

SAQ 23

How does the phenomenon of fluorescence work?

.....

.....

SAQ 24

Why are there suppression filters between the objective of the microscope and the eyepiece?

.....

.....

SAQ 25

Why are fluorite optics necessary in fluorescence microscopy?

.....

.....

6.7 THE TRANSMISSION ELECTRON MICROSCOPE

From this section we move on to the Electron Microscopy. You might have heard of Electron microscope, which is the same as the Transmission Electron Microscope about which you will study in this section.

It is a large instrument, consisting of a desk-sized console and a column, which is the microscope proper, that may be from about five to eight feet tall.

There are certain analogies between the TEM and the transmission light microscope (the one you have studied earlier). Both instruments rely on a source of energy which is focused on and through a thin prepared specimen, and upon the formation of a magnified image by an objective system which is further magnified by a viewing arrangement.

Despite these superficial similarities the two instruments are actually quite different. The reason for the development and success of the electron microscope is that it can give greater magnification because it uses a beam of high energy electrons instead of a beam of light. Taking the centre of the spectral range of human light perception (about 500 nm wavelength) the theoretical ultimate lateral resolution of the light microscope is about 200 nm. At magnifications greater than 1200 times, no more detail is resolved. However, using an electron beam at about 60 kV, giving a wavelength of around 0.005 nm, the theoretical resolution is approximately 0.5 nm, giving a usable magnification of the order of 1000000 times, (1000 nm = 1.0 μm).

The electron beam is produced by a device known as an electron gun apparatus, which is housed at the top of the column of the electron microscope (see Fig. 6.13). If a wire is heated electrically until it glows, it will 'boil off' electrons, which will form a cloud around it. If those electrons are then

subjected to an electrostatic or electromagnetic field they will move through space in the direction that the polarity of the field dictates, according to the normal rules for current and fields. Looking at Fig. 6.13, you can see the filament, f. The filament is actually of this shape, and the electrons are encouraged from the apex of the triangle it forms.

The anode plate with its central hole is maintained at some kilovolts positive to the filament, thus forming a considerable electrostatic field. This accelerates the negatively charged electrons towards the plate and through its central aperture. The action of the accelerating anode is moderated by the Wehnelt grid which is negatively biased with respect to the filament. The Wehnelt grid acts as an electrostatic lens which ensures that electrons are accelerated from only a small part of the filament and that they cross over before they reach the anode plate.

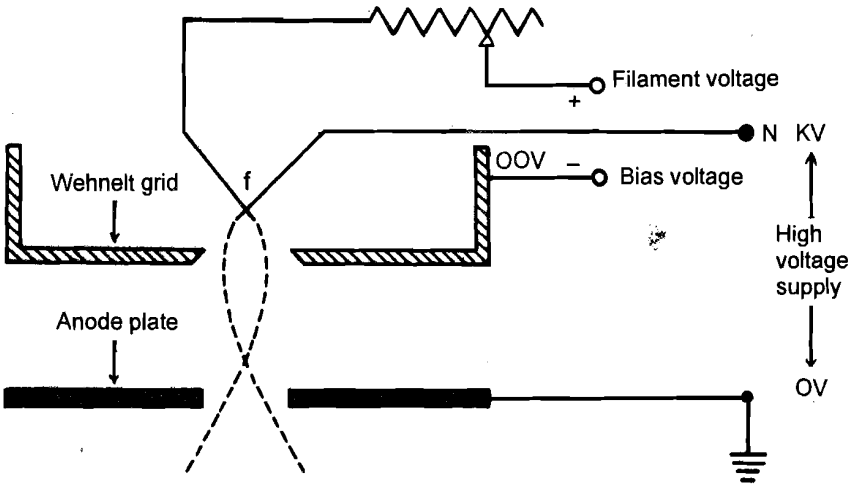


Fig. 6.13: The Electron Beam Gun.

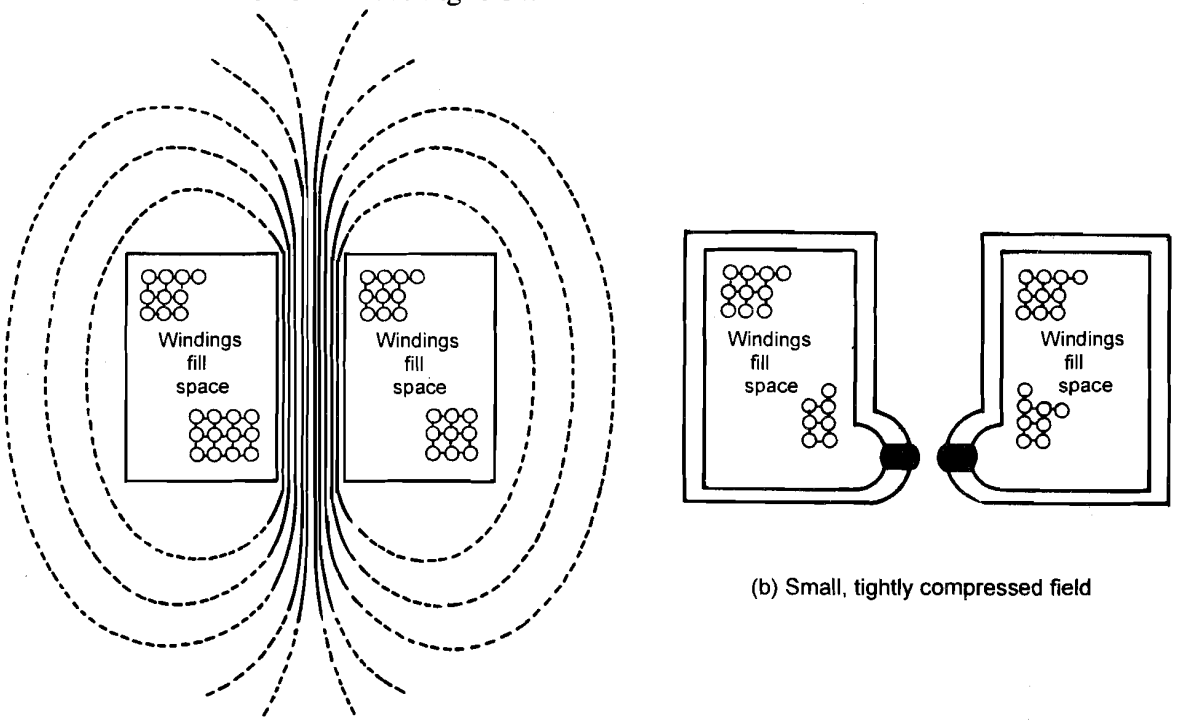
Changing the voltage applied to the filament regulates the temperature at which it operates and hence the size of the cloud of electrons available for acceleration. Changing the bias regulates the area or length of filament from which the electrons will be drawn. Control of these two parameters thus regulates the intensity of the beam. Changing the accelerating voltage, the high tension regulates the energy of the electrons, determining their penetrating ability.

The entire gun assembly acts rather like a triode valve and because of the crossover effect, the electron beam appears to come from what approximates to a point source known in electron microscopical terms as a 'virtual source'.

The electron beam cannot be focused with glass lenses but is brought to focus using electromagnetic lenses. These are, in fact, coils which are wound around the axis of the microscope and are designed in such a way that the action of their magnetic fields on the electron beam mimics that of glass lenses on a light beam. Since a beam of electrons travelling in space is a current, it is governed by the same motor laws as if it were travelling in a wire.

In a modern electron microscope there are up to about seven lenses. These should not be thought of only as simple coils (e.g., of the kind perhaps used as electromagnets for picking things up, or the cylindrical inductors used in school physics experiments) for they are provided with specially designed pole

pieces which direct the magnetic flux in such a way that the coils act like thin lenses on the electron beam. See Fig. 6.14.



(a) Large, relatively diffuse field

(b) Small, tightly compressed field

Fig. 6.14: (a) Unmodified coil; (b) coil with pole pieces.

These electromagnetic lenses are subject to the same kinds of aberrations as are their glass counterparts, exhibiting chromatic, spherical and astigmatic defects. In glass lenses, defects can be counteracted by the use of lenses made of two different glasses. This cannot be done for magnetic lenses and so only the very centre of effectively thin magnetic lenses can be used.

Since it is very difficult to design the 'perfect' magnetic lens, the microscope has a few main lenses, and several auxiliary lenses to recondition the beam that is, to focus it. For example, there exist 'stigmator' coils which are quadrupole or octopole magnets arranged to counteract the tendencies of the beam to become asymmetrical. These are not major beam-forming magnets, but relatively small corrective magnets which can be arranged to produce an asymmetrical field in opposition to the field error by strengthening the fields of some poles and weakening the fields of others.

There are also such things as 'wobbler coils' used to deflect the beam from side to side for setting up purposes. The two images formed are brought together by adjustments like a split image viewfinder. Figure 6.15 on the next page shows the main magnets placed in the microscope as well as some of the auxiliary devices.

As you can see from Fig. 6.15, the magnetic lenses are named in a fairly similar way to those in a light projection microscope.

The electron beam is produced by the electron gun and focused upon the specimen by the condensers. The specimen is magnified by the action of the objective and the intermediate lenses, and the magnified image is conditioned and brought to a focus on a phosphor screen by the projector lens. The electron

bombardment of the phosphor screen causes it to glow where the electrons hit, much as the image is formed on a television screen. Often a further ten times magnification is provided by means of a binocular viewer of the light image.

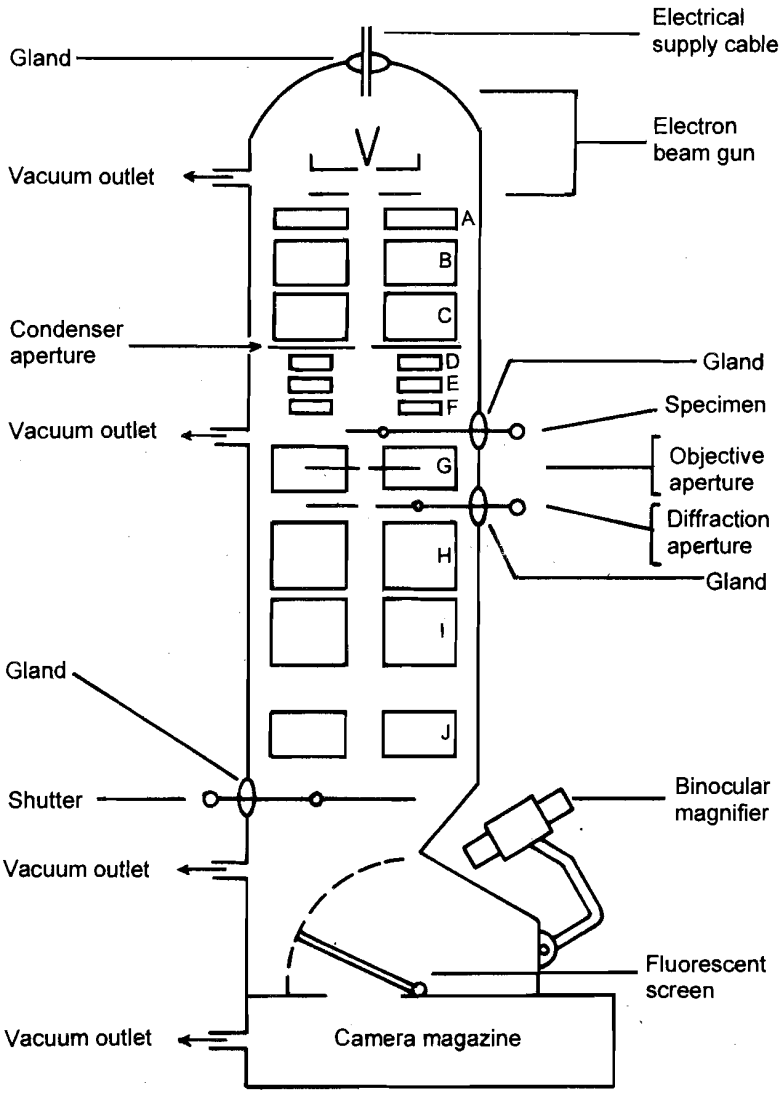


Fig. 6.15: Diagram of a Transmission Electron Microscope.

Key – Abbreviations used:

A – Gun Deflector Coil
B – Condenser Lens 1
C – Condenser Lens 2
D – Stigmator (Condenser)
E – Alignment Coil
F – Stigmator (Objective)
G – Objective Lens
H – Intermediate Lens 1
I – Intermediate Lens 2
J – Projector Lens

Now try the following SAQs

SAQ 26

What is the purpose of the electron gun?

.....

.....

.....

SAQ 27

Why is the crossover point important in the electron gun?

.....

.....

.....

SAQ 28

Why is it important that the electron beam should come from a point source?

.....

.....

.....

.....

SAQ 29

How are spherical and chromatic aberrations dealt with in the electron microscope?

.....

.....

.....

SAQ 30

How is astigmatism dealt with in the electron microscope?

.....

.....

Image Formation

In Fig. 6.15, you can see the position of the specimen in relation to the lenses of the microscope. You can also see a series of apertures which are crucial to image formation. The specimen is generally a very small and very thin section of material, mounted on a grid which allows electrons to pass through the apertures of its meshes.

Electrons encountering the specimen may pass through undeviated. However, they may either be elastically deflected losing no energy or they may be elastically deflected losing energy and causing the emission of secondary electrons or X-rays. There is clearly a potential here for damage to the specimen by the electron beam. If the electrons are simply focused they will not form an image, at least not a very useful one: it will have very little contrast because all the deflected electrons will be brought to a focus with all the undeflected ones.

If this was allowed to happen, we would just see a bright area on the phosphor screen with perhaps a few random looking, ill-defined, slightly darker areas. In

order to separate the deflected from the undeflected electrons, the objective lens aperture is introduced into the back focal plane of the objective (see Fig. 6.15). This aperture stops all those electrons which have been deflected by more than about half a degree.

Now try the following SAQs.

SAQ 31

Discuss how the deflected electrons are stopped by the aperture to form the image.

.....

.....

.....

SAQ 32

From your answer to SAQ 31 explain what effect this will have on the image?

.....

.....

The objective aperture is variable in diameter so that differing contrast effects may be selected for different subjects.

Electron opacity of the specimen is usually enhanced by treatment of the section with heavy metal salts.

By the time the first image is formed, slightly beyond the back focal plane of the objective, it has been magnified about fifty times. The diffraction selection aperture (see Fig. 6.14) is situated at the back focal plane. This aperture can be moved about in the image plane to allow specific portions to be selected for further magnification.

SAQ 33

Why do you think the area selection is done at this stage and not at the specimen itself?

.....

.....

.....

The intermediate lenses and the projection lenses are responsible for the further magnification of the image before it is focused on the phosphor screen. The magnification in these last stages is varied by using two of the lenses with the third switched off, or by using all three lenses together, and/or by varying the current flowing through the coils and thereby altering the characteristics of the lenses. The current is usually applied to the coils by a control moved in a series of click stops marked in magnification. Each increase in magnification brings about a reversal of the image.

Theoretically this is all very well, but electrons can only travel a few millimetres in air before being stopped by the gas molecules. Because of this the whole electron path must be evacuated to a very low pressure before the apparatus can work at all. Therefore the specimen also must be able to withstand very low pressures (see vacuum outlets, Fig. 6.15).

This implies that the whole structure of the column must be airtight and that pumps will be needed to realize and maintain the low pressure. In fact, the pumping arrangements are a large part of the electron microscope system, and generally consist of a heavy duty rotary vane pump backing a diffusion pump to attain the low pressures required. The diffusion pump is sometimes replaced by an ion pump, which is a cleaner device but which still needs the backing of the rotary vane pump.

One of the problems concerning with electron microscopy is that of contamination within the column. This contamination is due to the presence of extraneous molecules such as gas particles which interfere with the electron beam. In the reduced atmosphere of the column, old molecules which have been introduced with the specimen, or possibly cracked from it by the electron beam, tend to drift about and generally get in the way. Because of this, the area enclosing the specimen is set about with surfaces which are cooled by liquid nitrogen, on which renegade molecules tend to condense, losing their energy as they do so. This system is known as *cryogenic pumping*.

To further reduce contamination, careful handling of all introduced components must be practised as well as dry nitrogen purging.

Now try the following SAQ.

SAQ 34

It would be very time consuming to have to pump out the whole column each time a new specimen was placed into the microscope, or each time the specimen was re-orientated. How might this long procedure be avoided?

.....

.....

.....

.....

Looking back at Fig. 6.15, you will notice that there is a camera device at the foot of the column. To use it, the phosphor screen is hinged out of the electron beam to expose the camera, and so direct photography of the image at the same magnification as seen on the screen is possible.

SAQ 35

How is contrast achieved when the specimen is essentially of negligible contrast?

.....

.....

SAQ 36

What is the diffraction selector aperture used for?

.....

.....

6.8 SUMMARY

In this unit you have studied about several aspects of microscopy. Let us recall the salient points:

- Some of the properties of lenses like resolution and numerical aperture are also expressed as:
  Numerical Aperture (NA)

  $$NA = \text{Refractive index} \times \sin U$$

  Resolution (R)

  $$R = \frac{\lambda}{2n \sin U}$$
- Magnification and the tube length are important attributes of a compound microscope. The former is represented as:

  $$M = \frac{\text{Size of image}}{\text{Size of object}}$$

  M = Magnification of eyepiece x Magnification of objective.
  The tube length, that is normally 160 mm, is fixed at the time of manufacture of the microscope.
- A compound microscope is a very common instrument in biolabs. It consists mainly of a stand and the optical system. The latter is composed of series of optical units that are aligned such that the optical axis passes through the centre of each unit. The objective lens system of the microscope forms an enlarged, real inverted image of the object. This image is further magnified by the eyepiece to form a virtual image.
- In phase contrast microscopy the minute differences in the phase changes in the transmitted light are converted into perceptible light, in various shades of grey. Its key components are the annular substage disc placed below the condenser and an objective phase plate.
- Polarising microscope involves the principle of polarisation of light. The anisotropic materials have the ability to rotate light, which can be detected when placed between crossed polaroids.
- In fluorescence microscopy the energy medium (type of light) is ultraviolet or near ultraviolet light, which may make some parts of the specimen fluoresce and either render them more visible, or render them visible in a different way. In addition, parts of the specimen may be induced to fluoresce artificially by tagging them with fluorochromes. Fluorochromes may give off more than one colour of visible light, depending upon the energy of the light which excites them.

- In the transmission electron microscope, electrons are formed into a beam which is directed down an evacuated column by the electron beam gun. The electron beam is focused on and through the specimen by the condenser lens, and contrast is achieved by the objective aperture in the back focal plane of the objective. The beam is focused by the two intermediate lenses and the projector lens on the phosphor screen, where it is turned into a light image. The phosphor screen produces light (photons) in response to bombardment by electrons. The diffraction selection area aperture is used to choose the portion of the image which is to be viewed. Tendencies of the electron beam to be asymmetrical are corrected by the use of stigmator coils, and aberrations of the lenses are obviated by using only the centre portions of well-designed thin magnetic lenses. Magnification changes are achieved by varying the current in the last three lenses and each click stop of magnification reverses the image.

6.9 TERMINAL QUESTIONS

1) What is meant by the term 'resolution'?

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2) What is the magnification of a microscope fitted with a 5x objective and a 6x eyepiece?

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3) Compare and contrast the operating principle of the compound microscope with that of the transmission electron microscope.

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4) Comment on the utility of the 'phase contrast system' in viewing the biological specimens.

5) How does the polarizing microscopy differ from fluorescence microscopy? Discuss (i) the principles on which they are based; (ii) the types of images/information provided by them; and, (iii) some examples of the materials that are best studied with their help.

6) What are the major structural differences between a light microscope and an electron microscope?

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6.10 ANSWERS

Self-assessment Questions

1. (a)

Eyepiece	Objective					
	3.7x	8x	10x	20x	40x	90x
7x	25.9	56	70	140	280	630
10x	37.0	80	100	200	400	900
15x	55.5	120	150	300	600	1350

(b) Write your answer based on the specifications of the instrument available to you.

2. Your answer will depend on the specifications of your microscope.
3. The higher the magnifying power the greater the NA. If two lenses have the same magnifying power, the one that is more expensive of the two, is probably because it has a higher NA.
4. a) 3
   b) 2
   Statement – 1, is the definition of numerical aperture (NA).
5. The correct answer is 2).
6. Magnification is defined as the theoretical ratio between the apparent size of an object when it is viewed directly (without the aid of an optical system) and the apparent size when it is viewed under a microscope.
7. The NA of a lens is important because it is a measure of the objective lens's:
   (1) resolving power,
   (2) clarity,
   (3) definition, and
   (4) brightness, or more simply, how much light it allows in.
8. A micrometre is a millionth of a metre, i.e.,
   $1\mu m = 10^{-6}$. (or 10^{-3} mm).

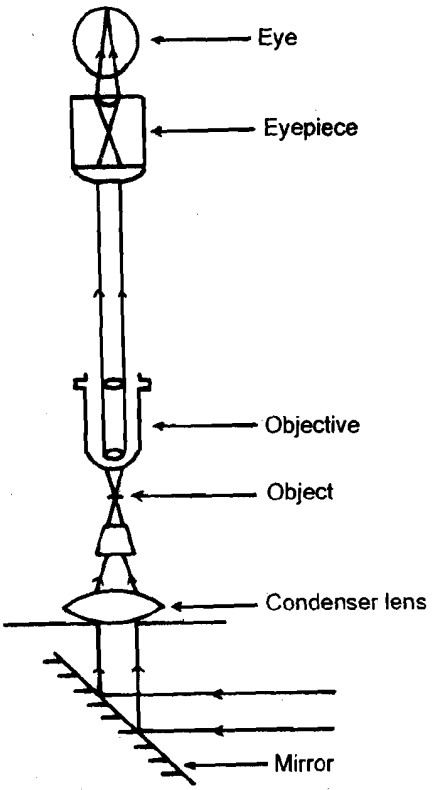
9. 3) If you didn't get this correct and have difficulty identifying the $^{-9}$ index, with the prefix nano think of nine and nano together.
10. Your answer is correct if you've written any two of the following:
    i) The microscope limb cannot be inclined when using wet preparations.
    ii) They are less comfortable to use.
    iii) Focusing is by movement of the limb. This is mechanically less efficient than stage focusing.
11. The 'modern' microscope: (i) may be uncomfortable for tall users; and (ii) is more difficult to service than the simpler 'traditional' type.

Activity 2

Component	Function
(1) Eyepiece	Magnification of objective's image.
(2) Tube	To provide fixed or movable support for optical components.
(7) Objective	Forms enlarged, real, inverted image of object.
(8/9/10) Condenser	Focuses light on object.
(18) Stage	Supports the object and substage optical components.
(21) Limb	Supports all components of the microscope.

Self-assessment Questions (Contd)

12. (1) and (4)
13. Reversal of movements occurs because the image seen is inverted.
14. After labelling the diagram in SAQ 14 it should look like this.



15. The phase plate, and the annular disc.
16. It improves the contrast and visibility of unstained material, in particular living material which can be observed in a near natural state.

17. All the light waves are in the same plane (though not necessarily in the same phase).
18. One beneath the substage and the other above the specimen.
19. Whichever one is capable of being turned. It is usually above the specimen.
20. Isotropic ones.
21. Isotropic substances do not rotate polarised light whereas anisotropic substances can rotate polarized light.
22. A substance with which part of or all of a specimen can be impregnated or dyed and which will fluoresce in a high energy light.
23. High energy light excites the electrons in fluorochromes to a higher than normal energy level. When these electrons drop back to their normal level or energy, the extra energy is emitted as lower wavelength light.
24. To prevent excess high energy light reaching the eyes of the microscopist and possibly causing blindness.
25. Ordinary lenses might absorb too much UV and might also be autofluorescent.
26. It produces a beam of electrons which is the 'light' of the electron microscope.
27. Because it gives rise to the 'virtual source' effect by which the electron beam seems to come from a point source.
28. The smaller the source of illumination, or electrons, the smaller the point to which it can be brought to focus. The image of a filament is likely to be as large as the filament, the image of a point is a point.
29. By the careful design of the lens coils and pole pieces to give the effect of thin lenses and then using only the very centres of those lenses.
30. By the addition of small quadrupole or octopole stigmator magnets which can be made to counter the tendency of the main field to asymmetry.
31. The more electron dense (or electron opaque as it is sometimes called) an area of the specimen is, (and therefore the more likely to deflect electrons through a considerable angle), the less will be its contribution to the final image, since most of the electrons encountering that area will have been deflected and stopped.
32. The more electron dense areas will look much darker than the less electron dense area, effectively increasing the contrast.
33. While the illuminated diameter of the specimen might be about 1 μm, the image at this stage is about 50 μm across and it is therefore easier to select the area that is required.
34. The specimen area, or rather volume, is isolated by shutters from the rest of the instrument so that only a small volume of vacuum is discharged when the stage is opened. Considerable ingenuity is exhibited by manufacturers in their efforts to reduce this volume to the minimum possible.
35. The objective aperture prevents the most deflected electrons from proceeding down the column and contributing to the image, thus the more electron dense parts of the specimen show up as dark areas. Electron opacity may be enhanced by treatment with heavy metal salts.
36. Selecting the area of the specimen which is to be observed.

Terminal Questions

1) The resolution of an optical system is expressed as the distance between two points which are just far enough apart to be seen as two points. If they were any closer together they would be seen as one point only.
2) 30x

3) In answering the first part of the question you will need to dig out the salient facts concerning the operating principles of the two basic systems and write about what they have in common and where they differ markedly. Do not attempt an 'advantages vs disadvantages' table. The instruments are used for purposes too diverse for this.
4) Refer to Section 6.4.
5) Refer to Sections 6.5 and 6.6. Your answer should focus on the three points asked in the questions.
6) See Sections 6.3 & 6.7.

UNIT 7 BASIC TECHNIQUES OF SLIDE PREPARATION

Structure

7.1 INTRODUCTION

In this unit you will be introduced to the terminology used in slide making, and you will also learn about a variety of techniques which will enable you to make slides. Similar techniques are widely and routinely used in biological laboratories in schools, colleges, research institutions as well as in hospital pathology labs to examine tissues and micro-organisms and to look at chromosomes.

In general biology, microscopy can be used to observe cellular processes such as cell division and the growth of embryos. It can also be used to discover and understand the arrangement of tissues and organs within organisms. These studies have helped to understand the relationships between cell differentiation processes and the adaptive link between a cell's structure and the function(s) it performs. It is now realized that the cells of all advanced organisms have the same internal structures or organelles. Larger organelles were studied using optical microscopy and the fine structures and small organelles were studied using electron microscopy. These pieces of evidence on the similarity of structures offer further confirmation of the common origins of all species through evolution.

In good lighting conditions, preferably side lighting against a dark background, the unaided human eye can see objects as small as 0.1 mm (100 μm) diameter, e.g. an amoeba cell or mammalian ovum. Of course, we cannot discriminate any details within these minute objects nor can we observe smaller objects such as the normal cells of plants, animals and micro-organisms. For these reasons microscopical methods are widely used in biology, medicine, veterinary medicine, agriculture, genetics, microbiology, forensic science and other areas.

In Unit 6 you have learnt about the compound microscope and that the most common illuminating system used in microscopy is the bright field method. In

this method the object is illuminated from behind so that light shines through it to the observer's eye(s). The object, therefore appears in colour, if stained, or in silhouette against a bright background. The object to be observed is normally mounted on a thin slip of glass, the slide. Various types are available as you will see if you consult suppliers' catalogues – for example it is possible to obtain slides with a 'frosted' portion on which temporary labels can be written in pencil. Another type you may have encountered is the cavity slide which has a small central depression in which thick specimens or small living organisms can be mounted.

This unit has been designed to introduce you to the care and preparation of microscope slides. It first describes the methods you can use in the care of these important and relatively fragile objects. The blank slides must be properly cleaned before use and section 7.2 examines these techniques. Section 7.3 considers the methods for preparing temporary slides and their care and section 7.4 describes the method for preparing a hand-cut temporary mount.

Objectives

After completing the study of this unit you should be able to:

- understand the importance of the care of slides,
- clean and store slides,
- list the solvents used for cleaning slides,
- state the importance of clear labelling and dust-free storage of prepared slides,
- distinguished between temporary and permanent slide preparations,
- describe some simple techniques for slide preparation.

7.2 CLEANING, CARE AND STORAGE OF SLIDES

Plain glass slides for use in mounting specimens and sections or for making smears must be free of dust and grease, and wettable. If dust is present, it will appear as specks or threads on the slides and interfere with the view of an object. Grease prevents adhesion to the slide of smears or when mounting wax ribbon microtome sections. The slide must be wettable so that smears and squashes spread evenly without coalescing into droplets.

Even most new slides must be cleaned before use, and, of course, those used for making temporary preparations must be cleaned before reuse. We will first describe a schedule for cleaning used slides.

7.2.1 Washing up used slides

(1) Rinse off debris with tap water.
(2) Soak the slides in detergent solution, e.g. or Teepol, at the manufacturers recommended concentration.
(3) Rinse the slides in tap water.
(4) Rinse the slides in distilled water using two complete changes of water.
(5) Wipe the slides dry on a lint-free cloth and store them in a dust-free box. This may be all that is required for general purpose use in a laboratory. For more exacting use, however, it may be necessary to give the slides a solvent wash as follows to ensure that they are completely de-greased.

(6) Soak the slides overnight in 1.2-dimethylbenzene/industrial ethanol (xylene/ methylated spirit) 1/1 solution in a closed jar (appropriately labelled).
(7) Remove the slides and rinse in pure methylated spirit.
(8) Drain and place the slides over a spirit lamp for a fraction of a second so that alcohol is burnt and fungal infection and germs are removed and they dry the slides with a lint-free cloth.
(9) Store the slide – see step (5). The slides must be stored in a dust-free box. Handle them only by their edges to prevent grease contamination from your fingers.
(10) Some lab workers store their slides in 1,2-dimethylbenzene/industrial ethanol (xylene/methylated spirit). See step (6), and perform steps (7) and (8) immediately before use.

CAUTION: During draining and drying, the working area must be well-ventilated and free from sources of ignition. Fumes from this process are toxic and flammable and the liquids are harmful.

7.2.2 Cleaning routine for new slides

These are usually acid clean and may simply need to be polished with a lint-free cloth before use to remove dust. For more exacting work, carry out the procedure described in the previous paragraph, steps (6)-(10). These routines can also be used for cover-glasses (also called coverslips or cover-slides). It is possible to purchase, at a premium, ultraclean slides.

7.2.3 Storage of prepared slides

Prepared permanent slides are valuable. Even mass-produced slides are expensive these days at least from lab suppliers. They are widely used by educational establishments in the teaching of histology (the study of tissues of plants and animals) and cytology (the study of cells). Slides which are individually prepared 'in house' may represent the investment of considerable time and care on the part of the person who made them. In some cases the preparation may be uniquely valuable. It could for example be an important reference slide or a slide of the diseased tissues of a hospital patient.

So the message is clear – take great care of prepared slides. The following is a short list of some important pointers towards good practice.

(1) Freshly prepared permanent slides must be stored flat until the mountant has hardened.
(2) Special thermostatically controlled warming plates are available, but alternatively an incubator may be used.
(3) Once the mountant is hard the slide may be stored flat in a card tray or upright in a slotted box or cabinet drawer.

Now work through the following SAQs. You will find it helpful to refer to suppliers' catalogues. Quote suppliers' references in your answer where appropriate.

SAQ 1

If you had to send five slides through the post to a fellow technician, what sort of container would you use?

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SAQ 2

If you had a small personal collection of forty slides, how would you store them?

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Check your answers with ours at the end of the unit before continuing.

7.2.4 Labelling of Slides

Slides act as a permanent record of tissues, organs and specimens. They may be of pathological origin, e.g. hospital patients, purchased material, prepared 'in house', etc. In all cases it is essential that the slide is properly identified by adequate labelling.

Labels should therefore carry the following information:

(1) The name of the organism – if the whole organism is mounted then the slide can be marked **WM = whole mount** or **E = entire**.
(2) The part of the organism used, e.g. **liver, root**.
(3) The type of preparation, e.g. **smear**; **squash**; **TS = transverse section; VS = vertical section; LS = longitudinal section**.

The following information is desirable but not essential:

(4) Stain(s) used, e.g. **H.E.** = haematoxylin/eosin.

If the slide is prepared 'in house' then it should be:

(5) Dated
(6) Signed.

It is common to use two labels, one on each end. Self-adhesive or gummed slide labels pre-printed with lines are available from lab suppliers – alternatively use ordinary self-adhesive or gummed labels. You will find it easier to write the label before you stick it on the slide. If the label is gummed (rather then self-adhesive) you must not lick it but use a wet sponge.

7.3 PREPARATION OF SLIDES

Slide making is an important part of many areas of biological, medical, veterinary and forensic sciences and you will often be required to prepare different kinds of slides in-house. Specimens may be smears of fluids, thin sections or whole mounts of all or part of an organ or organisms. In all cases the material is mounted on a glass slide prior to its examination.

Two main types of preparation are used:

(1) Temporary, and
(2) Permanent.

Temporary preparations are examined and then discarded, usually on the same day; whereas permanent preparations may remain in good condition for years. The techniques involved in preparation are described here.

7.3.1 Temporary Preparations

These preparations may be needed for a matter of minutes or hours only. They are mounted in water or dilute 1,2,3-propanetriol (glycerol) or other fluid of low volatility. After examination they are discarded.

The material under examination can be fixed and stained or even examined in a living state, for example protozoa such as amoeba. In this case a harmless aqueous stain such as 1% methylene blue can be used – it would then be known as a 'vital stain'.

If it is necessary to keep the slide for a matter of hours it is possible to reduce evaporative losses from the edges of the coverglass by painting a ring of gum or molten wax or nail polish around its edge.

It is always desirable to use a cover-glass over your temporary preparation. If it is omitted, the curvature of the drop of liquid in which the object is mounted causes optical distortions. An additional problem is the danger of contaminating your microscope's objective lenses.

The preparation of stained specimens for microscopical study either temporary or permanent generally involves the following three processes (additional processes are used in making permanent preparations which we are not going to discuss in this couse).

(1) Fixation

For fresh tissues, the main aim of fixation is to kill tissues rapidly by precipitating proteins. This minimizes post-mortem changes. The reagent used is called a fixative, the most commonly used being 70% alcohol. Other common fixatives are Bouin fluid and formalin. Different fixative are used depending on the nature of the tissue whether soft or hard. You will learn about fixatives in Unit 8. Tissues should be washed well after fixation, using the same solvent as the stain, in order to remove all traces of the fixative. If this is not done, tissues may not stain properly and some types of fixative may crystallize out (fixation is not necessary if the material is already preserved).

(2) Staining

The object of staining is to accentuate the distinction between the different components of a tissue or organ. You will learn about fixation and staining techniques in unit 8 of this course.

(3) Mounting

Mounting media employed for temporary preparations include water and 1,2,3-propanetriol (glycerol) 30%-50% aqueous solution.

7.3.2 Permanent Preparations

If the slide is to be kept for long-term reference, for days or even years, it must be made as a permanent preparation. This is achieved by:

(1) Dehydrating the specimen after staining (usually with a graded series of alcohols);
(2) Clearing it – treating it with a solvent that is mutually soluble between alcohol and the mounting medium [traditionally clove oil or 1-2 dimethylbenzene (xylene)]; and
(3) Mounting it under a coverglass in a preservative which dries hard and has a refractive index similar to glass, e.g. Canada balsam or Euparal or DPX mountent which gets dried up easily as opposed to Canada balsam which takes longer time.

The making of a permanent stained preparation mounted in Canada balsam involves five processes:

(1) Fixation,
(2) Staining,
(3) Dehydration,
(4) Clearing, and
(5) Mounting.

Fixation and staining have already been mentioned in the earlier sub-section and the purpose of dehydration, clearing and permanent mounting is outlined below.

(1) Dehydration

The purpose of dehydration, i.e. the removal of water, is to allow complete infiltration of tissues with Canada balsam. Unless all traces of water are removed, infiltration is incomplete, the tissues appear opaque and bacterial decay ultimately sets in.

If carried out too rapidly, dehydration causes distortion and shrinkage, especially of delicate tissues, by setting up violent diffusion currents. It should therefore be done gradually and sufficient time allowed for the complete extraction of water. Dehydration is commonly effected by the passage of the stained specimen or slide through successively stronger solutions of ethanol (ethyl alcohol) ending with immersion in absolute alcohol 100% ethanol (2-changes).

CAUTION: Alcohol is a highly flammable material. Adequate ventilation and no naked flames are essential safely precautions.

(2) Clearing

Where the dehydrating agent is immiscible with the mounting medium, it is necessary to introduce an intermediate fluid that is miscible with both. Such a fluid is known as a clearing agent. The main purpose of clearing is to remove all traces of alcohol, thus allowing the tissues to be infiltrated with the Canada balsam or other mountant. Examples of clearing agents are 1,2-dimethylbenzene (xylene) for small soft tissues; clove oil and cedar-wood oil for thick tissue. Incomplete dehydration is indicated by cloudiness in the clearing agent and the slide should be returned to absolute alcohol. Toluene is another good clearing agent but it is a bit costly.

(3) Mounting

Permanent preparations are obtained by enclosing tissues in solid, resiniferous media such as Canada balsam/DPX. After the tissues have been cleared, they are mounted in a semi-fluid 1,2-dimethylbenzene (xylene) balsam mixture. The 1,2-dimethylbenzene (xylene) subsequently evaporates and the balsam hardens. However, there are some disadvantages of using this mountant. Xylene is inflammable and toxic; the drying process is prolonged and this mountant discolours with time. It is advantageous to use DPX because it dries up easily. To hasten the process of drying up, slides may be kept in oven at lower temperature for sometime.

7.3.3 Use of stains

A great variety of stains are in common use. In all cases the aim is to give different parts of the specimen different colours and colour density and hence increase optical contrast between them. Two different staining methods are in common usage.

(1) *Progressive staining*: This involves leaving a tissue in the stain until nuclei, for example, are deeply stained and cytoplasm is only faintly stained. It is a good plan to dilute the stain beforehand.
(2) *Retrogressive (regressive) staining*: This entails deliberate, over-staining followed by destaining which results in differentiation. Best results are obtained when overstaining is prolonged. This method depends on differential rates of stain extraction. For example, acid alcohol removes haematoxylin (haematoxylin is a nuclear stem that stains only the nucleus) more rapidly from the cytoplasm than the nuclei.

Other important aspects of staining procedures are as follows.

(1) Stains may be used singly or in combination i.e. *counterstaining.*
(2) The use of two stains together is known as *double staining.*
(3) Stains used in combination should be complementary in colour, e.g. light green and safranin (green and red) used in plant tissue staining.

Stains are either general or specific. A general stain, e.g. borax carmine, stains all parts of a tissue usually in different densities or shades of the same colour. A specific stain acts on one or more components of a tissue or organ in a selective manner, e.g. safranin stains lignified plant cell walls red.

SAQ 3

Can you briefly describe the difference between progressive and retrogressive (regressive) staining?

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7.3.4 'Smear'/ 'Squash' Preparations

If you are to see cells clearly, they should be in a monolayer that is, a film one cell thick. If they overlie one another, as in a thick section, you will find that their structure is difficult to see under the microscope. In this subsection we discuss how the 'smear' and 'squash' methods are used on cell suspensions and certain soft tissues respectively. You will learn to prepare these slides in your practical sessions (Experiment – 9).

Cell Suspensions

Cell suspensions such as blood, cheek scrapings, tissue cultures, seminal fluid, throat swabs and cultures of micro-organisms (agar cultures are suspended in a drop of water on the slide) can be examined when spread in a thin layer on a clean slide. The smear is stained after spreading on the slide and for this reason the cells must be fixed, i.e. treated to make them adhere to the slide. Heat fixation is usual for slides of bacteria – the smear is allowed to dry in air and then passed several times high over a Bunsen flame. (It must not become so hot that you cannot rest it on the back of the hand.)

Suspensions of animal cells or plant cells such as pollen or spores are spread thinly onto a clean slide and allowed to dry in air and then fixed with 90% ethanol. The ethanol fixative allows substances to escape from the cells which then glue the cells to the slide. The smear is then stained. (Leishman's blood stain is made up in 100% alcohol; so fixation and staining are performed in one operation.)

If the smear is to be temporary, a drop of 30-50% glycerol and a coverglass are added after staining. If a permanent preparation is required, then the dehydration, clearing and mounting schedule described in Experiment – 9 is performed. Blood smears, when dry, can be examined directly under oil immersion without a coverglass, but a coverglass, with suitable mountant, is usual for permanent preservation.

Apart from bacterial and blood smears it is best to prevent the smear drying out during the preparative process – this reduces shrinkage/distortion effects.

Squash Preparations

Squash preparations are widely used in chromosome studies in which the process of nuclear division by mitosis can be observed in root tips (such as onion and crocus). Grasshopper testis tissue (locust hoppers) and early anthers from flower buds can be used to observe the mechanism of nuclear 'reduction division' by meiosis. Squash slides are usually observed in temporary preparations, tissue being firmly squashed in stain solution under a cover-glass. Such preparations can be made permanent by removing the coverglass after freezing on solid carbon dioxide or by inverting the slide in a bath of alcohol/ethanoic and fixative. The material on the slide and coverglass is then separately dehydrated, cleared and mounted.

7.4.5 Hand-cut sections

You have learnt that squash technique is used to prepare slides of soft tissues which can be spread on a slide. Some tissue like plant stems, roots, cartilage in animals are firm enough to be held in the hand directly or supported in a matrix

while cutting a section. The traditional supporting matrix for young stems and roots and also leaves that are soft is pith. A recent substitute (which you can easily obtain) is expanded polystyrene such as that used in packaging or insulating materials. In use, the plant specimen is sandwiched between two rods of polystyrene (in which grooves may be cut to receive it) or pith which could be strips of pumpkin or banana stem or potato. The support is sectioned along with the specimen and they are then separated by flotation in water or preservative. The aim is to obtain thin slices of the specimen, preferably only one cell thick. Very skilled workers may be able to cut the complete section to the correct thickness. It is usual, however, to cut wedge-shaped slices so that at least some part of the section is of correct thickness (see Fig. 7.1).

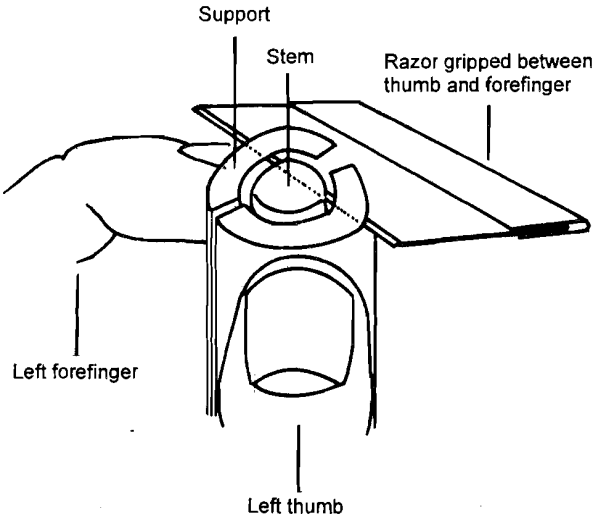

Fig. 7.1: Sectioned wedges.

The single hollowground botanical razor is the ideal instrument for cutting sections. However, it is difficult to sharpen the blade adequately. A satisfactory substitute is a single-edged disposable razor blade. The length and size of the botanical razor makes it easier to use than the small disposable blades. Both types are used with a slicing action in which the blade is pulled sideways whilst being pushed through the tissue (Fig. 7.2).

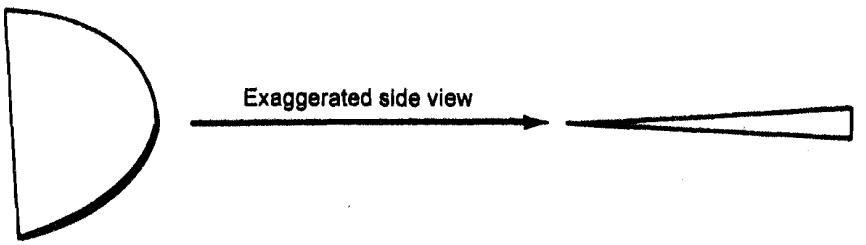

Fig. 7.2: Sectioning a thin soft tissue.

Such sections are then floated on water if the specimen is fresh or in 70% alcohol if the material is preserved. The thinnest sections are selected and stained in aniline sulphate or aniline chloride and mounted in dilute glycerol. If the slide has to be preserved for future use then the sections are stained,

dehydrated, cleared and mounted in canada balsam or any other suitable mountant.

7.5 SUMMARY

In this unit you have learnt about:

- The care and cleaning of slides using different solvents.
- The importance of dust free storage and correct labelling of prepared slides.
- Temporary slide preparation involving simple techniques for fixation, staining and mounting of cell suspensions, soft tissue as well as hard plant tissue,
- Permanent slide preparation involving dehydration, clearing and mounting.
- The use of progressive and retrogressive staining.

7.6 TERMINAL QUESTIONS

1. Complete the table below to compare temporary and permanent slide preparations.

Feature	Temporary	Permanent
(1) Ease and speed of preparation.		
(2) Keeping time.		
(3) Examples of mountant.		

2. A lab has a stock of 2150 slides. How would you suggest that these were stored?

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3. What mountants do you use in your laboratory? List the reasons why they are used.

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4. Under what circumstances would you use:
   1) A squash preparation;
   2) A smear preparation?

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7.6 ANSWERS

Self-assessment Questions

1. Either a small, stout slotted box or a flat pack of folded card as described in equipment catalogues.
2. Preferably in a slotted box or in flat trays.
3. Progressive staining is achieved by soaking the specimen with stain until a suitable colour intensity is reached. Retrogressive staining involves overstaining followed by careful removal of excess stain whilst observing under a microscope.

Terminal Questions

1.

	Feature	Temporary	Permanent
i)	Ease and speed of preparation	Rapid and simple	Time consuming
ii)	Keeping time	minutes to hours	several years
iii)	Examples of mountants	water 30% glycerol	Canada balsam, DPX

2. A metal or wooden lockable cabinet of slotted drawers. These can be designed to be dust-free.

4. 1) Squash preparations are used for soft tissues such as root tips (acid treated), anthers and grasshopper testes.
   2) Smear preparations are suitable for fluid suspensions of cells such as blood, bacteria, and spores.

UNIT 8 FIXATION AND STAINING TECHNIQUES

Structure

8.1 INTRODUCTION

In the previous unit you have already studied about basic techniques of slide preparations. You learnt how slides should be cleaned, cared for, labelled and stored. You also learnt the preparation as well as staining of temporary mounts of plant and animal tissues using squash technique (root-tip) and smear techniques (human cheek cells).

The present unit deals briefly with the steps involved in preparing permanent slides of tissues and whole mounts of plants and animals. This unit however deals mainly with the various chemicals that are used for fixing and staining of animal and plant tissues for both temporary and permanent mounts. So in this unit you will study the theory, nature, preparation as well as storage of fixatives, alcohol series, mordants and stains that are used in the preparation of whole mounts or tissue mounts of plants and animals. You will also learn that some fixatives and stains may be used for both temporary as well as permanent mounts, while some may be used exclusively for either temporary or permanent mounts.

While going through this unit you may wonder why you are being taught about the steps involved in preparing permanent (whole and tissue) mounts of plants and animals as well as the preparation of alcohol series, fixatives and stains that are used exclusively for preparing such permanent slides or mounts since the preparation of such mounts is not a part of this certificate course. The need for you to learn how to prepare these chemicals is that in all probability you will be asked to prepare them for the various practical exercises being conducted by teaching and research faculty.

Objectives

After going through this unit you will be able to:

- briefly list the steps involved in preparing permanent tissues and whole mounts of plant and animal,
- explain what a fixative is and describe its mode of action,
- explain the importance of the alcohol series and their mode of action in preparation of permanent mounts,
- describe the theory for the basis of various stains termed as acid, basic, amphoteric stain and their mode of action,
- describe progressive and regressive staining methods,
- define mordants and explain their use in permanent slide preparations, and
- prepare the commonly used fixatives, alcohol series and stains and mordants that are used in plant and/or animal tissue preparations.

8.2 INTRODUCTION TO STEPS INVOLVED IN PERMANENT MOUNTS OF PLANTS AND ANIMALS

In order to keep the slide of whole mount or tissue mount of plant or animal for long term reference in terms of days and even years, it must be made as a permanent preparation. This is achieved for (1) tissue and (2) whole mount by the following steps.

8.2.1 Tissue Processing

1. **Fixation** and preservation of the tissue material.
2. **Dehydrating** the material with increasing concentrations of alcohol (30%, 50%, 70%, 90%, 100%, absolute alcohol) in order to prepare it for infilteration with paraffin wax.
3. **Section** cutting of tissue by microtome.
4. **Clearing** the wax infilterated section with a solvent like chloroform or xylene that is mutually soluble with alcohol and wax and which causes minimal hardening and shrinkage.
5. **Hydrating** the tissue for preparing it to take up stain, by treating it with decreasing concentrations of alcohol – 100%, 95%, 90%, 80%, 70%, 50%, 30%, distilled water.
6. **Staining** with required stain.
7. **Dehydrating** the tissue again as before, for mounting by using increasing concentrations of alcohol - 30%, 50%, 70%, 90%, 100% (absolute alcohol).
8. **Clearing** the tissue once again with a solvent like clove oil or xylene which is mutually soluble with alcohol and the mounting medium .

9. **Mounting** the tissue on a slide, under a coverslip in a preservative like Canada balsam or Euparol or DPX which dries hard and has a refractive index, similar to glass.

8.2.2 Processing whole mounts

The reagents and stains are similar but the process is shorter and involves the following steps:

i) **Fixation** or preservation of whole mount.
ii) **Staining** with required stain.
iii) **Dehydrating** the mount by using increasing strengths of alcohol.
iv) **Clearing** the tissue with clove oil or xylene which is a solvent that is mutually soluble with alcohol and the mounting media Canada balsam or Euparol.
v) **Mounting** the whole animal or plant on a slide under a coverslip in a mounting media like Canada balsam or Euparol or DPX which is a preservative.

8.3 FIXATIVES AND THEIR ACTION

Tissue is prepared for microscopic study in various ways. You are aware from Unit 7 of this course that if the mount you make is to be anything but very temporary, the tissue within the mount must be preserved in some way. Fixation is a special case of preservation, special in that the biologist aims to use specific fixatives and related stains that tend to preserve and stain those tissue structures which he/she plans to study.

You should also be aware that to make permanent microscopical mounts, the sections that you cut must be dehydrated in order to be made compatible with mounting media (like Canada balsam) which can be dissolved in solvents such as xylene. Xylene is a very efficient solvent for fats.

Tissue which has been newly removed from the plant or animal and has not been preserved in any way is called fresh and may be justifiably said to be in the living state. Fresh tissue has all of its cellular structures (tissue elements) present, and if it were to stay that way indefinitely you would not be reading this course! It can stay in this state for a short time if kept in physiological saline or Ringer's Solution. However, it is a fact that fresh tissue does not remain in its fresh state for very long despite saline or Ringer's solution, because in the cell there are membranes, mitochondria, Golgi apparatus and a host of other structures which the cell's own enzymes start to break down as soon as the tissue is excised from its source. The process is called *autolysis*, and soon renders the tissue so unlike its living state as to make it useless for detailed examination.

The first purpose of fixation then is to prevent the tissue from breaking down by its own enzyme systems. It may therefore also be said with some justification that the fresh tissue, by being fixed, is also killed, indeed small protistans and not so small metazoans, to say nothing of plants, are often killed by immersion in, or treatment with fixatives.

If slides were to be prey to bacteria and other agents of decay we should not expect them to last for long in any usable stage. Many of the chemical agents we use as preservatives, simply to prevent the microbiological decay of tissue, are also used as fixatives, or are mixed with fixatives to produce a fluid which will ensure the attainment of both aims.

Above and beyond the fulfilment of these purposes, we further expect to be able to colour or stain the material on which we are working, so that we can actually see the different tissue elements we have taken such great pains to preserve. This implies some preparative function of the fixative, and it is indeed well known that certain fixatives are conducive to good results with certain stains while others are not. It is also known that certain fixatives will preserve certain tissue elements with greater success than others, which means that a fixative can be 'designed' to do a specific job, i.e. to pick out, when used in combination with the appropriate stain, a particular tissue element.

To sum up, we can therefore say that the general effect aimed for during fixation is that of rendering the tissue in a state which is as close as possible to the living state (i.e. with minimum distortion). It would be of little use to look at slides which bear no resemblance to the living material, so clearly we must ensure that each tissue element is at least visible in the finished mount, or, if it is not visible, we should know the reason as to why it cannot be seen.

Fixatives then, are, chemical agents which:

(1) preserve tissues in as life-like a manner as possible for microscopical examination, with each required tissue element at least represented; and
(2) prepare them for subsequent processing so they are receptive to the appropriate stains and so that the required elements will not be dissolved from them by such processing.

Since we have already said that fixatives are also preservatives, we expect that the tissues will remain in that state for a long period of time.

SAQ 1

If the first aim of a fixative is to prevent the autolysis (self break down) of tissue by its own enzyme systems, what do you think might be the second aim?

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8.4 PRIMARY FIXATIVE GROUPS

Fixatives are generally classified into two primary groups by the effect that they have on the protein constituents of cells. Thus they are classified as:

(1) Coagulating fixatives, which precipitate proteins, and
(2) Non-coagulating fixatives, which bind proteins.

We shall first work through the primary fixatives to give a brief sketch of the action of each individually, then we shall examine some composite fixatives.

8.4.1 Coagulant Fixatives

When a protein is precipitated, or coagulated, it is set, rather like the white of an egg which has been cooked. In fact 'cooking' is sometimes used as a means

of fixing smears of protein on slides. This generally leaves the protein insoluble and immobile and with its chemical reactions to reagents altered. It has been found that using a coagulant fixative renders tissues more open to infiltration by molten paraffin wax, so most fixatives for tissues which are likely to be wax infiltrated contain at least one coagulant.

The coagulant fixatives in general use today are:

(1) Ethanol (ethyl alcohol),
(2) Picric acid (trinitrophenol),
(3) Mercuric chloride, and
(4) Chromium trioxide (chromic acid).

(1) Ethanol

Ethanol penetrates the tissue quickly but causes shrinkage and hardening. Mitochondria are destroyed, lipids may be dissolved. It does not affect subsequent staining greatly.

(2) Picric acid

Picric acid is maintained under water and dispensed as a saturated solution. It is explosive when dry. It may also be used as a dye.

Picric acid penetrates rather slowly with almost as much shrinkage as ethanol, but leaves tissues soft and less liable to hardening by ethanol.

Chromosomes are well preserved, lipids are not dissolved in this acid.

(3) Mercuric chloride

Mercuric chloride penetrates moderately quickly with slight initial shrinkage but gives about 70% shrinkage on embedding in paraffin. It hardens moderately, distorts the cell less than any other coagulant fixative. It preserves mitochondria, nuclear membrane, nucleolus and the external shape of the cell particularly well and is often regarded as a good mucopolysaccharide fixative. It produces black pepper-grain crystals in the cytoplasm, which are removable by treatment with iodine solution. It is a pre-eminent fixative for producing brilliant staining effects.

(4) Chromium-trioxide

Chromium trioxide penetrates slowly and gives moderate shrinkage and moderate hardening. It converts polysaccharides to aldehydes and provides good fixation of chromosomes. It is a good neutral fixative in the presence of isotonic sodium chloride.

8.4.2 Non-Coagulant Fixatives

In the non-coagulant fixative the protein is bound and so it is not necessarily set or changed in any way which you can see, but its chemical reactions and solubility in common solvents may be changed. The alterations in the chemistries of the proteins differ from fixative to fixative.
The non-coagulant fixatives in general use today are:

(1) Formalin (formalin is a 40% solution of formaldehyde gas in water),

(2) Osmium tetroxide (osmic acid),
(3) Potassium dichromate, and
(4) Acetic acid.

(1) Formalin

Formalin penetrates moderately quickly, producing some initial swelling, often with considerable attendant shrinkage on paraffin embedding. It hardens almost as much as ethanol. It is a good preservative of lipids. Furthermore it is a very good general fixative when mixed with isotonic sodium chloride but gives poor staining response when tissues are paraffin embedded.

(2) Osmium tetroxide

Osmium tetroxide penetrates slowly with little shrinkage and no hardening. Mitochondria are exceptionally well preserved, lipids are rendered less soluble in certain solvents by the use of this fixative; Osmium tetroxide preserves the structure of the cell better than any other fixative which is why it is used in electron microscopy. However, it gives poor staining reactions and crumbly sections when used with paraffin embedding.

(3) Potassium dichromate

Potassium dichromate penetrates quickly but without much fixing action on its own. It produces great shrinkage when used with paraffin embedding, but causes no hardening. By itself it is a very poor fixative for paraffin work. It is very sensitive to pH in its staining reaction.

(4) Acetic acid

Acetic acid penetrates quickly with great attendant swelling and almost no hardening. It precipitates nucleic acids and mitochondria are not seen in paraffin work.

Both these coagulant and non-coagulant reagents may not be very good as fixatives if used separately, and so they are combined, often with non-fixative additives, to give specifically useful fluids. Some of the above have both advantageous and disadvantageous properties: acetic acid, for example, penetrates very fast but causes excessive swelling of the tissue. Ethanol penetrates rather slowly and tends to both harden and shrink tissue. These two are often used together, each offsetting the disadvantages of the other. Glycerol, a non-fixative, softening additive, is often added to offset the hardening effect of ethanol.

SAQ 2

What are the two basic or primary types of fixative?

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(2) .....

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SAQ 3

What special advantage do coagulative fixatives have which makes it easier to cut sections from wax blocks?

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8.5 COMPOSITE FIXATIVES

As we stated in section 8.4, fixative are seldom used singly but are mixed, often with non-fixative salts (sometimes called 'indifferent salts') to make complex fixative mixtures designed for particular jobs.

Though the fixation problems for proteins are more or less the same for both plants and animals, these two groups do differ. Structurally, plant tissue may contain cellulose, lignin and chlorophyll, whilst animal tissue may contain collagen, cholesterol and blood, so the jobs that the fixatives are designed to do, differ somewhat for these two groups namely plants and animals.

Fixatives for plant material were first formulated to harden the tissue so that it could more easily be cut by a hand-held razor; other constituents were added later to give better cytological preservation.

Animal material presented different problems in that any fixative capable of hardening the tissue enough to be cut with such a razor destroyed all the fine cytological detail anyway. The development of cytological preservation followed the discovery that the tissue could be supported by paraffin wax or celloidin.

It was also discovered that treatment with various salts and compounds affected the subsequent staining reactions of dyes. This was in fact a known phenomenon in other fields. For example, the textile industry had used chrome salts what were known as 'mordants' for centuries in order to make the colours of dyed textiles brighter and/or faster. Some fixatives possess mordanting qualities in themselves, but others require certain salts to be added to the fixative mixtures in order to prepare the tissue for the dyes which are to be used on the sections.

There are several fixative mixtures which have stood the test of time because of a basic efficiency in doing their jobs. These are general fixatives, of use in many situations. A list of some of these follows, along with an indication of the kind of uses to which they may be put.

8.5.1 Plant Fixatives

(1) *Formalin-alcohol*

This is very good for woody twigs, and material may be left in the fluid indefinitely without harm.

(2) *Formalin-acetic-alcohol (F.A.A or AFA)*

This is very good for stems, leaves and roots. Material may be left in the fluid indefinitely without harm.

8.5.2 Animal Fixatives

(1) *Formal saline*

If you don't know what you are going to do with a piece of tissue, this formulation will keep it safe for months without harm.

(2) *Zenker's fluid*

This is an excellent general fixative giving a good picture with many stains, useful for almost any kind of tissue for which there is no specific requirement.

(3) *Helly's fixative*

This has essentially the same formula as Zenker's, differing in only one respect, and is recommended for use with tissue containing large quantities of red blood cells, e.g. liver.

(4) *Bouin's fluid*

Bouin's fluid gives excellent results with a wide variety of tissues and organisms. Material can be stored in this solution for a considerable time without over-hardening. Fixation for 12 hours to several days is recommended.

With the above formulations you should be able to cover most eventualities reasonably well but there are many more formulae which are general, as well as many which are specific. Some of these may be found in section 8.8 of this unit but for more formulae, ask your counsellor to recommend good sources. Although we have given separate formulae for plant and animal fixatives you must not think the two are totally distinct. Zenker's may be used for plant material as well as animal material (both contain proteins after all) but the picture given with a certain stain will be different in the two materials because of their differing constituents. It is often worthwhile experimenting with fixatives and subsequent staining since there is a large subjective element in the craft, and no two workers will exactly agree on all aspects of what may be correct.

SAQ 4

What is the difference between a plant fixative and an animal fixative?

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SAQ 5

What is a mordant?

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SAQ 6

Why are different formulations of fixatives used, i.e. why do we not use just one general formula?

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8.5.3 Fixatives and Safety

While working with fixatives you have to be very careful because a large number of the chemicals used are very toxic. Mercuric chloride, for example, is a highly toxic material which is cumulative in the body. It is very easily absorbed as an aerosol when being weighed out since it is generally supplied in the form of an extremely fine powder. Osmium tetroxide gives off a highly irritant and toxic vapour which fixes the mucus membranes of bronchi and the alveoli of the lungs with great alacrity, preserving them beautifully!

WARNING: AT THIS POINT IT MAY BE PRUDENT TO POINT OUT THAT ALL THESE AGENTS ARE ALL OF THE TYPE WHICH AFFECT TISSUES MARKEDLY IN SOME WAY OR ANOTHER. FIXATIVES IN GENERAL ARE TOXIC, OFTEN IRRITANT, AND SO BRING WITH THEIR USE ATTENDANT HAZARDS, SOME MORE SO THAN OTHERS. ALSO ANY CHEMICAL WHICH IS CAPABLE OF FIXING TISSUE WILL ALSO QUITE CLEARLY FIX YOUR BODY PARTS ON CONTACT. THEREFORE, YOU SHOULD BE PARTICULARLY CAREFUL IN ITS HANDLING.

8.6 ALCOHOL SERIES

Alcohol series are essential for preparing and staining permanent mounts. They are as you will recall from section 8.1, first used for dehydrating the fixed tissues in ascending strengths – 30% - 50% - 70% - 90% - 100% so that they can be embedded in paraffin wax for section cutting. Later the same series is

used to rehydrate the tissue in reverse or descending alcohol strengths 100% - 90% - 70% - 50% - 30% for staining. After this once again the alcohol series are used to dehydrate the stained tissues so that they can be mounted in DPX or Euparol or Canada Balsam.

After the fixation period is complete and any washing that may be necessary is also complete (24 hours for Zenker-fixed tissue, and a ten minute wash for Bouin's fixed tissue) the tissues are dehydrated by exposure to varying concentrations of alcohol.

The reason for this is that the tissues are full of water, which is not miscible with the molten paraffin wax into which they are to be set for preparing section for permanent mounts, and alcohol is a good solvent for water. The alcohol usually chosen for this task is ethanol, but since pure ethanol is probihitively expensive often Industrial Methylated Spirit (IMS) is used. IMS is mainly ethanol to which a small percentage of methanol has been added, thus removing the temptation to drink it.

WARNING

IT SHOULD BE EMPHASIZED HERE THAT IMS IS EVEN MORE TOXIC THAN PURE ETHANOL. DRINKING IT MAY KILL ONE OR LEAVE ONE SIGHTLESS.

8.6.1 Use of Alcohol Series in Slide Preparation

In order to remove the water from the tissues without causing the enormous distortion which would occur if the tissues were air-dried, the water must be replaced gradually by a water solvent. Ethanol is just such a solvent, and a series of alcohol concentrations is made up which start at a low percentage of alcohol to water and end up with no water at all. The concentrations used are as follows:

30% Alcohol; 50% Alcohol; 70% Alcohol; 90% Alcohol; 100% or Absolute Alcohol

Stock bottles of these concentrations are made up and added to the tubes containing the tissues over the course of some hours. 100% Alcohol is usually known as 'absolute alcohol'. A second stock of 70% Alcohol and 90% Alcohol may also be made up, to which a crystal or two of iodine has been added for the purpose of removing mercury deposits from the tissues. Such deposits are produced by mercuric fixatives, which actually precipitate crystalline mercury in tiny particles through the tissue. This may be removed during the course of dehydration by the use of iodized alcohol solutions instead of normal alcohol of the same concentrations. Alternatively, the tissue may be simply dehydrated and the mercury deposit removed by treatment with Lugol's Iodine at the staining stage.

Initial dehydration of tissue is started by adding 30% Alcohol to the tubes containing the washed tissues and they are left for 2 hours. At the end of two hours, the 30% Alcohol is poured off and replaced by 50% Alcohol, and again after two hours by 70% Alcohol and so on. When the tubes are filled with absolute alcohol they are left for one hour only after which the alcohol is replaced by fresh absolute alcohol and again left for one hour. This ensures

that even the last tiny remnant of water is removed leaving the tissues totally dehydrated.

Since water is being removed from tissue by the alcohol series, it follows that the alcohol series will become gradually diluted, and will eventually no longer be at its required concentrations. When this happens the series is moved up one, i.e., 70% is re-labelled 50%, and 50% is re-labelled 30% and so on. Absolute alcohol becomes 90% and the absolute is replaced with fresh alcohol, while the 30% is thrown away. In this way, the alcohol is used economically.

Alcohol has proved to be a most effective agent for the dehydration of tissues, but its one important disadvantage when used as a precursor to embedding in paraffin wax is that the molten wax is only slightly more miscible with the alcohol than it would be with the water originally contained in the tissues and so the alcohol is not suitably wax miscible and is removed with yet another solvent before the tissue is embedded in wax. This new solvent also called cleaning agent must, therefore, be capable of removing alcohol present in the dehydrated tissue and at the same time be miscible with moltan paraffin wax in which the tissue is to be embedded. Some important clearing agents used at present are xylene, cedar wood oil and chloroform. Xylene is used the most in Indian Laboratories since it is safe though more messy than cedar wood and not as toxic as chloroform.

One might ask why alcohol like IMS or ethanol is used and not some solvent which is miscible with both water and wax, should such a solvent exist! Such solvents do indeed exist and they are indeed used for just this purpose. However, the concept of dehydration by alcohol is fundamental to the history and development of microscopy, and has the definite advantages of relative cheapness, ease of use and understanding, and relatively low-hazard rating, in terms of toxicity.

WARNING

CHLOROFORM IS A HAZARDOUS MATERIAL CAUSING AMONG OTHER THINGS CIRRHOSIS OF THE LIVER, AND SHOULD BE HANDLED WITHOUT UNDUE BREATHING OF THE VAPOUR OR SKIN CONTACT.

SAQ 7

Why can the tissues not be simply dried out in air?

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8.6.2 Basis for Procedure of Staining of Permanent Mounts

Once sections embedded in wax have been cut with the microtome and mounted on slides and properly dried, the next step is to stain them. For this another alcohol series is used, along with a clearing agent before staining can be done.

For staining, the wax must first be removed from the section. This is achieved by dissolving out the wax with xylene or some other recommended solvent. This leaves the section free of wax, but full of xylene, which is immiscible with the water soluble stains which are used for staining the tissue. The xylene, therefore, has to be removed and to do this the slide is now immersed in absolute alcohol which dissolves out the xylene but adds no water.

Water is added by stages to the tissue section on the slide, since if the water were to be added to the tissue all at ones, distortion and tissue damage would result because of the stresses involved. So water is added gently, the slide is placed in 90% alcohol and then in 70% alcohol, etc. down to distilled water, at which level the stains are generally added. Having stained the material, it must again be dehydrated by means of descending alcohol series, then mounted in a suitable mounting medium and covered by a coverslip.

Stains tend to dissolve out in the lower percentage alcohols and for this reason it is usual to 'come up' from water rather quickly to absolute alcohol. Thus two absolute alcohol jars are used when it comes to staining and mounting sections. This is because the very last vestiges of water must be removed before the section can be cleared, since the clearing agent is not tolerant of water. Since many slides may be taken up or exposed to the same alcohol series, the first absolute alcohol may become contaminated with carried over water. The second absolute alcohol is the remedy for this and is used only in the final dehydration. From the final absolute alcohol, the slide is transferred into xylene to clear it optically and to remove the alcohol and then mounted.

The mountant that is the mounting medium may be Canada balsam, DPX, Euparol, etc, is miscible with the xylene. There are many mounting media. A good mountant or mounting medium is one which is non-discolouring and remains transparent without crazing or changing the colours of stains. Crazing of a mounting medium is the formation of many tiny cracks within the hardened medium, due to tensions within it brought about by slow shrinkage. Colour changes occur in sections mounted in a medium whose pH changes. If this happens, electrostatic changes may occur in the mounted tissue which affect the dyes therein.

8.7 SOME STAINING THEORY AND METHODS

You already have some knowledge about stains in unit 7. Stains, or dyes as they may more properly be called, fall into three broad categories as follows:

(1) Basic dyes,
(2) Acid dyes, and
(3) Amphoteric dyes.

8.7.1 Basic Dyes

If you find the concept of electrical charge difficult in this context, simply think of the dyes and substrates as acidic or basic. The electrostatic terms come from the theor of electrolysis.

These are so called because they are essentially alkaline. The dye ions are positively charged and are therefore attracted to and stain acidic tissue constituents such as the chromatin in nuclei. A typical basic dye will be positive at all pHs (Fig. 8.1). Basic dyes are also classified as cationic dyes since they are positively charged and will move toward a negative charge.

8.7.2 Acid Dyes

These are so called because they are essentially acidic. The dye ion are negatively charged and are therefore attracted to and stain alkaline tissue constituents such as cytoplasm generally. Acid dyes are also classified as anionic dyes since they are negatively charged and will move towards a positive charge (Fig. 8.1).

8.7.3 Amphoteric Dyes

These dyes are capable of both acid and basic reactions, because the negativity or positivity of their charges changes with pH, and they will stain different tissue elements in different pH solutions.

The classic amphoteric dyes are carmine and orcein. Carmine is used as a basic dye in the formulation known as aceto-carmine in which it is positively charged, and as an acid dye in the formulation known as borax-carmine in which it is negatively charged. The isoelectric point of carmine (i.e. the point at which it has no charge at all) is at about pH 4.2. Aceto-orcein is similarly used to stain chromatin of the nucleus of the cell. An amphoteric dye will be thus positive at some pHs and negative at others (Fig. 8.1).

This deceptively simple Fig. 8.1 is in practice complicated by the fact that many tissue elements also change their charge when the pH of the medium is changed and are hence amphoteric themselves.

In general, what is required of a dye is that it renders some element or elements of tissue more visible: in other words, it increases the contrast between the sought element and its background. In practice, it is normal to emphasize more than one tissue element by using a combination of at least two stains, one basic and one acidic.

The classic combination, used for general histology and pathology for many years, is the combination between haematoxylin, a basic nuclear stain, and eosin Y, an acidic cytoplasmic stain. This is not by any means the only combination available, but it is typical in that the basic stain, haemtoxylin, is dark, and stains nuclei blue, and the acid stain, eosin Y, is light and stains the cytoplasm a range of pink through orange to crimson.

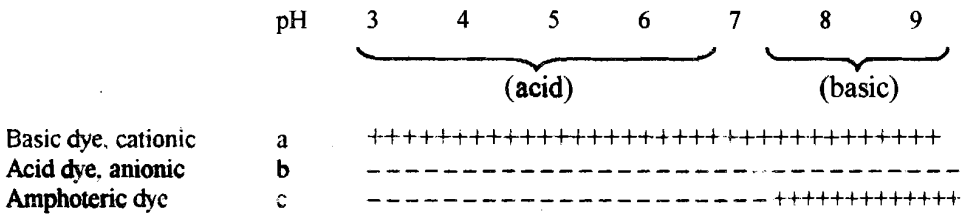

Fig. 8.1: pH of stains. a) a typical basic dye (+) will be positive at all pHs. b) a typical acid dye (–) will be negative at all pHs. c) an amphoteric dye (+ –) will be positive at some pHs and negative at others.

8.7.4 Types of Staining Methods

A large number of stainsing are used in the histological study of tissue of plants and animals. Different stains highlight different tissues, some stains are good at staining the nuclei while others are effective in staining the cell organelles. Thus often specific stains are needed and used to study specific structures. So stains are often used singly or in combination and as a result staining of tissues may be classified as

(i) **Single Staining**: When only one stain is used to give a single colour.
(ii) **Double Staining**: Where two stains are used, on the same tissue, each staining a specific area or structure.

(iii) **Multiple Staining**: When more than two stains are used on the same tissue, each being used for a specific area, or structure.

SAQ 8

Are the ions of a basic stain negative or positive?

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SAQ 9

What is the general electrostatic term for an acidic dye?

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Progressive Staining

This term is given to the method of staining where dyes are added as a relatively weak solution to the tissue and the tissue is allowed to take up the stain over a fairly protracted period. As the stain is being taken up, the process may be monitored by watching the tissue that is being stained under the microscope as the colouration deepens. When the colour density seems to be correct, the stain is washed off and the tissue is dehydrated, cleared and mounted. Stain is actually taken up into a susceptible tissue element against a concentration gradient, i.e. the stain in the tissue being dyed may be more concentrated than the stain in solution.

Regressive Staining

This method is based on the fact that the fastness of a basic dye is decreased in acid solution, and that the fastness (setting) of an acid dye is decreased in alkaline solution, or simply that a dye will dissolve out from tissues under the right conditions. Regressive staining consists of overstaining and then removing the excess stain until the density or colour is just right.

A dye, for example, haematoxylin, which is used as a basic dye, is applied to tissue at a pH at about its isoelectric point. The dye thus stains all the tissue. The dye is then 'set' (i.e. made fast) in the tissue by placing the slide in alkaline solution and the result viewed down the microscope.

In the case of haematoxylin, the tissue will be in varying shades of blue all over, but really it is only the nuclei which are deeply stained and these will appear deep blue. The slide is placed in an acid solution, which releases the dye's hold on the tissues, and this is watched and monitored under the microscope. The dye turns red and leaves the cytoplasm first and the nuclei last. The progress of the washing out can be easily followed.

When it is thought that all the haematoxylin has left the cytoplasm and only the nuclei remains stained, the slide is again returned to an alkaline medium and 'blued'. If the dye is still too dense on bluing, the acid treatment is repeated.

This adjustment of the stain density in regressive staining is known as 'differentiation'. When the basic stain is correct, the slide is generally counterstained with an acid dye so that staining is completed. You will be able to try this out when you attend your practical session.

SAQ 10

What is meant by the term 'progressive staining'?

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SAQ 11

What term is used in regressive staining to mean the removal of stain from the tissues?

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8.7.5 Mordanting

Mordanting is effectively the conditioning of tissues so that they will accept basic dyes more readily than they would otherwise. Mordants are usually metal salts, most often 'alums', e.g. potash alum, (which is aluminium potassium sulphate), potassium permangnate, ammonium chromate, ammonium dichromate.

The salts are used sometimes in advance of the stain and sometimes with the stain. They attach to the tissues selectively and enhance the action of the stain by 'lake formation' which is a subtle changing of the dyestuff from an aggregated, or granular, to a more diffuse and soluble form. The dye lake or stain is generally more pervasive, more dense, and brighter in colour than the unmordanted dye.

The reactions involved are not simply a matter of positivity or negativity, but firm and highly stable complex chemical bonding between the tissue and the dye and the mordant. Hence the lake is attached more tenaciously to the tissue than the simple dye would be. Differentiation is often carried out with mordant or modified mordant solutions which are used as specific differentiators for specific dye lakes.

SAQ 12

Name the compound which is formed from the mordant with the dye.

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SAQ 13

What extra property do you think a dye lake has, which a dye does not have?

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8.8 THE FORMULARY OF REAGENTS AND STAIN

Before we start reading about the various preparation methods of fixatives, alcohol series, mordants and stains it is essential to be aware of certain important aspects of these preparations. Thus before looking through the formulary, carefully read the following points.

General rules of preparation

(1) Where no units are indicated against the stated quantities, the amounts are in 'parts by volume' and may be any fluid measure, e.g. for Farmer's Fluid, the quantities have no units; however, 3.0 ml of ethanol mixed with 1.0 ml of glacial acetic acid will make 4.0 ml of Farmer's, and 3.0 litres of ethanol mixed with 1.0 litre of glacial acetic acid will make 4.0 litres of Farmer's; similarly 3 gallons of ethanol and one gallon of glacial acetic acid will make 4 gallons of Farmer's.

(2) Notes appearing after a formula are specific to the formula used as intended by the formulator and may not be applicable to a new situation, e.g. plant material may be left indefinitely in FAA without harm but it is doubtful whether this would be true for animal material.

(3) Where 'ethyl alcohol' appears in a formula, unless otherwise stipulated an identical concentration of industrial methylated spirit (IMS) may be substituted without affecting the result.

(4) IMS may be obtained as IMS or as IMS anhydrous: either will do. IMS is ethyl alcohol (ethanol) to which has been added a small but significant quantity of methyl alcohol (methanol) to make it poisonous to drink.

(5) Where 'formalin' appears in a formula this is taken to mean a 40% solution, i.e. saturated, of formaldehyde gas in water. Where a percentage of formalin is stipulated, it is a percentage of this solution, thus 25% formalin is made up from 25 parts of the saturated formaldehyde solution and 75 parts of water.

8.8.1 Fixatives in Common Use

Plants and animal tissue are usually fixed in relatively small pieces which do not exceed 6 mm in any direction. 'Standard-sized' pieces of tissue particularly in animal tissue may be taken to mean cuboids of tissue whose longest dimension is no greater than 6 mm. Staining times are given for standard-sized tissue samples.

(1) Fixatives for Plant Material

Formol Alcohol

Ethanol	15.0 ml
Formalin	1.0 ml
Distilled water to be added	to 100.0 ml

General fixative, good for woody material. Material may be left in this fluid indefinitely, without harm.

Formol Acetic Alcohol (FAA or AFA)	Formula 1	Formula 2
70% ethanol	90.0 ml	85.0 ml
Glacial acetic acid	5.0 ml	5.0 ml
Formalin	5.0 ml	10 ml

Formula 1 is a general fixative for stems, roots, leaves and all soft parts. Material may be left in this fluid indefinitely without harm. Formula 2 is also a general fixative of plant. However it is also used for fixation of nematodes.

King's Formula

95% ethanol	150 ml
5% formalin	102 ml
Glycerol	50 ml

General fixative for delicate subjects. Material may be left in this fluid indefinitely without harm.

Carnoy's or Farmer's Fluid

Ethanol	3.0 parts
Glacial acetic aicd	1.0 parts

Useful for rapid smear techniques

Champy's Fixative

3% potassium dichromate	7.0 parts
1% chromic acid	7.0 parts
2% osmic acid	4.0 parts

Mix the constituents immediately before use. Fix plant material for 6 to 24 hours then wash in running water for an equal time. Good for cytoplasmic structures, especially mitochondria.

Navaschin's Fixative

Solution A:

Chromic acid	1.0 g
Glacial acetic acid	7.0 ml
Distilled water	92.0 ml

Solution B:

Neutral formalin	30 ml
Distilled water	70 ml

Mix equal quantities of A and B just before use.

(2) Fixatives for Animal Material

Formol Fixative

Formalin	10 ml
Distilled water	90 ml

Fixation is improved, if physiological saline is added to the formalin solution.

Formol Saline

Formalin	10 ml
Sodium chloride	0.9 g
Distilled water	to 100 ml

General fixative, extremely good for tissue whose future is uncertain. Tissues may be left in this fluid for long periods without undue harm. Fixation is usually complete in 24 hours. For amphibia, the quantity of NaCl is reduced to 0.65 g.

Invertebrate Fixative

Formalin	5.0 ml
Sodium chloride	0.7 g
Distilled water	to 100 ml

This is a safe fixative for whole mounts of small metazoans. Marine forms should be placed in 5% formalin in the sea water from which the animals came, or 5% formalin in 3.5% sodium chloride.

Carnoy's Fluid	Formula 1	Formula 2
Glacial acetic acid	25 ml	10 ml
Absolute alcohol	75 ml	60 ml
Chloroform		30 ml

The various ingredients of Carnoy's fixative should be combined only before use. Formula 1 is in more general use than formula 2. Chloroform increases the rate of penetration. Both these solutions are used for the fixation of dense impervious objects such as arthropods and nematodes, particularly *Ascaris* and *Ascaris* eggs. Fixation is very rapid. Small block should be removed in 30 minutes, larger blocks after several hours. Formula 2 is sometimes used for marine crustaceans, especially for injection in large species.

Zenker's Fluid

Mercuric chloride	5.0 g
Potassium dichromate	2.5 g
Sodium sulphate	1.09 g
Distilled water	to 100 ml
Immediately before use add glacial acetic acid	5.0 ml

A fairly fast routine fixative giving good results with stains. Fixation is complete in about 24 hours.

Helly's (Zenker's formol) fixative

As Zenker's but substitute	
Formalin instead of glacial acetic acid	5.0 ml
Formalin to be added immediately before use.	

A good general fixative for use with tissues containing much blood. Fixation is usually complete in 24 hours.

Bouin's Fluid

Picric acid (saturated aqueous soln:)	75.0 ml
Formalin (40% formaldehyde)	25.0 ml
Glacial acetic acid	5.0 ml

Rapidly penetrating fixative with little shrinkage. Material can be stored in this solution for a considerable time without over-hardening but the best staining results are achieved if the material is processed as

soon as possible after fixation. Transfer tissue to 70% ethanol in order to stabilize the picrates formed. Often gives an exceptionally bright staining picture

(3) Other Useful Formulae

Neutral formalin

Formalin usually contains formic acid and so is slightly acidic but it can be neutralized by the addition of sodium carbonate or calcium carbonate. If excess calcium carbonate is placed in the formalin bottle (so that some undissolved salt always remains) it will keep the formalin approximately neutral. The excess calcium carbonate is allowed to settle out and the solution is filtered before use.

Lactophenol

Lactic acid	25 ml
Glycerol	50 ml
Distilled water	25 ml
Crystals of phenol	25 g

Warm all ingredients on a water bath until the phenol crystals have dissolved, and store in a dark bottle.

Mayer's albumen

White of egg	50 ml
Glycerol	50 ml
Sodium salicylate	1.0 g

The egg white is separated from the yolk, beaten to break up the membranes. The sodium salicylate is dissolved in a little water and beaten with the white. The glycerol is added and the whole egg mixture is beaten again and filtered through muslin cloth. This stock solution will keep well in a stoppered bottle. It is diluted by ten with distilled water for use.

Polyvinyl lactophenol

Solution A **Polyvinyl alcohol stock solution:**

Add polyvinyl alcohol powder to a little cold water and stir thoroughly. Heat on a water bath with stirring until the solution becomes thickened like treacle (syrup). Keep in a closed bottle wherein it will clear. This may be used as a mountant in its own right.

Solution B **Polyvinyl lactophenol**

Polyvinyl alcohol stock solution	56 ml
Phenol	22 g
Lactic acid	22 ml

Solution B is available commercially as well.

8.8.2 Stains in Common Use

Aceto-carmine

Dry carmine stain	0.5 – 1.0 g
Acetic acid, glacial	45 ml
Distilled water	55 ml

Mix the glacial acetic acid and distilled water and heat to boiling. Then add the carmine stain, shake well, cool and filter. This is one of the most commonly used stain for chromosomal studies.

Aceto-orcein

Orcein	1.0 g
Glacial acetic acid	45 ml
Distilled water	55 ml

Mix the above three things together and warm to dissolve stain. Cool and filter. It is an ideal stain for the study of chromosomes.

Aniline (Cotton Blue) Lactophenol

Cotton blue	0.1 g
Lactophenol	100 ml

Crystal Violet Stain

Crystal violet (Gentian violet)	2.0 g
Ethyl Alcohol 90%	20 ml
Distilled water	180 ml

Dissolve the crystal violet in the alcohol and add the water. This is the simple substitute for Gram's stain.

Giemsa's Stain

Giemsa's powder	0.5 g
Glycerine	33 ml
Absolute methyl alcohol	33 ml

It takes 1 to 2 hours to dissolve the powder in the glycerine. Then add the acetone free absolute methyl alcohol. This stock solution is diluted 1:10 with distilled water before using.

Delafield Haematoxylin

Haematoxylin crystals	4.0 g
Absolute (ethyle alcohol) ethanol	25 ml
Saturated solution of Ammonia alum	400 ml
Glycerine	100 ml
Methyl alcohol	100 ml

Dissolve 4 gm of haemotoxylin crystals in 25 ml of absolute alcohol in warm water bath and filter. Add to it 400 ml of saturated solution of ammonia alum and mix thoroughly. Leave it exposed to light and air in unstoppered bottle for about a week. Filter and add 100 ml of glycerine and 100 ml of methyl alcohol. Allow the solution to stand uncorked for ripening until the colour is sufficiently dark and filter. This stain is used for histological studies and may be ripened immediately by the addition of:

Potassium permanganate	0.2 g
Warm distilled water	5 ml

Though this will ripen the stain quickly, it will affect the keeping qualities of the final stain greatly by eventual over ripening.

Ehrlich's Acid Haematoxylin

Haematoxylin crystals	2.0 g
Absolute Ethyl alcohol (ethanol)	100 ml
Saturated solution of Ammonium alum (mordant)	8.0 g

Distilled water	100 ml
Glycerol (glycerine)	100 ml
Glacial acetic acid	10 ml

Dissolve the haematoxylin crystals in absolute alcohol on warm water bath and filter. Then add glacial acetic acid and then glycerine and water. Let the mixture ripen in light until it aquires a dark red colour. This stain is quite stable and will keep for years if kept in well stoppered bottle. Alum haematoxylin is generally more useful in histological preparation in combination with counter stains such as eosin or erythrosin.

Aqueous Eosin

Powdered, water soluble eosin	1.0 g
Distilled water	100 ml

Mix and add four or five drops of chloroform to preserve

1% Alcoholic eosin

Eosin powder	1.0 g
70% or 90% alcohol	100 ml

Eosin should impart a clear pink colour to cytoplasm.

Leishman Stain

Leishman stain powder	0.15 g
Pure methanol	100 ml

Dissolve the stain in absolute alcohol and leave it for ripening for 48 hours in a stoppered bottle. Leishman stain is very good for the staining of protozoans. Filter before use.

Lacto Fuchsin

Acid fuchsin	0.1 g
Lactic acid	100 ml

Light Green in Clove Oil

Light green	1.0 g
Clove oil	100 ml

Heat the mixture to dissolve on a water bath, then cool and filter. It is a good cytoplasmic stain and is used for staining cellulose wall and some filamentous algae.

Lugol's Iodine

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	100 ml

Add iodine and iodide to about 25 ml water. When dissolved, add the rest of the water.

Mallory-Heidenhain Rapid One Step Stain

Phosphotungstic acid	1.0 g
Orange G	2.0 g
Aniline blue	1.0 g
Acid fuchsin	3.0 g
Distilled water	200 ml

Add ingredients serially and individually to the water, dissolve each before the next is added. This stain is used for protozoa and paraffin sections.

Alcoholic Methylene blue

Methylene blue	1.5 g
Ethyl alcohol 95%	100 ml

Aqueous Methylene blue

Methylene blue	1 g
Distilled water	100 ml

This stain is used in staining small live animals.

Neutral Red

Neutral red	1.0 g
1% acetic acid	2.0 ml
Distilled water	1.0 litre

Dissolve and filter.

Noland's Solution

Gentian violet	0.20 g
Phenol (saturated solution in distilled water)	80 ml
40% formalin	20 ml
Glycerol	4 ml

Dissolve the gentian violet in the mixture of phenol, formalin and glycerol.

Saturated Phenol

Phenol crystals	6.0 g
Water	90 ml

Dissolve crystals for saturated solution. Keep container well closed and protected from light.

WARNING: DO NOT HANDLE PHENOL CRYSTALS WITH BARE HANDS. DEATH HAS RESULTED FROM AS LITTLE AS 1.5 GRAM ABSORPTION. NEVER APPLY TO LARGE PORTION OF THE BODY SURFACE.

Polychrome Toluidine Blue

Lithium carbonate		0.5 g
Toluidine blue		1.0 g
Distilled water		75 ml
Glycerol	Mix	20 ml
95% ethanol		5.0 ml

Warm the water and stir in the lithium carbonate. When dissolved, add toluidine blue, dissolve and add glycerol-alcohol mixture. Leave to 'ripen' for one month before use.

Alcoholic Safranin

Safranin	1.0 g
Distilled water	50 ml
Absolute Ethanol	50 ml

Dissolve the dye in the water and add the absolute alcohol and filter

Aqueous Safranin

Safranin	1.0 g
Distilled water	100 m

Safranin is popular for its brilliancy and permanency in balsam.

Toluidine Blue

Toluidine blue	1.0 g
Distilled water	100 ml

Osmium tetroxide for EM (Palade)

A. Veronal acetate buffer:

Sodium (veronal) Barbitone	2.89 g
Anhydrous sodium acetate	1.15 g
Distilled water	to 100 ml

Should be kept cold in a refrigerator where it will remain stable for some months.

B. Osmium tetroxide

Osmium tetroxide (2.2% osmic acid)	1.0 g ampoule
Distilled water	50 ml

Break the clean ampoule into a dark, scrupulously clean bottle in subdued light. Stopper and keep in a refrigerator. If precautions for cleanliness are not taken the solution will quickly deteriorate, but will remain stable for some weeks if made correctly.

Add 5.0 ml of the buffer to 2.5 ml of distilled water, and add sufficient 0.1 N HCL to make the pH 7.4. Mix this total volume with an equal volume of 2% osmium tetroxide. Fixation is from 30 to 90 minutes.

WARNING: ALWAYS HANDLE OSMIUM MATERIAL INSIDE A FUME CUPBOARD.

8.8.3 Grades of Alcohol

The alcohol grades are usually prepared from rectified spirit which contains approximately 95% of alcohol. Here however we have given the preparation of grades of alcohols using absolute alcohol.

Alcohol Series	**30%**	**50%**	**70%**	**90%**
Absolute Alcohol (ethanol or methanol)	30 ml	50 ml	70 ml	90 ml
Distilled water	70 ml	50 ml	30 ml	10 ml

Acid Alcohol

70% ethyl or methyl alcohol	100 ml
HCl	1 ml

8.8.4 Animal Ringer's Solution

Fresh tissue can be studied for a short period of time if it is kept in or moistened with Ringer's solution specific to its group. Ringer solution is thus used for moistening tissues to be observed in the living state under the microscope or for keeping the fresh tissues for a period of time before fixation. The preparation of Ringer's for invertebrates and cold blooded vertebrate is different from that of warm blooded vertebrates.

Ringer's solution	Invertebrates + cold blooded vertebrate	Warm blooded vertebrates
Sodium chloride (Analytical)	6.5 g	8.5 g
Potassium chloride	0.25 g	0.25 g
Calcium chloride	0.25 g	0.25 g
Sodium bicarbonate	0.25 g	0.25 g
Distilled water	1000 ml	1000 ml

8.8.5 Animal Physiological Saline Solution

A simple physiological saline solution is only used for moistening or washing tissue before fixation. The physiological saline for invertebrates and cold blooded vertebrates is different from that of the warm blooded vertebrates in the amount of sodium chloride.

Physiological Saline solution	Invertebrates + Cold blooded vertebrates	Warm blooded vertebrates
Sodium chloride (analytical)	0.75 g	0.9 g
Distilled water	100 ml	100 ml

8.9 SUMMARY

In this unit you have studied that:

- Small animals and fresh tissues of animals can be washed or moistened with animal physiological saline. The physiological saline for invertebrates and cold blooded vertebrates differs from the physiological saline of warm blooded vertebrates in the quantity of sodium chloride (NaCl) used.
- Small animals and fresh tissue of animals can be kept in and studied for a period of time in Ringer solution. The Ringer's solution of invertebrates and cold blooded vertebrates is different from that of warm blooded vertebrates.
- It is essential for histological or microscopic study of small animal, animal tissue and plant and plant tissue to make permanent slides.
- Steps involved in making permanent slides or mounts are:
  - i) Fixation – a) kills and preserves structures of tissue and b) prepares material for future treatments.
  - ii) Dehydration – washes out the fixative from the tissue and prepares it for embedding in paraffin wax. This is accomplished by passing tissues through increasing strengths of graded series of alcohol – 30%, 50%, 70%, 90%, 100% (Absolute alcohol).
  - iii) Embedding – hard substance such as paraffin is used for embedding the tissue sample (piece) so that it can be sliced into thin sections.
  - iv) Section cutting – (a) hardened paraffin block containing tissue is trimmed and (b) mounted on cutting machine called microtome for cutting
    (c) section of desired thickness are cut with microtome and placed on slides smeared with adhesive such as Mayer's albumin.
  - v) Rehydration – section are cleaned in xylene and rehydrated with decreasing alcohol grade series (90%, 70%, 50%, 30%, distilled water) in order to prepare them for staining.
  - vi) Staining – suitable stain is used for staining the sections.

vii) Dehydration – the sections are again dehydrated by increased alcohol grade series - 30%, 50%, 70%, 90%, 100% (Absolute alcohol) in order to prepare it for mounting.

viii) Mounting – the dehydrated section is cleaned in xylene or clove oil and mounted in DPX or Euporol or Canada Balsam.

- Fixatives may be of (i) coagulant type which coagulate the protein or (ii) non-coagulant type which bind to the protein.
- Coagulant type fixatives are – ethanol, picric acid, mercuric chloride and osmic acid. Non-coagulent type fixative are – formalin, osmic tetroxide, pottasium dichromate and acetic acid.
- Fixatives are seldom used singly but often mixed with indifferent salts to make complex fixatives that preserves the tissue better and prepares it in a better manner for staining. Some common complex fixatives are – i) plant fixatives – Formol acetic acid (FAA) and Formol alcohol ii) Animal fixative – Formol saline, Zenker's, Helly's and Bouin's solution.
- Alcohol grades are used in (i) increasing strengths - 30%, 50%, 70%, 90%, 100% (Absolute alcohol) for dehydrating tissue and in decreasing strengths 90%, 70%, 50%, 30%, distilled water for rehydrating tissues.
- Stains may be of three broad types (i) Basic whose ions are positively charged (ii) Acidic whose ions are negatively charged (iii) Amphoteric – whose ions may be negative or positive depending on the pH.
- Staining of tissue may be done by (i) one stain (ii) two stains (iii) multiple stains.
- Tissue staining may be done by (i) Progressive staining – where dyes are added as a relatively weak solution to tissue, which is allowed to take up the stain over a long period of time. (ii) Regressive staining – The tissue is allowed to overstain and then the stain is gradually removed till the right colour is obtained.
- A large number of fixative and stains are used in the histological study of small plants, small animals and tissues of plant and animals.

8.10 TERMINAL QUESTIONS

1. What is meant by 'fresh' tissue?

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2. What is the difference between binding fixative and coagulation fixative?

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3. Why are fixatives used in combination rather than individually?

4. Give an advantage and a disadvantage of the hardening effect of fixatives.

5. What is an 'indifferent salt'?

6. Why must water be removed from the fixed tissues?

7. What does the term amphoteric mean when applied to dyes? Upon what condition is the positivity and negativity of an amphoteric dye dependent?

8. What is a mordant? What kind of salts are generally used as mordants?

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8.11 ANSWERS

Self-assessment Questions

1. The second aim of the fixative is to prevent the breakdown of the tissue by the enzyme systems of invading living organisms such as bacteria and fungi.
2. The two basic types of fixatives are:
   i) Coagulant fixative which is protein coagulating.
   ii) Non-coagulant fixative which is protein binding.
3. Coagulation fixative causes the tissue to be more easily penetrated by molten paraffin.
4. There is not much difference between animal and plant fixative. Generally the same fixative may be used for both (possibly except for some specialized fixatives which have been designed for compatibility with specific tissues or dyes to highlight constituents that are specific to the plant or animal).
5. A mordant is a substance which may be a fixative or may be added to a fixative solution so that it makes the tissue more receptive to certain dyes in later processing. The action of a mordant may also make a dye appear brighter in colour, change its colour or make it less liable to wash out or fade.
6. Different formulations of fixatives are used because certain components of fixatives fix certain tissue elements better than others. This means we can design a fixative to show up a chosen aspect of a given tissue. If we tried to mix all the fixatives so that they showed up every aspect of every tissue, the components of the fixatives would interact negatively with each other, e.g. some fixative components are acidic and some are basic.
7. Tissues cannot be simply dried out in air because once the water is dried off, the tissue would shrivel up since there would be nothing to support them.
8. Positive.
9. Anionic
10. The tissues on a slide are allowed to take up colour from a weak solution of stain until the colour is dark enough. The process is watched using a microscope.
11. Differentiation

12. A dye lake
13. The dye is transformed from an aggregated form to a more mobile form which can penetrate the tissue more easily.

Terminal Questions

1. Fresh tissue is one that has been recently excised or cut from its own source and so still retains many of the attributes of the living tissue.
2. In coagulant fixative the protein is coagulated and so the visible aspect of the tissue is changed as well as the chemical structure and reactions, whereas in non-coagulant fixatives the protein is bound so the chemical reactions of the tissue may be different, the visible aspect remains more or less the same.
3. Individual fixatives tend to be weak fixatives. This is because on their own individual fixatives often have disadvantageous effects which can be balanced or counteracted by the different disadvantageous effects of other fixatives.
4. An advantage of hardening effect of fixative, particularly in the case of animal tissue, is that the tissue, instead of being floppy and rather different to handle, takes on a firm structure and can thus be handled more easily. A disadvantage is that the tissue, if left in a hardening fixative for too long, may not only lose its fine cytological detail but may also prove impossible to cut into sections.
5. This is a substance which is not itself a fixative, but when added to a fixative formula, makes the fixative work better in some way. It may be sodium chloride to make the fixative isotonic, it may be a salt which enables a particular component to penetrate better, or makes a formulation less acid (a buffer), or it may be a mordant, etc.
6. Water must be removed from the tissue so that it will accept the molten paraffin wax, which would not enter if water was present, as wax and water do not mix.
7. It means that they are capable of acting both as an acid and a basic dye. It depends on the pH.
8. A mordant is a substance which forms a complex compound between the tissue, the dye and itself and enhances the fastness and brilliance of the dye. Metal salts, usually such as alums of potassium, chromium or ammonium are usually used as mordants (All alums are metal salts since they all contain aluminium).

UNIT 9 CULTURE OF MICRO-ORGANISMS

Structure

9.1 INTRODUCTION

You will be aware that micro-organisms can cause infectious diseases. We are exposed to a variety of bacteria, viruses and fungi daily and a small proportion of these can cause infection. In addition, all of us carry a 'normal flora' of micro-organisms on both the external surface (e.g. skin) and internal surfaces (e.g. mouth) of our bodies. Micro-organisms also called microbes are therefore, all around us.

However, in choosing to handle bacteria or fungi in the laboratory, you are deliberately choosing to work with still larger quantities of micro-organisms: for example, a single colony of bacteria contains many millions of individual cells. The higher the number, the greater the risks. You do, of course, offset this risk by learning correct handling techniques and choosing to work with non-pathogenic (safe) organisms. It must, however, be stressed that every micro-organism has the potential to be pathogenic (cause disease). In this unit we will study the types and characteristics of micro-organisms. You will also be introduced to the preparation of culture media for study of bacteria, fungi and *Paramecium*. *Paramecium* is a microscopic protozoan that is found in fresh water rich in decaying matter. The overriding safety principle must be that every micro-organism is potentially harmful. Therefore, you must never be tempted to leave a culture or contaminated equipment where untrained personnel may handle them. The best answer to this is to decontaminate and sterilize as soon as possible. However, this may not always be possible. In which case the equipment and cultures should be labelled clearly as hazardous and stored in a safe, designated area until time is available for sterilization. You have already studied about safety measures in course LT-01 of this programme. In this unit you will also be introduced to decontamination and disposal methods.

Objectives

After studying this unit. You should be able to:

- identify bacteria and microbial fungi by their body form (morphology),
- explain the characteristics of bacteria and microbial fungi,
- list the nutritional requirements and the factors affecting growth of micro-organisms,
- maintain and preserve the stock cultures of microorganisms in the laboratory,
- decontaminate used glasswares and dispose off the microbial cultures.

9.2 CHARACTERISTICS AND TYPES OF BACTERIA AND FUNGI

9.2.1 Characteristics

Bacteria are prokaryotic organisms. Some of the characteristics of bacteria are as follows:

a) They are unicellular
b) Have a variety of shapes
c) Have a variety of feeding habits: such as parasitic, saprophytic and autotrophic.
d) Reproduce asexually (binary fission)
e) Vary in their gaseous requirements. Some are aerobic (require oxygen) for extraction of energy from the food i.e., respiration and others are anaerobic (don't require oxygen for their energy needs for life processes)
f) Some are motile (are capable of movement) and others are non-motile (not capable of movement). Movement is by the use of flagella.
g) Can be differentiated through the use of different staining reactions such as gram stain.
h) Grow in groups called colonies on solid medium.

Fungi are eukaryotic organisms. The basic characteristics are as follows:

a) Some are unicellular. Most are multicellular.
b) They are either parasitic or saprophytic in food habits.
c) May have a sexual or asexual reproduction. Asexual reproduction is mainly by spores, sometimes by flagellate motile cells.
d) They grow as colonies on solid medium.

9.2.2 Types

Bacteria have size range from 0.5 to 15 μ in diameter. They are of the following types (Fig. 9.1) based on their forms.

Coccus: Round or oval forms. They can occur in pairs (Diplococci), in chains (Streptococci) or in groups or clusters (Staphylococci)
Bacillius: Rod like or stick-like with rounded or square ends.
Spirillum: These are thin regularly coiled organisms.
Vibrio: Small curved like commas. They may have a flagellum.

Some bacteria possess flagella. On the basis of number and position of flagella bacteria can be of the following types:

i) Monotrichus: Single flagellum at one end, e.g., *Cholera vibrie.*
ii) Lophotrichus: Many flagella at one end e.g., *Spirillum undulla.*
iii) Amphitrichous: Flagella arise from both ends e.g. *Spirilla.*
iv) Peritrichous: Flagella present all round the cell e.g. *Salmonella typhosa.*

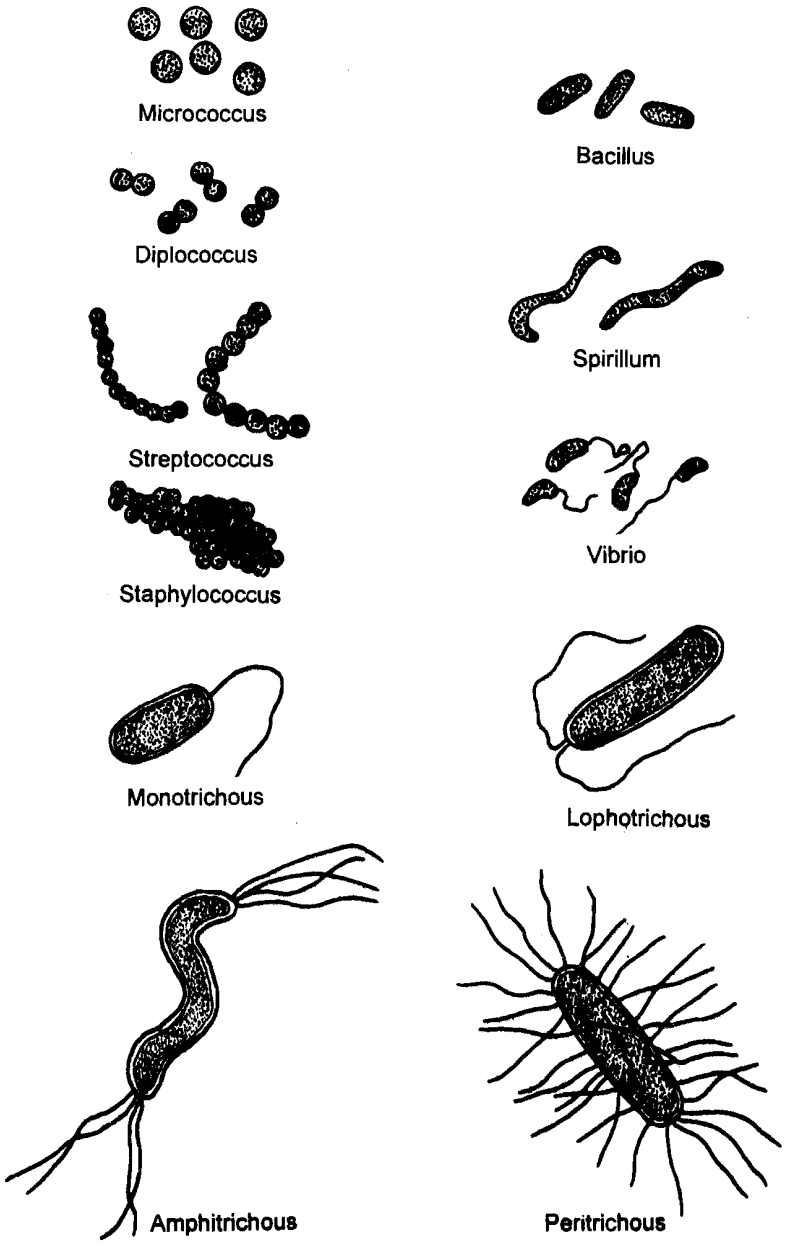

Fig. 9.1: Types of Bacteria. Also shown is different types of flagellation.

The majority of the *fungi* are microscopic except for some like mushrooms and puff balls that are visible to the naked eye. Certain fungi can have single-celled bodies e.g. yeast (Fig. 9.2 a). However, most fungi are multicellular and their bodies is made up of filamentous structures called hyphae (Fig. 9.2 b). These hyphae branch freely and join each other to form a tangled mass called myclium e.g. Penicillum (Fig. 9.2 c).

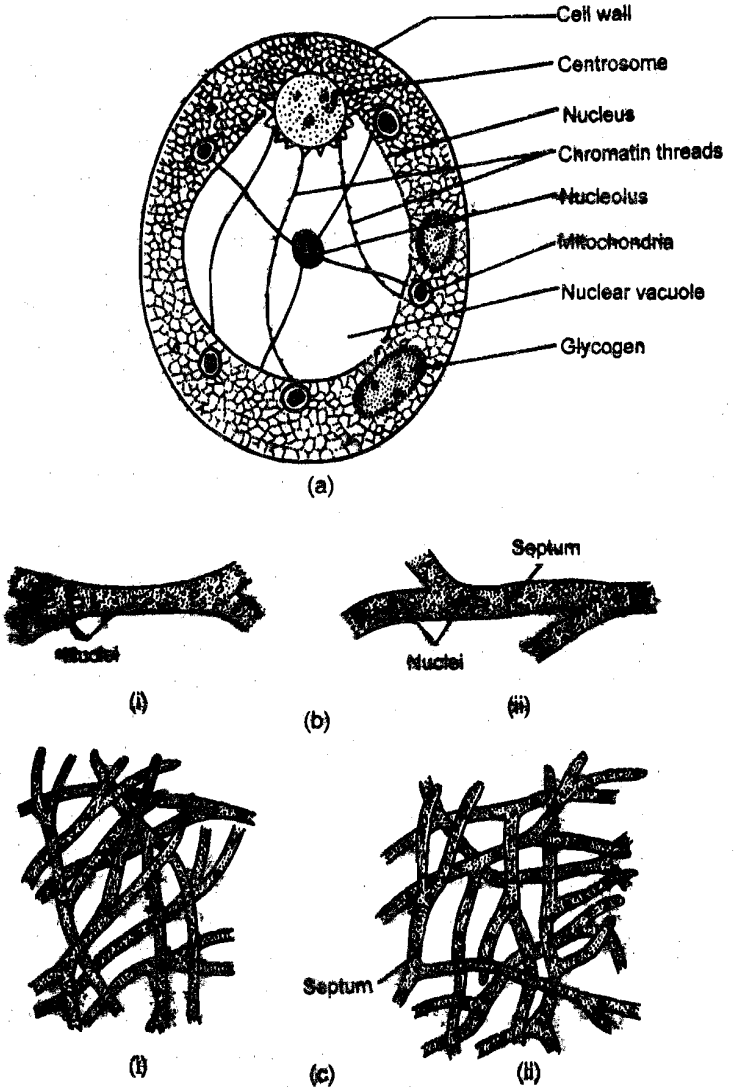

Fig. 9.2: (a) Yeast (unicellular fungus), (b) hyphae, (c) Mycelium.

Let us now do the following SAQ

SAQ 1

1. List three characteristics that are common to bacteria and fungi.

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2. List the types of bacteria according to their shape.

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3. Draw the structure of fungal mycelium.

9.3 FACTORS AFFECTING GROWTH OF MICROORGANISMS

Bacteria and fungi are no different from ourselves: in order to grow they require a combination of the correct nutrients and the correct physical conditions. You should note, however, that there are many species of bacteria and fungi, each one requiring its own specific growth conditions. It is possible, nevertheless, to describe those nutrients which are commonly required by the common micro-organisms likely to be met in the laboratory. The individual nutritional needs of a particular species often required in identification of single type of micro-organisms are not discussed here. We will also discuss the factors that affect the growth of the microbes.

9.3.1 Common Nutritional Requirements

(1) *Carbon (C) Source*

Carbon is an essential element that is required both in organic as well as inorganic forms by living beings. Four chief types of C-containing organic compound i.e. fats, proteins, nucleotides and carbohydrates are required by living beings. These compounds are essential for cell structure as well as cells' metabolic activities, e.g. for energy production. Microorganisms which need carbon in the form of such organic compounds are termed *heterotrophs. Autotrophs* on the other hand, take up carbon dioxide, the inorganic form of C, and build up their own organic compounds. All fungi and the majority of bacteria display heterotrophic nutrition, whereas all green plants are autotrophs.

(2) *Nitrogen (N) Source*

Nitrogen is a constituent of proteins and nucleic acids. Proteins are composed of amino acids and are important both as structural components of cells and as enzymes. Nucleic acids carry the hereditary information of the cell. Dietary nitrogen may be obtained from one of three sources.

(a) Inorganic salts, e.g. nitrates or ammonium salts.
(b) Atmospheric nitrogen gas.
(c) Organic compounds, e.g. amino acids.

Commonly, a bacterium or fungus will require a mixture of inorganic and organic nitrogen. Only a very few bacteria can utilize *atmospheric* nitrogen and these are termed *nitrogen-fixing* bacteria.

(3) *Hydrogen*

Hydrogen is an essential component of organic compounds and, of course, water. It is obtained either by splitting water or as a component of organic compounds of the diet.

(4) *Oxygen*

Oxygen is also an essential component of organic compounds and water. It is obtained from the atmosphere or as a component of dietary organic compounds.

(5) *Phosphorus*

Phosphorus is found in some important organic compounds, for example combined with lipid in the cell membrane. It is also involved in the production and storage of energy. Again, it may be acquired as a pre-formed component of an organic molecule or as an inorganic ion, commonly phosphate.

(6) *Sulphur*

Sulphur is a component of some amino acids. Micro-organisms obtain it in the form of sulphate ions or from the sulphur containing amino acids: cysteine and methionine.

(7) *Trace elements*

Other inorganic ions are required in small amounts and so are termed trace elements. These are required for various metabolic activities for example as the components of the enzymes. These include ions of sodium (Na^{+}), potassium (K^{+}), magnesium (Mg^{+++}), copper (Cu^{++}), calcium (Ca^{++}), zinc (Zn^{++}), manganese (Mn^{+++}), iron (Fe^{++}), cobalt (Co^{++}) and chloride (Cl^{-}).

(8) *Growth Factors*

Some organic compounds may be required in very small amounts to encourage growth. They may be essential components of some enzymes. As such they are termed *growth factors* and are the vitamins.

Finally, it should be remembered that water is an essential nutrient, primarily as a solvent to provide a medium for chemical reactions to take place.

This is only a general picture of nutrition and we must bear in mind that each species of fungus or bacterium has its own individual requirements.

9.3.2 Environmental Factors

Micro-organisms will only be found where prevailing environmental conditions are adequate for growth. Every bacterial and fungal species has its own specific range of conditions within which it will grow and outside which it will die. These physical conditions have a direct limiting action on the metabolism of microbial cells in that they control the rate and efficiency of a cell's chemical reactions. Most species of micro-organism encountered within

the laboratory will share predictable, narrow environmental growth conditions, but micro-organisms as a whole display a surprisingly wide range of adaptation to extreme physical conditions.

(1) *Temperature*

Each microbial species possesses a characteristic temperature range, determined by a maximum and minimum temperature, within which it can grow. The best growth and reproduction occurs at an *optimum temperature* which is a specific value within this general range.

The majority of micro-organisms encountered in the lab will be able to grow within the temperature range of 25-40°C. At these levels, cellular metabolism is most efficient because the controlling enzymes can function properly. Optimum temperatures differ amongst common bacteria and fungi. Fungi grow best at 26-30°C whereas bacteria from the human body prefer 37°C. Some bacteria can grow well at temperatures below 20°C, often growing in refrigerators at 4°C and some bacteria require high temperatures of 55-80°C.

(2) *Oxygen*

Oxygen may be considered an environmental growth factor as well as a nutrient. Although it is an essential nutrient for all micro-organisms, their requirement for *gaseous atmospheric* oxygen varies. Strict or *obligate anaerobes* can only grow in the total absence of atmospheric oxygen: Oxygen gas kills them. They can only obtain oxygen as a component of organic compounds. In comparison, obligate aerobes can only grow in the presence of atmospheric oxygen. Fungi are obligate aerobes. They require oxygen to grow. Many bacteria are *facultative*, that is, able to grow both in the absence or presence of oxygen. Certain bacteria grow best at oxygen concentrations below that found in the normal atmosphere.

(3) *Carbon dioxide*

All bacteria require the presence of a small amount of carbon dioxide and this is usually provided by the metabolism of the bacteria themselves. Some bacteria require a much higher concentration, 10-20% in the surrounding atmosphere.

(4) *pH*

pH is a measure of the hydrogen ion concentration of the environment. Most common bacteria grow best at neutral pH or very slightly alkaline pH of 7.2-7.6. Some prefer a considerable degree of acidic pH 6.0-6.5, and are termed *acidophilic*. Fungi generally are slightly acidophilic.

(5) *Osmotic pressure*

Micro-organisms, as with all plant and animal cells, possess a semi-permeable plasma membrane. They are susceptible, therefore, to the movement of water in or out of the cell across the membrane, in response to the osmotic pressure of the environment. If sodium chloride (NaCl) concentration is taken as a measure of osmotic pressure of the environment,

then most species prefer a concentration of 0.5 to 0.9% NaCl, although wel able to withstand a slightly higher concentration of NaCl.

(6) *Light and other radiations*

Ultraviolet radiation from direct sunlight or a mercury vapour lamp can be bactericidal (kills bacteria) and fungicidal (kills fungi). Micro-organisms are best grown, therefore, in darkness. Other radiation will also destroy micro-organisms, and can be used as agents of sterilization.

Now try the following SAQs.

SAQ 2

1. List the three elements which are the most important components of organic compounds.

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2. List three potential sources of nitrogen for micro-organisms.

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3. Match the terms with the correct definitions.

Terms		Definitions
1) Obligate anaerobe	(a)	grow on acidic pH.
2) Optimum temperature	(b)	is killed by atmospheric oxygen
3) Facultative bacterium	(c)	induces best growth and reproduction
4) Acidophilic	(d)	grow both in the absence and presence of oxygen.

9.3.3 Culture Media

When the micro-organisms are grown on laboratory medium, they are called a culture. Culture medium is a mixture which is made by mixing all the essential nutrients in appropriate amounts. The ingredients of a culture medium consist of essential or basic nutrients, which are required by all micro-organisms, and additional ingredients, which vary accordingly to the type of bacteria or fungus that is being cultivated or isolated. A variety of media have been devised for specific purposes in microbiology. Let us read about some of these.

Simple medium also known as **minimal medium (MM)** contains basic essential nutrients of a carbon source, a nitrogen source and inorganic salts and supports the growth of undemanding microorganisms.

Complete medium (CM) contains additional amino acids, nucleotides and growth factors and is used when MM is not enough to support the growth of the microbe.

Basal medium (BM) is the simplest form of laboratory medium for example, peptone water.

Selective medium has an additional inhibitory substance in the MM, e.g., antibiotics that kill some types of microbes and let other types grow so that these can be isolated from the mixture of microbes.

Indicator medium has substances added to the MM that change colour due to the metabolic activities of particular organisms. This helps in identifying the microbes.

These media are chemically defined synthetic media prepared exclusively from pure chemicals so that their exact composition is known.

Routine laboratory medium is routinely used for microbial cultures. Though the exact chemical composition of such medium is not known, it provides the correct nutrients. Aqueous extracts of potatoes and meat are some such type of media. Such media are known as non-synthetic media.

There are two further points which need to be considered:

(1) A medium may be *liquid* or it may be *solid*. In the liquid medium the growth of bacteria and unicellular fungi and other unicellular microbes looks like a soup. The colonies of multicellular fungi grow in the form of balls, each ball being an isolated colony growing from a single spore. If we require single colonies of unicellular micro-organisms it is necessary to use a solid medium. Solid medium can be made by putting *agar* into the ingredients of a liquid medium. Agar is an extract, in powdered form, of some species of seaweed. It has gelling or setting properties like gelatin. It dissolves in water by heating to above 90°C, and causes the medium to set when it cools to below 45°C. Agar is chemically inert, neither encouraging nor inhibiting the growth of micro-organisms.

(2) Laboratory media are routinely made from a list of ingredients – no different from following a cake recipe. The ingredients are weighed out, dissolved in water and distributed into appropriate containers. Often, a particular medium can be made simply by mixing a commercially manufactured powder, consisting of a mixture of all ingredients, with water. This is quicker but more expensive. Urea broth, MacConkey agar, Sabouraud agar are some of the examples of the culture media available commercially. However, the art of cultivation and isolation is to know the correct medium for the particular bacteria, fungi or other microbes under examination.

Now try the following SAQ to check what you have learnt in this section.

SAQ 3

Define the following types of media:

(1) Basal medium

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(2) Indicator medium

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(3) Selective medium

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9.4 *PARAMECIUM* CULTURE (HAY CULTURE)

Paramecium belongs to phylum protozoa. The type of medium required to culture any protozoan in the laboratory depends upon its habits and habitat. As said earlier in this unit the important factors responsible for successful culture of any protozoan would be:

- adequate/abundant supply of food.
- required inorganic and organic substances in suitable amounts, and
- appropriate temperature required for growth and reproduction.

Paramecium can be found in fresh water that is rich in decaying organic matter. In the lab a small amount of pond water containing *Paramecium* is added to the boiled and cooled mixture of wheat grains and hay. The optimum temperature for *Paramecium* culture is between 22°-25°C. Hay culture technique will be discussed in the experiments. (Fig. 9.3)

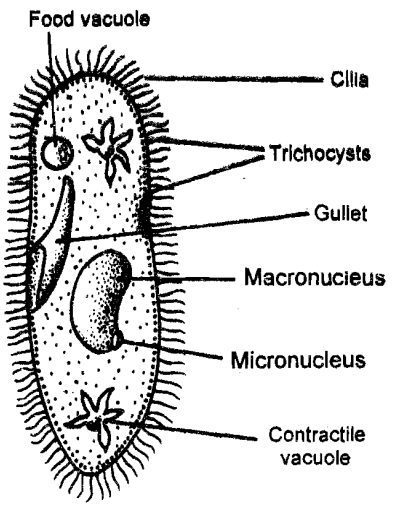

Fig. 9.3: Unicellular protozoan *Paramecium*.

State the main ingredients of culture medium required for growing *Paramecium*.

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9.5 MAINTENANCE AND PRESERVATION OF CULTURES

The large collection of microbial cultures kept in the laboratory from which micro-organisms are taken for study from time to time are called **stock-cultures**. These cultures need to be maintained and preserved properly so that all the characteristics of the microbes are conserved. The problems associated with improper maintenance and preservation can be: growth of mixed microbial populations in the culture, which means that culture is no more pure; inability of the old cultures to grow and reproduce, etc. In the following sections we will study the ways to maintain and preserve the cultures.

9.5.1 Maintenance

The only reliable way to maintain purity of a stock culture is to repeat a set routine of the following steps each time you handle them:

(1) The stock cultures should be handled and stored quite separately from other cultures.

(2) If the stock culture is to be used in an experiment, it should be sub-cultured into two culture tubes. One is incubated, then stored in a refrigerator to act as the new stock, the other is used as the supply for the class.

(3) Simultaneously, a sample of the original stock should be plated (streaked) out on solid medium in petriplates to check for signs of contamination. ('Streaking' will be demonstrated and practised in your practical session).

(4) **A culture used in an experiment should never be used again as the stock.**

(5) **Most stock cultures can be maintained quite successfully at 4°C in the refrigerator.**

(6) Most bacteria and fungi can be kept as stock cultures on agar slopes in screw- capped bottles. These should be inspected at regular intervals so that when the agar is to dry out, they may be sub-cultured on fresh medium.

9.5.2 Preservation

You may want to preserve cultures for long periods whilst ensuring that they do not change in any way. Some micro-organisms, especially fungi, do change with age, e.g. they may lose the ability to form spores.

The methods of preservation fall into two groups:

(1) Preservation in the moist state; and
(2) Preservation in the dried state.

Moist State

In this technique, time to time subculturing of microbes is required. Therefore, it is best to use media which require as infrequent sub-culturing as possible. This has two advantages:

(1) A reduction in the likelihood of contamination, since each time a culture is used the risk of contamination rises; and
(2) Prevention of the likelihood of mutants arising which might replace the original culture.

Two such commonly used media are:

(1) *Semi-solid nutrient agar*: That is routine nutrient agar but with the agar content reduced to 0.75%, producing a semi-solid state. The medium should be inoculated right to the bottom of the bottle that is screw-capped. Sub-culturing is required every 10 weeks.
(2) *Cooked medium*: This is produced by boiling small pieces of ox heart in an alkaline solution draining off the fluid and placing the meat in screw-capped bottles. The meat is then covered with nutrient broth and then sterilized. This medium provides anaerobic conditions at the base and aerobic conditions at or near the surface. Another medium that can be used for such culture is made by using cooked and mashed potatoes. This also requires sub-culturing.

Dried State

Dried cultures have an advantage over moist cultures as sub-culturing is eliminated in these cultures. The micro-organisms are unaltered during the process, and if successfully dried, such cultures may remain viable for years.

The commonest method is *lyophilization* which means rapid drying of cultured micro-organisms from the frozen state under high vacuum. The high vacuum rapidly draws off the moisture, while the frozen state prevents the detrimental effect of an increase in salt content which is caused due to the elimination of water from the liquid state.

Lyophilization or 'freeze-drying', however, requires a specialized and expensive piece of laboratory equipment known as a freeze-drier. In practice, use of moist cultures should be adequate to meet most laboratory requirements.

For a detailed explanation of the methods and applications of freeze drying you can read the reference books.

Now try the following SAQ to check your progress.

SAQ 5

1. What is meant by purity of the culture?

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9.6 DISPOSAL METHODS

You all know that micro-organisms are agents of diseases and infections and are also capable of destroying materials like wood, textiles etc. Therefore, it is very important to inhibit their unwanted growth or kill them. Decontamination of equipment/apparatus/glassware used in the experiments and disposal of cultures are the activities that control the growth of the micro-organisms. You have already learnt about autoclave, incubator and oven and how to operate these instruments in this course. These are the equipment essentially used when disposing off the microbial cultures and sterilizing the apparatus/glassware.

9.6.1 Decontamination of Equipment

Contaminated glassware should be immersed for a period of 18 hours or overnight in a suitable disinfectant. These glassware are washed with water again soaked in a soap solution for 2-3 hrs and then washed thoroughly using soap solutions. Finally these glassware are subjected to dry heat at about 160°C in a hot air oven for about over night i.e. 16 to 18 hours. Some equipment like glass pipettes that do not come into direct contact with microbes will need to be decontaminated, or at least placed somewhere safe during performing an experiment. For decontamination laboratory discarded jars or pots containing a suitable disinfectant should be available at the time of doing the experiment where such glassware can be placed immediately after use. The glassware that are directly used in handling the micro-organisms need to be autoclaved before being put into the disinfectant. It is because these equipment come into direct contact with microbes.

Some of the general properties of disinfectants are as follows:

1. Disinfectants are chemicals which act by destroying living cells, including our own! They therefore, require careful handling.
2. As disinfectants can be both damaging and toxic two other factors must be considered when choosing a disinfectant:
   (a) The nature of the article to be treated; and
   (b) The use to which the article is put.
3. Once made up to the correct dilution, the potency of the disinfectant falls with time and will also be reduced by the presence of organic material, e.g. dead bacteria, soil, other chemicats etc. Therefore, solutions must be changed regularly. You must, follow the manufacturer's instructions also.

9.6.2 Disposal of Cultures

No viable microbial cultures should leave the laboratory. Cultures in glass bottles, test tubes and glass petriplates should be destroyed in an autoclave or pressure cooker. Loosening the bottle caps beforehand will ensure adequate penetration of heat. Many cultures will be in plastic petri dishes which may also be autoclaved (This is beyond the scope of a pressure cooker).

All the culture material should be taken out from plates, tubes and bottles and placed in labelled, disposable bags and then incinerated. The emptied glassware should then be dipped in the disinfectant overnight, washed and oven dried before reuse.

Incineration is a form of dry heat sterilization, which requires culture to leave the laboratory. Though these cultures are autoclaved, the culture material is nutrient rich and can have viable cultures, so proper care should be taken to avoid the exposure, which means disposable bags carrying the cultures should be adequately sealed. The incineration should be supervised by trained laboratory staff and not left to non-laboratory staff who might be quite unaware of the contents of the bags they are handling. If incineration facility is not available in the institution, the next best way of disposal of cultured material is to bury it. (You will practise the way of disposal of culture material in the practical session).

9.7 INOCULATION ON SOLID MEDIUM

Large bacterial and fungal colonies can be obtained by inoculating these on agar surface of the solid media. There are two ways of inoculation on solid medium.

i) **Point inoculation:** Fungal spores or bacterial cells are taken from the stock culture on the tip of a sterilized dissecting needle (sterilized tooth-picks can also be used) and inserted at a point in the medium. This process is repeated thrice, each at a different point on the medium. Care should be taken that these inoculation points are almost at equidistance from each other. For this one can make three point marks on the back of the petriplate containing the medium. Two important precautions are:

   (a) The plate containing the solid medium should be inverted while inoculation. This is because the extra spores or cells will fall on the working bench and not spread on the plate.
   (b) For every inoculation a sterilized needle should be taken.

ii) **Streaking:** A loopful of spores or cells from the stock culture are taken on the tip of the inoculating metal loop and spread over the agar plate. Again two precautions are to be taken:

   (a) The sides of the plate should not be touched.
   (b) The loop should not be streaked back but only in a forward direction in such a way that every streaked area is almost perpendicular to each other. This is because by the time loop reaches the farthest end, the number of spores or cells is almost negated. The advantages of streaking (spreading) is that you can obtain/isolate single colonies. These single colonies are genetically pure colonies because they arise from one spore or cell. In the practical class you will do the inoculations. After point inoculation and streaking with microbes the culture plates are kept in the incubator set at the temperature required for their growth. You have already studied about the incubator and how to operate it earlier in this course. You will learn to raise the fungal culture by the streaking method when you go for practical work.

1. Why should you take a fresh disinfectant every time you decontaminate the glassware?

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2. What do you mean by inoculation?

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9.8 SUMMARY

In this unit you have studied that:

- Bacteria and fungi have varied morphological forms on the basis of which you can identify these. *Paramecium,* the unicellular protozoan, that is found in fresh water body containing decayed organic matter, can be cultured in the laboratory.
- Like all the living organisms including humans, microbes also have common requirements for nutritional supplements and growth conditions. Ofcourse there can be specific variations in the conditions for individual species. Microbial species have specific range of conditions required for growth outside which the microbes will die. The nutrients required for the culture of microbes can be mixed and prepared in the form of different types of media.
- Micro-organisms are preserved in the laboratory in the form of stock cultures from which these organisms are taken from time to time for study. These stock cultures need to be repeatedly cultured so as to maintain the purity of the cultures. Maintenance is an important activity also because microbes of old cultures tend to lose some of their characteristics such as reproduction etc.
- Since microbes can be pathogenic it becomes important to kill these organisms by decontaminating the glassware used directly or indirectly (not by direct touch) in handling these microbes. It is also important to kill the cultured microbes by autoclaving and disposing the killed cultures by incineration or burying.

9.9 TERMINAL QUESTIONS

1. List six trace elements.

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2. Which of the following statements is incorrect?
   (1) Water is an essential nutrient
   (2) Phosphorus is a trace element
   (3) Growth factors are the components of some enzymes
   (4) Nitrogen is a constituent of amino acids
3. pH is a measure of ..... (tick the correct answer)
   (1) Environmental osmotic pressure
   (2) Hydrogen ion concentration
   (3) Oxygen concentration
4. Micro-organisms grow best in darkness because
   (1) The absence of light helps maintain a constant temperture.
   (2) Lack of sunlight enables optimum enzyme action.
   (3) The absence of sunlight means an absence of ultraviolet radiation.
5. Where can you find *Paramecium*?

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6. Differentiate between preservation and maintenance of culture.

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7. Why is it necessary to decontaminate the glassware and dispose off the microbial cultures?

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9.10 ANSWERS

Self Assessment Questions

1. 1) (a) Unicellular
      (b) Have a variety of feeding habits
      (c) They both have asexual reproduction
   2) *Coccus, Bacillus, Spirillum, vibrio*
   3)

2. 1) Carbon, Hydrogen, Oxygen
   2) Three sources of nitrogen:
      (1) Inorganic salts, e.g. nitrates or ammonium salts
      (2) Atmospheric nitrogen gas
      (3) Organic compounds, e.g. amino acids.
   3) (1) b; (2) c; (3) d; (4) a.
3. (i) Basal medium is the simplest form of medium such as peptone water.
   (ii) Indicator medium helps to identify the microbes. Chemical substances are added to minimal medium that change their colour due to the metabolic activities of particular micro-organism.
   (iii) Selective medium has inhibitory chemicals such as antibiotics added to it. These chemicals allow only certain microbes to grow and kill others. Required microbes can be isolated from the growing ones.
4. Wheat grains, Hay, Water.
5. A pure culture is the population of cells that have been derived from a single parent cell.
6. (1) At each time of the decontamination process the potency of disinfectant is reduced by the presence of materials such as media components, dead bacteria, fungi, soil, chemicals etc. from the dipped glassware.
   (2) Transfer of a miniscule amount of microorganisms from stocks to fresh culture medium for getting fresh cultures is called inoculation.

Terminal Questions

1. Any six from the following:

   Ions of Sodium
   Potassium
   Magnesium
   Copper
   Calcium
   Zinc
   Manganese
   Iron
   Cobalt
   Chloride
2. (2) is incorrect. Phosphorus is not a trace element.
3. (2) is correct: pH is a measure of hydrogen ion concentration.
4. (3) is correct. The absence of sunlight means an absence of ultraviolet radiation.
5. *Paramecium* is found in fresh water body that is rich in dead and decayed organic matter.
6. Preservation means storing the cultures for a long time in such a way that the microbes retain all their characteristics whenever they are cultured again. Maintenance means repeated culturing of the microbes to maintain the purity of cultures.
7. The microorganisms have the potential to become pathogenic if they are not already pathogenic. Therefore, allowing uncontrolled growth of any microbe is a great risk. So the need for decontamination and disposal arises.

EXPERIMENT 1 HANDLING COMMON LABORATORY EQUIPMENT

Structure

1.1 INTRODUCTION

Each science lab i.e. Physics, Chemistry and Biology is equipped with equipment. In this unit, you will study about the equipments which are necessary for a biology lab. A lab technician should know about various types of equipments and their uses. As a lab technician you should also know about the working and maintenance of equipment.

Objectives

After performing this experiment you should be able to:

- use a pressure cooker and autoclave for sterilisation,
- operate hot air oven and water-bath,
- handle a centrifuge,
- operate laminar air flow system,
- use microtome knives.

1.2 MATERIALS

Autoclave
Pressure cooker
Microtome knives
Dissecting kit
Hot air oven
Incubator, Water bath, Centrifuge

1.3 PRESSURE COOKER

In Unit 3, you studied the working principle of a pressure cooker. In this experiment you will study about the procedure to use a pressure cooker.

Procedure

1. Pour 300 ml water into a cooker.
2. Place the container with materials to be sterilized inside the cooker and close the lid.
3. Lock the cooker making sure that it is sealed properly.
4. Place the cooker on a burning stove.
5. See that the air is expelled from inside the cooker and a clear and continuous steam comes out from the vent tube. Fit the vent weight on the vent tube immediately.
6. A hissing sound is heard in about 5 minutes and the vent weight (pressure regulating device) will produce a whistle. If there is no hissing sound after 5-7 minutes and the steam is seen escaping around the rim, it means the lid has not been properly fitted. In this condition take the cooker off the stove, adjust the lid with thumbs pressing down slightly.
7. Now the steam will lift the vent weight and will be expelled from the vent tube producing a loud hissing sound. This will indicate that the full cooking pressure (sterilizing pressure) has been reached.
8. Allow the cooker to remain on the stove for atleast 15-20 minutes for sterilization.
9. Later, take the cooker off the stove and allow it to cool of its own for a sufficient period without removing the lid or vent weight (Fig. 1.1).

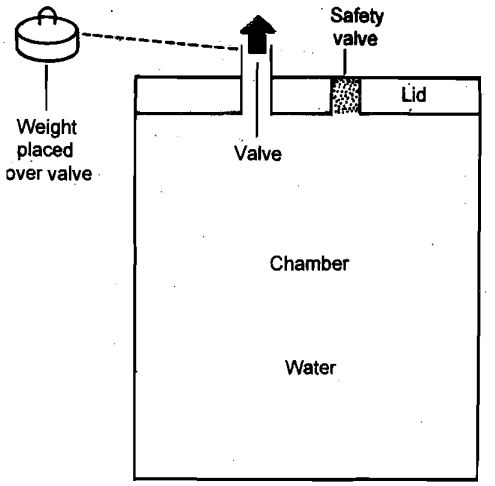

Fig. 1.1: Pressure Cooker.

1.4 AUTOCLAVE

These days sterilization is very conveniently done in the laboratories with the aid of an autoclave. These are of different types such as: (1) *Simple autoclave* (2) *Steam jacketed autoclave* and (3) *Automatic autoclave*.

You have studied the structure and design of autoclaves in Unit 3. In this experiment you will know how to use an autoclave.

1.4.1 Simple Autoclave

The following steps are to be carried out at the time of using an autoclave:

Procedure

1. Pour a sufficient amount of water in the autoclave.
2. Set the safety valve, put the articles to be sterilized in the container and load it inside the autoclave.
3. Open the steam outlet for passing it out.
4. Lock the door by tightening the bolts diagonally.
5. Set the autoclave pressure.
6. Place the autoclave on heat source ensuring that heat is maximum.
7. See that air is expelled from within the body of the autoclave and a continuous stream of steam comes out from the steam outlet. Now shut the steam outlet.
8. Allow the autoclave to come to the required pressure and adjust the amount of heat so that the needed pressure is maintained and then time it.
9. Let the materials remain at a necessary pressure for a definite time. Later cut the heat source and allow the autoclave to cool.
10. Open the steam outlet when the needle reaches zero in the pressure gauge.
11. Before opening the door of the autoclave and taking out articles which were sterilized, let the autoclave cool down for a considerable time (complete cooling is necessary).

Precautions

1. Do not open the valve before zero is reached because the boiling liquid will dampen the cotton wool plugs and there will be chances of contamination by bacteria which may enter through the moisture film upto the medium.
2. Do not open the valve when a vacuum has developed within because the air will rush suddenly inside the autoclave and will carry loose fitting plugs into the autoclave.
3. Do not allow the vacuum to remain for a long time because it will take out moisture from the medium kept inside the autoclave for sterilization.

1.4.2 Steam-jacketed Autoclave

Procedure

1. Allow circulation of steam through jacket continuously at a required pressure.
2. Put the articles inside the autoclave when the jacket has attained a working temperature.
3. Shut the door, lock it in position and see that discharge outlet, provided at the bottom of barrel, is open.
4. Close the discharge outlet when required temperature is reached. (In automatic jacketed autoclaves the discharge outlet is themostatically controlled which automatically closes when predetermined temperature is reached). Any drop in temperature due to condensation of steam during sterilization helps in opening of outlet. The condensed steam goes to waste allowing the fresh steam to enter inside the barrel and predetermined temperature is reached again.
5. Allow steam to enter from steam jacket through baffle fitted at the back of barrel.
6. Count the period needed for sterilization after required temperature has been reached and discharge outlet valve has closed (15-20 minutes are needed for sterilization of media).

7. Cut down the supply of steam to barrel after 15-20 minutes of sterilization and allow it to cool.
8. Open the door of autoclave when the pressure inside has become atmospheric and take out the sterilized articles (Fig. 1.2).

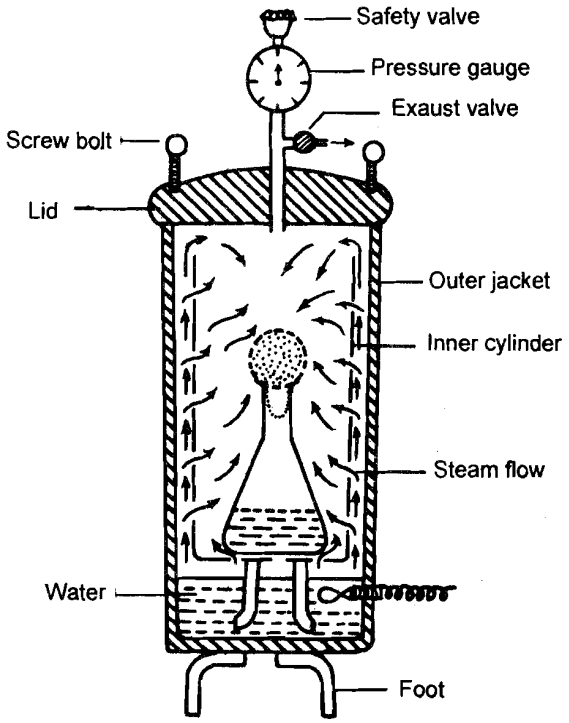

Fig. 1.2: Diagrammatic representation of an autoclave.

1.5 HOT AIR OVEN

You have studied about the structure of a hot air oven in Unit 3. In this experiment you will learn how to use one.

Procedure

1. Sterilize only dry petridishes singly or together in a metal can. If a metal can is not available, wrap the petridishes with paper. Do not bring out the sterile petridishes from can or unwrap them till they are used.
2. Flasks and tubes should be dried and plugged with cotton wool before sterilization. The tube should be placed together in iron, steel and heat-resistant glassware e.g. enamel tray, test tube stand and beaker.
3. Pipettes should be plugged with cotton wool at the mouth end after air drying and placed in an oven in a closed metal cylinder.
4. Do not exceed the prescribed temperature.
5. Do not overload. These may prevent air circulation and the glassware may not be properly sterilized.
6. Allow the temperature to rise upto 160°C and continue sterilization at this temperature for one hour.
7. Do not open the door of the oven immediately after the sterilization because glass may develop cracks due to sudden fall in temperature.
8. Use a towel to remove the glassware (Fig. 1.3).

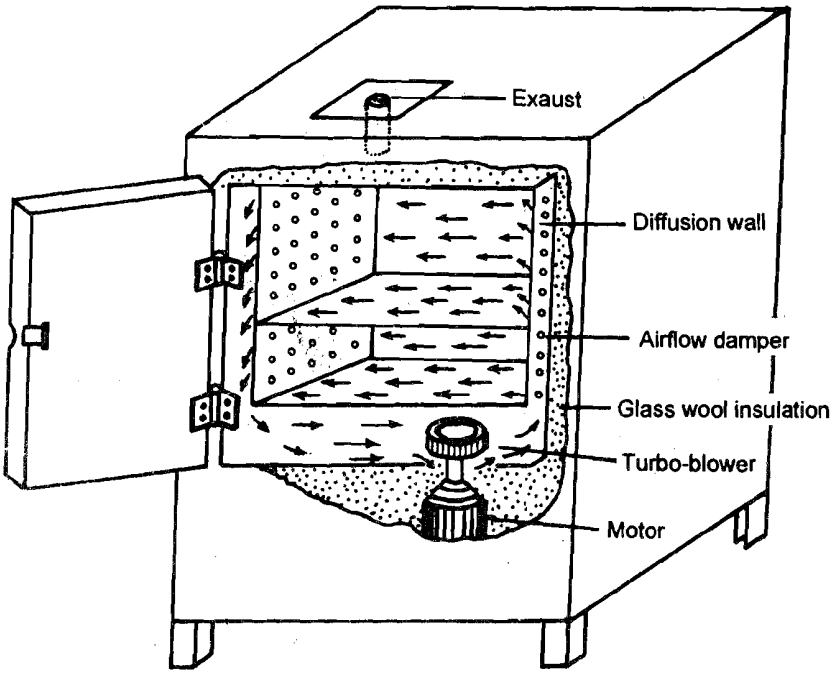

Fig. 1.3: Diagrammatic representation of hot air oven.

1.6 INCUBATOR

It is an electrically operated equipment designed to provide a controlled temperature for the growth and development of microorganisms in culture media. Its construction and operation are more or less the same as those of a hot-air oven. Only the operational range of temperature is lower in an incubator which lies between room temperature to a temperature of 50°C.

Incubators, hot air ovens and water baths require accurate temperature control. Required temperature in such apparatus is maintained by a thermostatic system.

1.7 WATER BATH

The liquid contents of tubes or flasks kept in a water-bath are raised to the required temperature much more easily and much more rapidly than in an incubator. The difference in the level of water in the water-bath and that of the liquid in the tube or flask causes a convection current which makes the liquid of the tube mix well and hasten reactions. A water-bath is equipped with thermostat, stirrers and cooling device. It is advisable to use distilled water in a water-bath to avoid chalky deposits on tubes.

1.8 CENTRIFUGE

A centrifuge is an important equipment of the biology lab. In most biological experiments generally low-temperature centrifuges should be used. This can prevent metabolism and loss of viability or enzyme activity during the operation of the centrifuge. You have studied about the structure of various types of centrifuge in Unit 3.

Precaution during centrifugation

1. Tubes must be put in pairs to balance.
2. Be sure that rubber cushions are in position at the bottom of the bucket before inserting the tubes.
3. Be sure that cotton wool plug if used are not forced down during centrifugation.
4. Make sure that the metal buckets are properly sealed on the ring and are free to swing.
5. Secure the lid properly.
6. Bring the rheostat to zero before start. Gradually increase the speed and bring the speed to required rpm.
7. After centrifugation, switch off the meter and then bring the rheostat to zero position allowing it to come to a stop. Do not apply hand to slow down the speed.
8. Take care to lubricate periodically.

1.9 LAMINAR AIRFLOW SYSTEM

Handling of microorganisms under a bacteriological safety cabinet may not always be suitable for many practical reasons.

To keep such a room free of all microbes carrying particles, a new kind of technology has been developed. This technique is known as laminar airflow technique. In this technique, air of a closed room or cabinet is allowed to pass through a high efficiency particulate air (HEPA) filter pack and the filtrate becomes free of all particles above 0.3 μm dimension. The technique involves sucking in room air and blowing it out through a bank of fitters with uniform velocity and in parallel flow line used in microbiological and pharmaceutical laboratories and in aerospace industries.

The advantage of the system is that in operation involving inoculation, transfer of culture and in opening of lyophilized culture, no closed chamber is necessary, instead, that operation can be done on a platform provided with the laminar airflow unit making it easier for handling (Fig. 1.4).

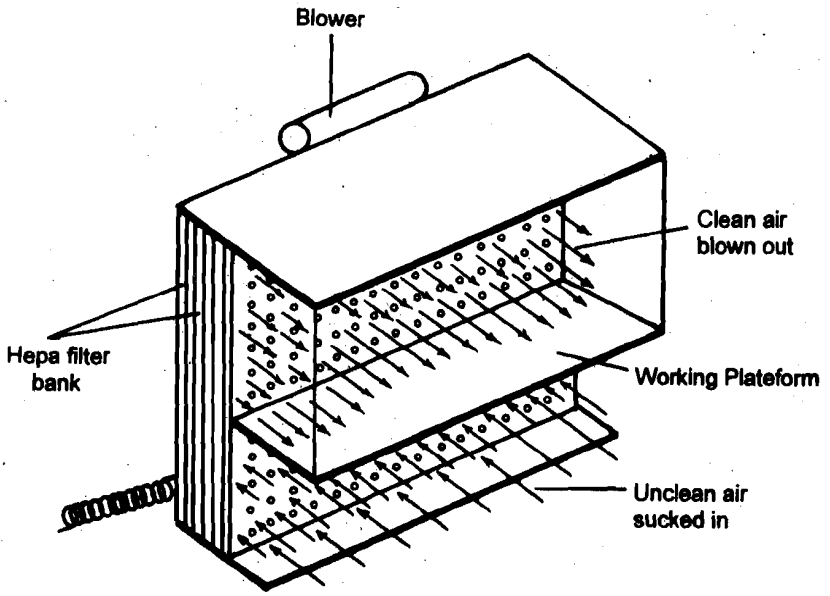

Fig. 1.4: Schematic diagram of a horizontal laminar airflow unit.

1.10 MICROTOME KNIVES

There are three basic cross sectional shapes of knives, wedge, plano concave and double concave, about which you have studied in Unit 3.

The best knives for the cutting of paraffin blocks are the double concave type, which should be of a heavy pattern cross-section to prevent vibration, i.e. the taper should be short and the base wide back down, to repeat the stroke. The knife is also moved laterally along its length as the stroke is made.

Strops are made of shell horsehide and require only a little light oil rubbed into the back of the leather occasionally to keep them in condition. There are strops which are fixed (hang) on a wood block to keep them rigid, and these require strop dressing applied sparingly to the front at intervals.

Hones are made of stone which is of an extremely fine texture and which is ground to a true flat face. Stones too are delicate and should be used over the full area of the face so that no inconsistencies develop in the glass-flat surface. Hones are generally dressed for sharpening with a little fine oil or water. Whether they are water or oil stones is stated on the manufacturer's instructions.

For **honing**, the action is exactly the same as that of stropping except that the knife edge is the other way round. Whereas the stroke for stropping is always made back first, the stroke for honing is always made edge first. Clearly if the blade were stropped edge first, the strop would last about a microsecond! If the honing is done the same way as the stropping, a foil edge is produced by the drawing off of material from the edge of the knife which is being ground.

The blade gradually becomes blunt in use. The care of blade should be taken properly, even if they are not being used.

Ordinarily, all the blade needs between cutting is stropping, which involves rubbing it on a leather strap made for the purpose, but if it becomes nicked it must be honed on a stone or plate glass hone. For stropping and honing, a knife is generally, though not inevitably, fitted with a 'back', which is a length of channel which slides over the thick part of the knife along its length, which increases the effective thickness of the back. The additional back determines the angle which the blade will make with a strop or hone when it is laid flat on either (Fig. 1.5).

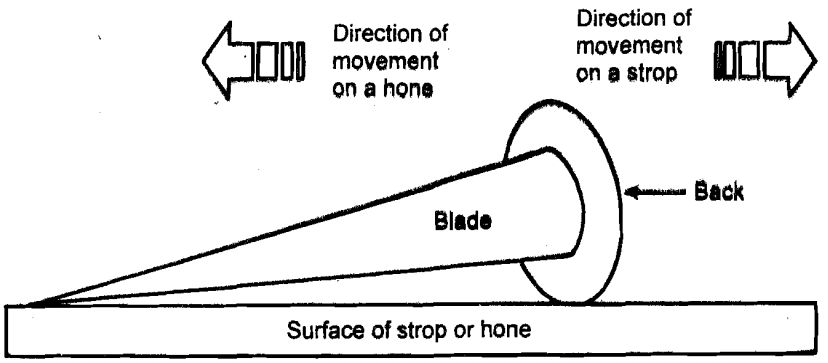

Fig. 1.5: Cross-section of Blade and 'Back'.

To strop a blade, it is placed over the strop and moved, back first from the near end to the far end of the strop whilst pressing down on the blade. The blade is then turned over, keeping the back on the leather, and the knife is drawn from the far end to the near end and then turned again keeping the back down to repeat the stroke. The knife is also moved laterally along its length as the strike is made (Fig. 1.6).

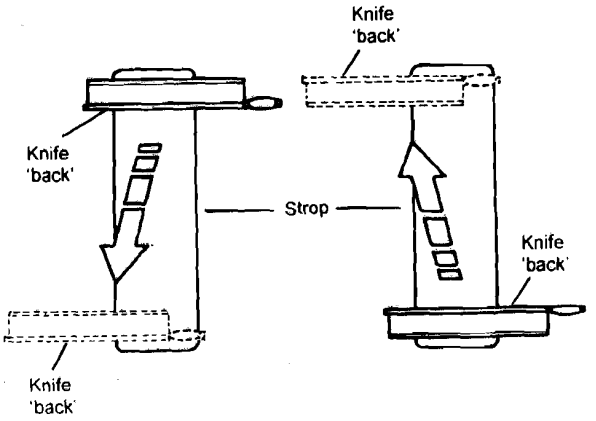

Fig. 1.6: Stropping the Knife.

1.11 DISSECTING KIT

Different components of dissecting kit are shown in the following diagram (Fig. 1.7).

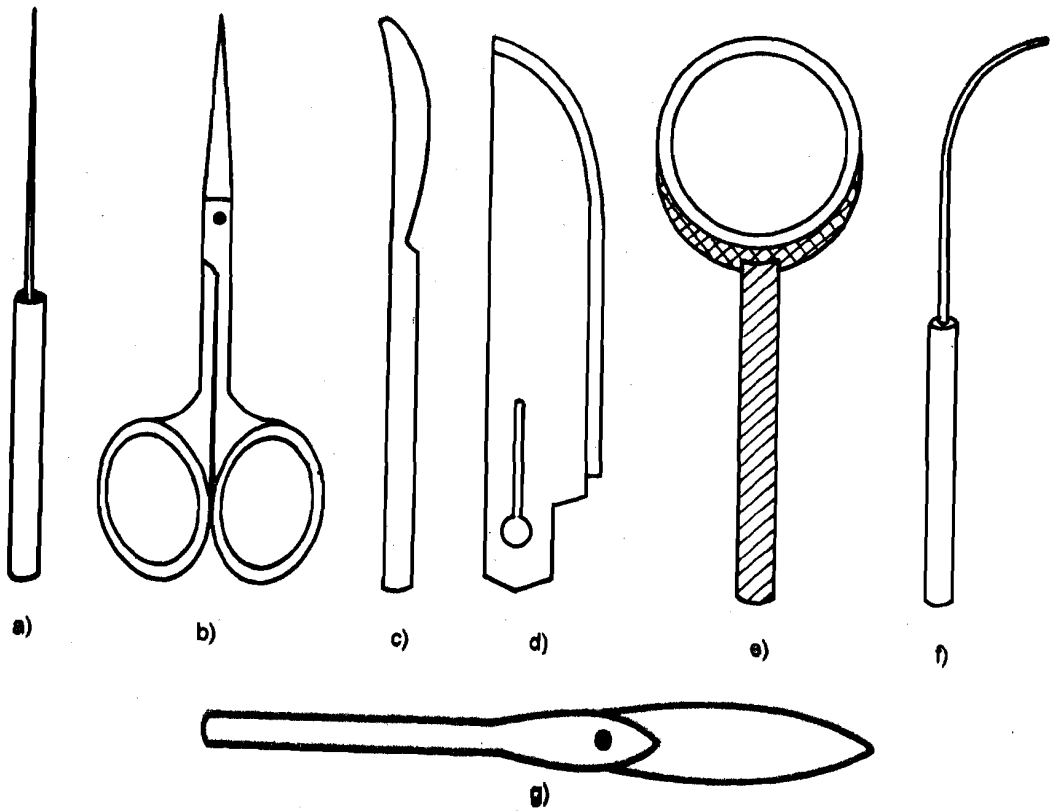

Fig. 1.7: Dissection kit components.

a) **Forceps:** for lifting/picking specimens or parts of specimens.

b) **Seekers:** for probing into the animal being dissected without causing displacement of the organs.
c) **Scalpels:** for cutting or making an incision on a specimen.
d) **Scissors:** for cutting specimens/small bone.
e) **Hand lens:** for magnifying the details of specimens.
f) **Dissecting needles:** for teasing out specimens/holding skins/appendages on dissecting board.

Though each student possesses his/her own dissection box some dissection boxes should be available in the laboratory. One dissection box should be compulsarily kept for the instructor. All the dissecting instruments should be clean and dry to use. Rusted instruments should be thrown off. After the dissection, instruments should be thoroughly washed and wiped clean to prevent rusting.

Dissecting trays : Dissecting trays should also be kept in a cabinet in the laboratory or in the store-room. It should be seen that wax is layered properly in the tray and there should be no leakage in the tray otherwise dissection cannot be done properly.

You will study in detail about the preparation for dissection in exp. 10 of this course.

You will study details of microscope handling and maintenance in exp. 7 of this course and temporary slide preparation in exp. 9 of this course.

1.12 SELF ASSESSMENT QUESTIONS

1. How will you use a simple autoclave?

.....

.....

.....

.....

.....

.....

.....

2. How will you do honing and stropping with microtome knife?

.....

.....

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.....

.....

3. What precautions should be taken while doing centrifugation?

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EXPERIMENT 2 LABORATORY ORGANISATION

Structure

2.1 INTRODUCTION

In Unit 2 and 3 of the LT-01 course, you have learnt about lab organisation and day-to-day management. In principle a well designed, organised, and aesthetically appealing lab is attractive to everyone. Besides, it is convenient to use, easy to clean and maintain. It elevates the mood, increases the enthusiasm of students and teachers alike, and is conducive for working. Unfortunately, many of us tend to neglect this aspect and do not make an effort in this direction.

Whether you will be allowed to reorganise a lab or not, through this exercise you will become aware of the approach and guidelines used for organising it. Besides, you may get a few tips for organising other places such as- home, work place, factory etc. and we would like that whichever place you are allowed to implement your ideas you can take the opportunity and do so.

In most jobs a well-organised place is primary for efficient functioning. Organising a place is almost always a collective activity and a lab is also organised by the joint effort of people using it. Though there are a set of guidelines common for most laboratories and dos' and don't are well-defined, but each lab is unique in its set-up and reflects the talent of the users.
It is seen that many people are uncomfortable at the thought of rearranging a place because it is quite demanding and involves hassles. Therefore they simply put up with any amount of disorder and lack of cleanliness. This attitude is undesirable and we must strongly discourage it.

In this exercise you will be doing the following activities with regard to laboratory organisation:

Activity 1 To plan the organisation of a laboratory
Activity 2 To display general instructions for laboratory use
Activity 3 To label specimens, reagents and slides

Objectives

After doing this exercise you should be able to:

- appreciate the need for an aesthetically organised, clean and well maintained lab,
- develop an attitude and talent for organising a laboratory,
- help others in organising and maintaining a laboratory,
- display instructions for the users of a lab so that it could be used effectively, conveniently, and safely,
- display instructions and necessary precautions required for using sophisticated instruments,
- prepare labels for biological specimens, slides and reagent bottles.

2.2 ACTIVITY 1 TO PLAN THE ORGANISATION OF A LABORATORY

It is important for a technician to participate in lab organisation since he/she is given the responsibility for its day-to-day management. He/she is expected to know about everything that is present in the lab and its purpose. In this activity you will learn how to plan the organisation of a lab.

First prepare a comprehensive account of the items 1 to 10, listed below. Examine the lab in which you are conducting the practical and find out what is available. To save your time we have listed the things commonly available in most labs. Add the missing things to complete the list and strike off what is not available. With regard to each item it is important to consider the size, number and suitable place for keeping them. You should classify small items and put them together.

2.2.1 Accommodation

No. of Main labs

Preparation room

Store

Office

Museum

Any other (animal house, green house, herbarium etc.)

.....

.....

.....

2.2.2 Persons to be Accommodated

Number of Permanent staff

Number of Teachers

Number of Supporting staff

Number of Research students

Number of Batches of students

Number of classes

Any other
.....

2.2.3 Furniture

Teacher's table, Chair

Work Tables/ benches

Revolving stools

Almirah for storage

Filing cabinet

Slide cabinet

Open shelves

Office tables

Any other
.....

2.2.4 Apparatus/Equipment

Dissecting Microscopes

Compound Microscope

Microtomes

Spectrophotometer

Incubators

Refrigerator

Centrifuge

Hot air oven

Hot plate

Water bath

Magnetic stirrer

Balances (physical and chemical)

Electrical balance

Distillation plant

Knife sharpner

Shaker

Autoclave

Pressure cooker

Safety cabinet

pH meter

Any other
.....

2.2.5 Glassware

Beakers

Test tubes

Conical flasks

Round bottom flasks

Volumetric flasks

Distillation flasks

Pipettes

Burettes

Measuring cylinders

Jars

Bell jar

Dessicators

Troughs

Petridishes

Watch glasses

Microslides

Cover slips

Cavity blocks

Any other
.....

It is important to know the quantity in each category to estimate the space requirement.

2.2.6 Chemicals

Solid chemicals

Liquid chemicals

Stock solutions, stains and culture media

Try to prepare a list of the above and estimate the space required.

2.2.7 Specimens, Charts and Models

Write details of each and estimate the space requirement and appropriate place for each.

2.2.8 Miscellaneous Items

Bunsen burners

Spirit lamps

Iron stands

Tripod stands

Wire gauges

Dissecting kits

Any other
.....

Complete the list and try to categorise them.

2.2.9 Provision for Disposal

Garbage cans

Incinerators

2.2.10 Files, Records and Catalogues

Determine the quantity and space available.

Once your list is ready examine the organisation of the lab. See if there is scope for improvement. It is also likely that you may come up with a completely new plan of organisation. In any case you cannot implement your ideas for this lab. Therefore put down your suggestions in table 2.1.

You must consider the following questions before your suggestions.

1. Is the present arrangement satisfactory?
2. Who all use the lab and for what purpose?
3. Is the furniture arranged in a manner that staff and students would be able to move around easily?
4. What items are to be used everyday?
5. Which of the experiments are routinely performed?
6. What precautions are required while placing electrical appliances?
7. Which is the best place for storing glassware?
8. Where could the different chemical (acids/photosensitive/heat sensitive) be safely stored.
9. Is the provision for storage most appropriate?
10. Are the provisions for safety adequate?
11. Is the arrangement of chemicals and equipment adequate for independent student activity?
12. Are the provisions for good housekeeping adequate?
13. **Is the arrangement of furniture, equipment, glassware etc. convenient and serviceable?**
14. **Is the area for conducting certain experiments identified?**
15. **Is it possible for the students to study the displayed charts, models and specimens?**
16. **Is there a convenient arrangement for frequent washing of the glassware by the students?**
17. Is the arrangement for disposal satisfactory?

Now complete the table given below:

List of Items	Placement		suggestions
	Appropriate	Not Appropriate	

After completing this exercise discuss your suggestions with your peer group and counsellor. Remember organising a place is a collective effort.

Remarks:

2.3 ACTIVITY 2 TO WRITE GENERAL INSTRUCTIONS FOR LABORATORY USE

2.3.1 Materials Required

Coloured chart paper
Scissors
Coloured pens
Adhesive tape

2.3.2 Procedure

Displaying instructions /rules is very important for housekeeping, convenience, ease and above all the safety of a lab. Preparing a display is partly a creative exercise since it requires a little bit of artistic ability. A display should be conspicuous, legible, neat, and attractive.

Here we have listed some laboratory safety general guidelines. Choose any 7 instructions that you consider more important than the rest for the display. Cut a chart paper of an appropriate size, write the instructions neatly using a colour pen that can highlight it and try to make it attractive without missing the purpose. When it is ready, display it at an appropriate place.

1. Keep the lab scrupulously clean and free of unnecessary things.
2. While entering the lab, place all books, notebooks and purses in the designated areas and not on the working benches.
3. Do not eat, drink or smoke in the laboratory.
4. Do not make a noise in the lab.
5. Do not wear short or loose clothes in the lab.
6. Always wear a lab coat.
7. Do not perform unauthorised experiments.
8. Do not use equipment without reading the instructions.
9. Report all spills and accidents to your instructor immediately.
10. Never leave heat sources unattended.
11. Keep containers of alcohol, acetone, and other inflammable liquids away from flame.
12. Leave the laboratory clean and organised for the next batch of students.
13. Wash your hands with powdered soap prior to leaving the laboratory.
14. Upon completion of laboratory exercises, place all discarded materials in the disposal area designated by your counsellor.

15. Do not allow any liquid to come into contact with electrical cords. Handle electrical connectors with dry hands. Do not attempt to disconnect electrical equipment that crackle, snap, or smoke.
16. Wash skin immediately and thoroughly if exposed to chemicals or micro-organisms.
17. Never pipette by mouth. Use mechanical pipetting devices.
18. Wear disposal gloves whenever necessary.
19. Use bandage on cuts or scrapes before attending the lab.
20. Do not taste the chemicals.
21. Do not lick the labels.
22. Decontaminate work surfaces after any spill of potentially dangerous chemicals/ micro-organisms
23. Wear safety glasses, gloves or other protective devices whenever necessary.
24. Turn off gas, electricity and water before leaving the lab.

2.4 ACTIVITY 3 TO LABEL SPECIMENS, SLIDES AND REAGENTS

2.4.1 Specimens Labels

Biology laboratories have a collection of a variety of plants and animals. In Unit 3.1 you have learnt how to collect and preserve biological specimens. After the collection it is essential that such specimens are appropriately and attractively labelled. Likewise, certain slides of tissues, organs or micro-organisms prepared by a teacher or student may be essential or valuable for permanent record. These need to be appropriately labelled for proper identification.

Specimen labels should therefore carry the following essential information:

1. Common name of organism
2. Kingdom
3. Phylum
4. Class
5. Order
6. Family
7. Genus
8. Species
9. Place from where it is collected (optional)
10. Name of the collector, date

Sample 1

Sea cucumber
Kingdom – Animalia
Phylum – Echinodermata
Subphylum – Echinozoa
Class – Holothuroidea
Genus – *Holothuria*
Vikas, 20/4/2000

Now prepare a label for any specimen.

2.4.2 Labels for Slides

They should carry the following information:

(1) The name of the organism – if the whole organisms is mounted then the slide can be marked WM = whole mount or E = entire. Examples : *Paramecium* WM, Frog blastula WM
(2) The part of the organism used, e.g. liver, root. Examples : Liver of Frog, Onion root
(3) The type of preparation, e.g. smear; squash: Examples : Human blood/smear.
(4) Type of section e.g. TS = transverse section; VS = vertical section; LS = Longitudinal section. Examples TS of kidney, VS of phloem.
(5) Other specifications e.g. type of cell division (mitosis, meiosis), stage of cell division (prophase/metaphase), sporulation.

The following information is desirable but not essential.

(6) Stain(s) used, e.g. H.E = Haematoxylin, eosin.

If the slide is prepared 'in house' then it should be:

(7) Dated
(8) Initialled

It is common to use two labels, one on each end. Self-adhesive or gummed slide labels pre-printed with lines are available from lab suppliers – alternatively use ordinary self-adhesive or gummed labels. You will find it easier to write the label before you stick it on the slide and remember if the label is gummed (rather than self-adhesive) you must not lick it. Instead use a wet sponge.

Prepare at least one label and stick it to a slide.

Sample Label

Onion root tip,squash
Mitosis, prophase
Vinita Sharma,
20th October, 1999

2.4.3 Labels for Reagents

We also need to label routinely prepared stock solutions, stains and culture media for identification. These should carry the following information.

Name of the Chemical(s)
Percentage/molarity
Aqueous/any other solvent
Date
Initial

Sample label

5% $CuSO_4$ (aqueous)
Lalit, 15/3/2000

EXPERIMENT 3 PROCURING PLANT MATERIAL

Structure

3.1 INTRODUCTION

Laboratory work is an integral part of learning science. In biological sciences living or preserved organisms have to be provided for the study of anatomy, physiology, taxonomy etc. As lab technical staff, you will have to provide various plant materials to the students for experiments. You have to collect lower and higher plants for study. In this unit we are going to describe all the techniques which will help in collection of plants only. For collecting the plants you should know what type of bottles, fixatives, solutions and equipments are needed. You can start by collecting the plants with the teacher who will help you identify them. In due course of time you will also be able to perform this activity independently.

Objectives

After studying this unit you will be able to:

- grow root tips of a few plant species for squash preparation,
- fix the root tip,
- collect algae, bryophytes, pteridophytes and gymnosperms,
- collect, press, dry mount, label, store, and preserve angiosperm specimens to make a herbarium.

Study Guide

Before doing this experiment kindly study Unit 5 of Block 1 of LT-2.

3.2 GROWING OF MATERIAL FOR SQUASH PREPARATION

The root tips may be obtained by allowing them to germinate on a wet blotting paper disc or/sand free from soil and debris. The seeds of *Medicago* (Hindi - Methi) have been found more suitable because these germinate soon and their chromosomes are also bigger in size. In India we generally grow root tips from onion so we are going to discuss both of them one by one.

3.2.1 Material Required

Onion bulbs
Seeds of *Medicago* (Methi)
Coplin jars or wide mouth bottles
100 ml beakers
Scalpel

3.2.2 Procedure

Methi Seeds

1. Soak the methi seeds overnight and then place them in sterilized wet filter paper cover them and leave them to germinate.
2. Cut the root tip, as soon as they are visible between 7-9 a.m. because during this time the cells are actively dividing and the chromosomes also duplicate and separate at the same time.
3. These root tips are ready for squash preparation.

Onion Bulbs

1. Take an onion and scrape off the dry roots from the bulbs to expose the disc.
2. Fill a coplin jar with tap water and place the onion bulb on it in such a way that the disc touches the water.
3. Place this near the window to get enough light for three to four days. Roots will start growing and the roots tips can be clearly seen.
4. The technique for squash preparation will be explained to you later in Experiment-9 of this course.

3.3 COLLECTION OF LOWER PLANTS

The collection of plant material is a simple job but one should take much care of collected plants or their parts so that they are preserved without any damage. The plants may be collected in vasculum, polythene bags or in bottles. You will need a pair of secateurs for cutting hard material, a sharp knife for cutting soft parts, pick for digging out underground parts like roots and rhizomes, scalpel and forceps for separating those plants which grow attached to the barks of trees and rocks.

The stems and roots are cut into pieces of size about 3 cm long with the aid of sharp razor or knife so that the tissues at cut ends do not get macerated. Bryophytes are made free from soil particles and debris before storing in some preservative. The smaller leaves can be preserved as such and larger ones can be cut in pieces, and then preserved.

We will now discuss the collection of algae, bryophytes, pteridophytes and gymnosperms specimens one by one.

3.3.1 Algae

Sources: The algae occur widely in nature viz. on the soil surface and below it, on the bark of trees, in fresh water, sea water, and a variety of other habitats.

Collection from Bark

i) In case of bark algae pickup the algal patches from the tree trunk with the help of iron spatula.
ii) Sterilize spatula by swirling it in spirit and then flaming it.
iii) Store various samples collected in separate sterilized bottles after fixing and labeling in their respective shelves.

Collection from Fresh Water

i) Collect the fresh water algae at the spot in sterilized specimen tubes containing some habitat water.
ii) Never fill the container more than a quarter so that the quantity of oxygen present in the water may be sufficient for respiration.
iii) Fix the material, label them and keep in respective shelves.

Collection from Sea Water

i) In India western coast is best for marine algae collection during low tides of winter season as during this period these are mostly in their reproduction stages.
ii) Collect marine algae in large bottles.
iii) Fix them and label them and keep them in their respective shelves.

3.3.2 Collection of Bryophytes

Now we will discuss the collection of bryophytes. Normally bryophytes occur in nature attached to wet soil, rocks, bark of living and dead trees, wood and humus rich in organic substances.

- Scrape the bryophytes from the place of occurrence with the help of a sharp scalpel or knife and keep them in polythene bags within which they remain alive to a number of days.
- Keep these bags loosely tied and in damp condition in laboratory.
- Wash the soil growing species with ordinary water to remove soil particles and dirt attached to plant.
- Keep the bags under illumination at 0°C - 5°C to keep the plant alive for a longer duration.
- Fix the material, label it and keep it in the cupboard.

3.3.3 Collection of Pteridophytes

Pteridophytes are commonly known as vascular cryptogams, these are spore-producing vascular plants. They possess the vascular tissues xylem and phloem. They grow in variable habitats. Most of the pteridophytes are terrestrial which thrive well in moisture and shade while others are found growing in xeric conditions. A few are epiphytes and some of them are found in aquatic habitats.

- Collect the pteridophytes from natural habitats in mature spore producing stage.
- Collect the plants with or without strobili or mature sporophyll in polythene bags loosely tied at mouth.
- If the material is large cut them into pieces, fix label and keep them in a cupboard.

3.3.4 Collection of Gymnosperms

Gymnosperm belong to seed plants but the seeds are naked with a very conspicuous and independent sporophyte which is the main plant and have very reduced gametophyte dependent on the sporophyte. They have xeric characteristics also.

i) Collect the root, stem, leaves, male and female gametophytes of the plant.
ii) Cut the material into small pieces of 3 cm, fix them, label them and keep them in a cupboard.
iii) You can collect the dry fruits and cones of gymnosperms and preserve them as such.

3.4 COLLECTION OF HIGHER PLANTS FOR MAKING A HERBARIUM

In this section you will study about preserving plants for a herbarium because as a technician you have to make and maintain a herbarium. Some plants are also grown in a botanical garden or specially maintained in green house for this purpose. Theoretical portion of this exercise you will read in Unit 5 of this course.

3.4.1 Collection of Plants

1. You should remember one important thing about collection – **Do not pick plants haphazardly**. Choose only those plants whose flowers are large and whose organs are easily seen.
2. If possible collect complete plants with flowers and fruits (sometimes they are necessary for recognising species of some families such as Cruciferae, Umbelliferae, Compositae revised name Asteraceae).
3. The underground parts (root, bolbs, rhizomes) are often interesting. Try to collect them. If the plant is large take just a branch with leaves, flower and fruits.

3.4.2 Carrying the Specimen

1. Do not mix up or damage the selected plant with others.
2. Place the plant quite flat between newspapers and place in a cardboard folder and fastened in a strap or keep the plant vasculum as you have already read in unit 5 of Block 1.
3. Attach a label to each plant stating details of its dwelling place, date, name of place, flower colour and any other interesting feature.

3.4.3 Examining the Plant

1. Identify the name of the plant with the help of your counsellor in due course of time you will be able to name the plant by yourself.
2. You can also keep a plant with some labelling with it and it could be identified with the help of flora.
3. Make a few sketches to point out the characteristics which helped you in identification of the plant.

3.4.4 Drying

1. Spread each plant carefully in between blotting paper or newspaper (Unit 5 Section 5.5).

2. Then place the plant in the plant press available with its label.
3. After a few days (the next day, if the plants are very moist) check that the plants are properly laid out and change the paper.

Fig. 3.1: Mounted specimens.

4. Check the specimens and if they are properly dried, place them in your herbarium.
5. Place the plant on herbarium sheet (a fairly large piece of special paper or on any fairly thick paper), fix the dried plants in their natural position with some cellotape.
6. If the plant is too big, break the stem cleanly or arrange fragments of the leafy stem, the floral stem and the fruits.
7. Rewrite the label which will be placed in the bottom right hand corner of the page. Copy out the notes you have already made on it. (It is not necessary that you should identify the family and name of a plant). The name and family of the plant can be identified with the help of the counsellor at your study centre.
   i) *Heading*: Country, state or province name, name of the institution.
   ii) *Scientific name* of the specimen followed by the author's name and the name of the family.
   iii) *Locality:* Specific locality should be mentioned, so that if another person wants to collect the same specimen, he should be able to reach the exact site without much difficulty.
   iv) *Habitat:* Vegetation type, moisture content of soil and atmosphere, soil type, elevation, direction of slope, etc. should also be mentioned.
   v) *Date of collection* should include the exact month and year to indicate when the specimen was collected.
   vi) *Name of collector*
   vii) *Collection number*: The literature on plant systematics identifies and refers to the specimen by the collector's name and collection number. Hence, the collection number is a must for any collector.
8. You can arrange the herbarium according to their surroundings (aquatic, from dunes, from mountain rocks) or according to their uses (edible, medicinal, ornamental, fodder plants .....; harmful plants, poisonous plants etc.).

EXPERIMENT 4 PROCURING ZOOLOGICAL MATERIAL FOR LAB EXERCISES

Structure

4.1 INTRODUCTION

One of the important duties of a lab technician is to collect, procure and preserve animal specimens for the use of the students. The live animals are collected with the use of accessories like nets, jars pans etc. from water bodies (aquatic animals) as well as from different land areas (terrestrial animals). The animal specimens are procured from the market. For this you have to place the orders with the dealers. In the previous experiment you have read how plant material is procured and preserved. The ways of procuring specimen and live animals and their preservation is dealt with in this unit. You will also learn how to identify male and female animals by studying the external features.

Objectives

After doing this experiment you will be able to:

- place the order with a dealer for purchase of specimen and live animals,
- collect live animals from their natural environments,
- preserve the surplus animal materials,
- identify male and female animals from their external features, if any.

4.2 PLACING ORDER FOR THE SPECIMENS AND LIVE ANIMALS

Specimens are to be procured from the market. For that you have to first invite quotations in sealed and marked envelopes for the articles to be purchased. The purchase committee in your institution will open all the quotations and decide from which dealer to purchase the specimens and/or live animals. You have already read in LT-01 course that the purchase order is given to the lowest bidder. For this activity two letters are to be written from your institute-one for

inviting quotations and the second for placing the order. The letters are signed by the Head of the institutions or the person designated by the Head. Given below are examples of these letters. The first letter is an example of the format used for inviting quotations from the dealer and the second is an example of a letter for placing the order. There can be certain variations in the formats used in different institutions.

Letter No. 1

Tel. No.....
Fax. No.....

EDUCATIONAL INSTITUTE
Vijay Garden
Ramapur
New Delhi – 110 000

M/s Vinay Chemicals
4, Jawahar Bagh
Delhi – 110020

Ref: EI/quotation/1999-2000/

Dated:

Dear Sir,

Quotations are invited for the supply of the apparatus/equipment as per the enclosed list quoting the price/discount (%) on the catalogue prices and the make of the apparatus/equipment.

It is requested that the following should be carefully observed in every detail while submitting quotations, otherwise they may not be considered.

1. Quotations should be sent under sealed cover addressed to the Principal, Educational Institute, Vijay Garden, Ramapur, New Delhi-110 000, so as to reach him not later than .....
2. The words "Quotations for apparatus" should be written prominently on the envelope.
3. Quotations should be for the supply of articles F.O.R. Educational Institute, Vijay Garden, Ramapur, New Delhi – 110 000.
4. Kindly quote the price/discount (%) you will give on the listed price.

Thanking you,

Yours sincerely,

(V.K. Singh)

Tel. No.....
Fax. No.....

EDUCATIONAL INSTITUTE
Vijay Garden
Ramapur
New Delhi – 110 000

M/s Vinay Chemicals
4, Jawahar Bagh
Delhi – 110020

Ref: EI/quotation/1999-2000/

Dated:

Dear Sir,

Regarding, your Quotation No..... you are requested to kindly supply the following ..... immediately.

Items	**Number**
1.	
2.	
3.	
4.	

Bill may be sent in duplicate to the undersigned for payment.

Yours sincerely,

(V.K. Singh)

4.3 COLLECTION OF LIVE ANIMALS

Live animals are generally collected from aquatic and land areas. For collection visit a nearby aquatic body (eg. pond) and land area (eg. park) along with your counsellor and other students of your group.

4.3.1 Collection of Aquatic Invertebrates

Sources

The sources can be ponds, lakes, rivers for fresh water animals and ocean/sea coasts for marine animals. However, in this experiment, as an example we will study the collection of animals from ponds.

Materials Required

Nylon nets (Fine weave for small animals and coarse weave for large animals)
Large clean jars or buckets
Shallow white pans or papers
Trowel

Method

1. Take a clean bucket or a jar and fill it upto about half with the pond water from which you are going to collect the samples.
2. With a trowel scoop a little amount of mud from the wet edge of the pond and put it in the bucket or jar having pond water.
3. Also put one or two small submerged branches of aquatic plants in the bucket or jar.
4. Take the suitable net and sweep through the water in the pond. You may have to sweep more than once (see Fig. 4.1 for different types of nets)
5. Take out the net. You will see the specimens trapped in the net. Transfer the specimen into the bucket or the jar.
6. Take some extra pond mud, submerged branches or aquatic plants along with some pond water and carry to the laboratory for subsequent use, if needed.
7. In the lab, transfer the live specimens into shallow white pans or place them on a large sheet of paper and spread them out for study.

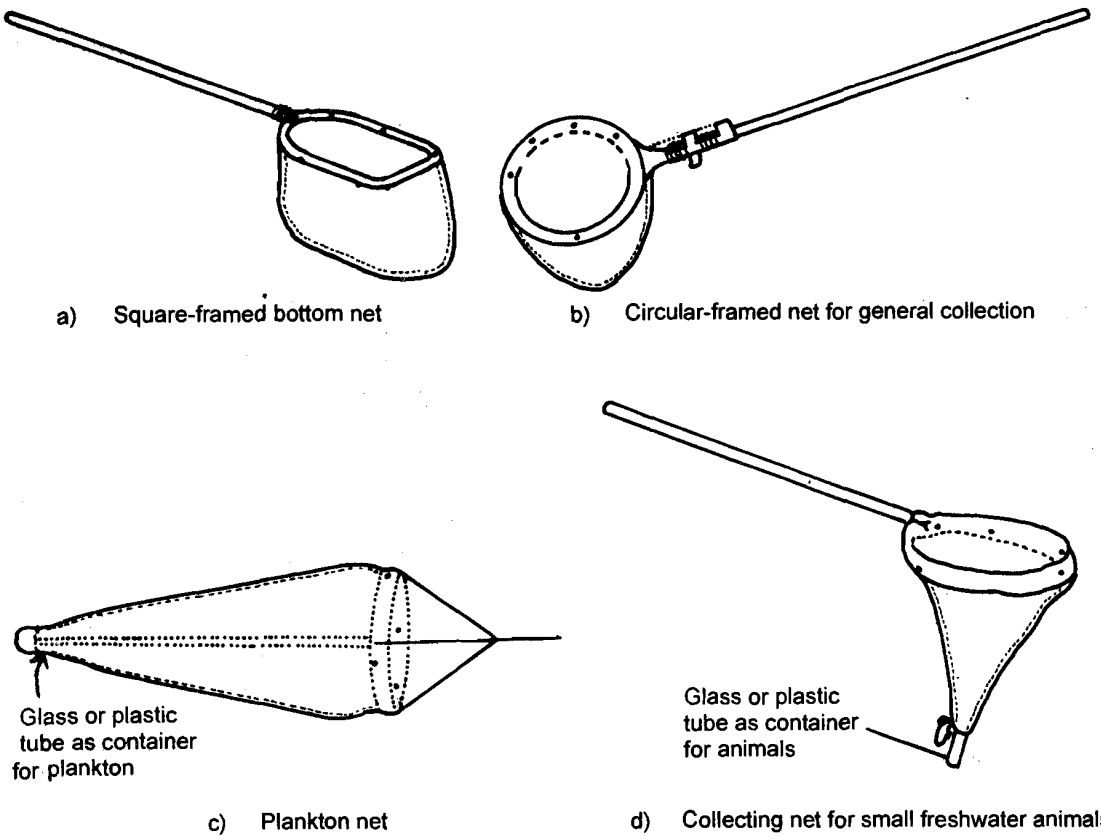


Fig. 4.1: Types of nets used for collection of various animals.

4.3.2 Collection of Earthworms

These annelids can easily be collected from the soil having organic matter especially at night and after a rain, when they come out at the surface of the soil. In winter earthworms are not easily available. So, they are preserved whenever available for use in winters.

Sources

Rich garden soils, lawns, agricultural fields especially after a rainy day or night.

Material required

A bucket
Flashlight torch (for night collection)
Blunt-end forceps

Method

Visit the collection site. Put some moist soil in the bucket. Pick up the worms with blunt-end forceps and put them in the bucket. Use flashlight torch if collection is to be done in the night. Take the worms to the laboratory.

4.3.3 Collection of Insects

Sources

Terrestrial insects are found in gardens especially during flowering seasons, in the fields and ofcourse indoors. Aquatic insects can be collected from water bodies like ponds, lakes etc.

There are several methods of collecting insects but in this experiment you will collect terrestrial insects by three methods using:

a) net,
b) light trap, and
c) aspiration

Methods

(a) Sweep Net Method

This method is suitable for collecting many insects.

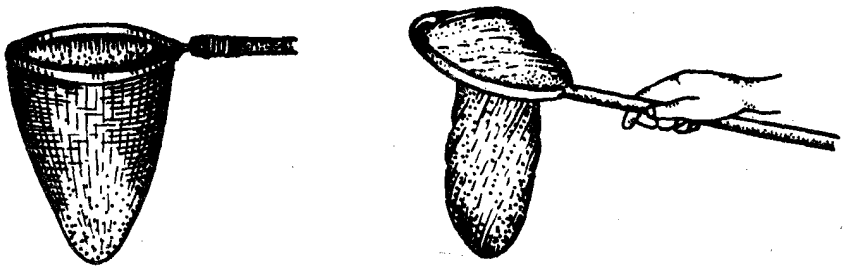
Materials Required

Insect-collecting net
Killing jar

Steps

1. Go to the garden/field and identify the insects to be collected.
2. Approach the specimen(s) very quietly. You should try to avoid chasing the insects overtly as it would alert the insects and make them fly/run away.

3. Sweep the net (Fig. 4.2 a) through the herbage over the specimen(s). You might have to sweep more than once.
4. When the insect(s) is trapped in the net, twist the net or your wrist so that net is closed (Fig. 4.2 b) and the specimen is not able to escape.
5. Transfer the collected insects into the killing jar.



a) Insect or butterfly net

b) Method of holding an insect net when catching an insect

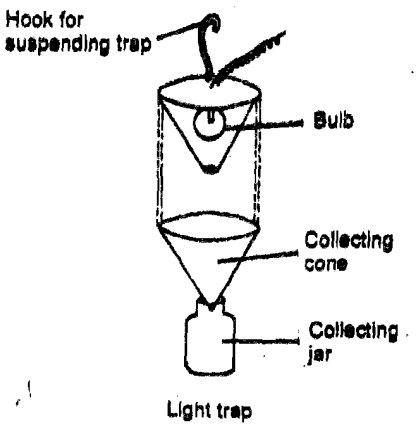
Fig. 4.2: Insect net: It consists of a steel wire loop of 30 cm diameter and a bag of thin muslin or mosquito netting (a). The bag is tapered towards the bottom. The length of the bag should be longer, at least double the diameter of the frame so that with the twist of the wrist the net can be closed on the frame (b).

(b) Light Trap Method

In this method the collector is not required to be present. It is mainly used for nocturnal insects like moths, midges, some beetles and delicate lacewings.

Materials Required

Light sources such as an electric bulb (~ 200 W) or a lantern lamp.
Large shallow container such as a basin sauce pan
White paper sheet
Killing jar



Light trap

Fig 4.3: Light trap for insect collection.

Steps

1. Select an area where insects are in abundance.

2. Hang the light source with the help of a hook.
3. Put the white paper as lining in the shallow container and set the container below the light sources so that electric lamp is shining in the middle of the container.
   (In the absence of an electric light keep a lantern lamp in the middle of the container)
4. Soon the insects will be attracted by the light and fall into the container.
   (In case the shallow basin saucepan is not available you can keep a collecting jar fitted with a cone made of white sheet under the light source as shown in Fig. 4.3. The most efficient light source for insect-trapping is a mercury vapour lamp)
5. Transfer the collected insects into the killing jar.

(c) Aspirator

An aspirator is a simple suction device used for collection of small insects such as mosquitoes, thrips, sandflies etc.

Materials Required

A transparent vial made of glass or plastic (transparent plastic is preferably used)
Rubber stopper with two holes
Two glass tubes each with a bend
Rubber tube
Small piece of muslin cloth

Steps

The aspirator described here (Fig. 4.4) is the one that is most commonly used.

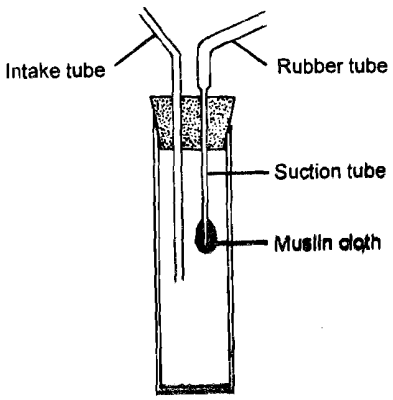


Fig. 4.4 : Most commonly used Aspirator.

1. Insert the two glass tubes (intake and suction tube) through the two holes in the stopper.
2. At one end of one glass tube attach a rubber tube. Cover the other end of this tube by tying a piece of muslin cloth. This tube acts as a suction tube. The other tube is the intake tube.
3. To the open end of the vial fix the rubber stopper (with inserted tubes). The stopper should be tightly fixed in the vial. The end of the suction tube that is covered by muslin cloth should be inside the vial. The aspirator is now ready for use.

4. Place the aspirator with the outer end of its intake tube facing the insect(s) and suck through the rubber tube. The suction creates a partial vacuum in the vial there by drawing the insect through the intake tube. The muslin cloth tied on the inner end of suction tube will prevent the entry of insects into this tube.
5. Plug the outer end of the intake tube to prevent the escaping of the insects caught in the vial and then transfer the collected insects into the killing jar.

4.4 KILLING, MOUNTING AND DISPLAY OF INSECT SPECIMENS

4.4.1 Killing

For killing, the insects are transferred into a bottle, containing killing agent. Though various killing agents such as ethyl acetate, chloroform, ether, tetrachloroethane etc. can be used, the safe and most efficient agent is ethyl acetate. You can make a killing bottle as given below (Fig. 4.5).

Materials Required

An empty glass bottle with an air-tight lid (you can take a jam or horlicks bottle)
Ethyl acetate
Cotton
Blotting paper
Forceps

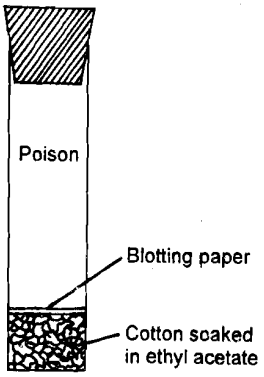


Fig. 4.5 : Killing bottle containing soaked ethyl acetate and blotting paper.

Steps

1. Soak a wad of cotton in ethyl acetate. You must hold this cotton wad with forceps and not with hands.
2. Place the soaked cotton at the bottom of the bottle and cover it with a piece of blotting paper. Blotting paper is used to avoid the direct contact of the specimens with the chemical because it will wet the specimens and spoil them. However, you can pour a few drops of killing agent over the blotting paper to make the bottle more effective. (Instead of cotton wool, plaster of paris can also be used)
3. Transfer the insects into the bottle. Close the bottle tightly. Take out the insects within 20 mins. otherwise they will decolourise and get unduly hardened. Do not overcrowd the bottle with insects. Overcrowding of bottle with tough and fragile insects or large and small insects may cause damage to the insects. You should, therefore, use separate killing bottles for separate types of insects.
4. Label the killing bottle as **'poison'** and keep it away from the reach of the others. The bottles that are no longer be able to kill should be buried or burned.

4.4.2 Mounting

After being killed the insects are pinned with the help of entomological pins on the pinning board. These pins are made of steel and do not rust. Such pins are available in a variety of sizes and thickness. You can also prepare entomological pins with sewing needles and coloured beads. Take thin sewing

needles, heat the eye of the needle on a spirit lamp flame and insert the heated end into a coloured bead. The bead forms the head of the needle. You can make many such entomological pins. Now you know that mounting means pinning the killed insect. It can be done by any one of the methods given below.

You should mount the insects immediately after killing them. If left for a long time in the killing bottle the insects become dry and stiff and one faces difficulty in pinning them. Under such circumstances insects must be first **relaxed**. For this you can make your own **relaxing box** by the procedure given below.

Steps

1. Take a plastic container with a lid of about 2′ x 1′ x 1′ size.
2. At the bottom of the container place a thin sheet of synthetic sponge or any other porous material of 2 to 4 cm thickness. Saturate this material with water.
3. In one corner of the container place a cotton wad soaked in ethyl acetate to prevent the growth of mold.
4. Place a sheet of blotting paper on the inside of the lid to absorb the moisture that may otherwise condense and fall over the specimen.
5. Put the insect in a petridish or envelop and leave those in the relaxing box. Though relaxing time depends on the size and type of specimens, most of the specimens are relaxed satisfactorily when left for one night in the box. Too long a period in the relaxation box will cause damage and discolouration to the insects.

Now follow the procedure for mounting.

(i) Direct mounting

As you have read earlier mount the insects immediately after their death.

Steps

1. The entomological pin is pushed through the thorax region of the insect. However, the exact point in the body of the insect through which the pin should pass differs in the different groups of insects. You can take the help of your counsellor in identifying that point in the insects you have collected.
2. Insert the pin vertically through the body or sloping in such a way that the front part of the body is raised very slightly.
3. Push the specimen up in the pin until its back is about 1½ cm away from the top. This distance helps in holding the pin freely without having any contact with the back of the insect body.
4. Mount these pinned insects on the board or on a pinning block. Take care to mount the insects uniformly so that specimens can be examined and compared easily.

(ii) Point Mounting

This method is especially used for mounting small and dried insects.

Steps

1. Take a stiff card paper and cut triangles from it. For a smaller insect the size of the triangle can be 6 mm long, 2 mm wide at base and 0.5 mm wide at the apex (tip). However, the size of the triangle varies depending upon the size of the insect.
2. Attach the dried specimens to the apical tip of the triangle with the help of a quick drying adhesive like quick-fix. The best places on the insect body for adhesion can be at the sides of thorax below the wings, margin of the tergum and above or between the bases of the legs.
3. Insert the entomological pin in the broader end of the triangle and pin this triangle with mounted insect on the display board.

(iii) Spreading

To display the head, abdomen, wings and legs you have to spread the freshly killed insects on the spreading board. In the freshly killed insects the internal parts are soft to allow the pin in and appendages are pliable. The pin is pushed through the thorax region. A spreading board is available in the market but can also be made locally. A simple method is to take a thick sheet of cork or thermocol and cut a groove in it for the body of the insect.

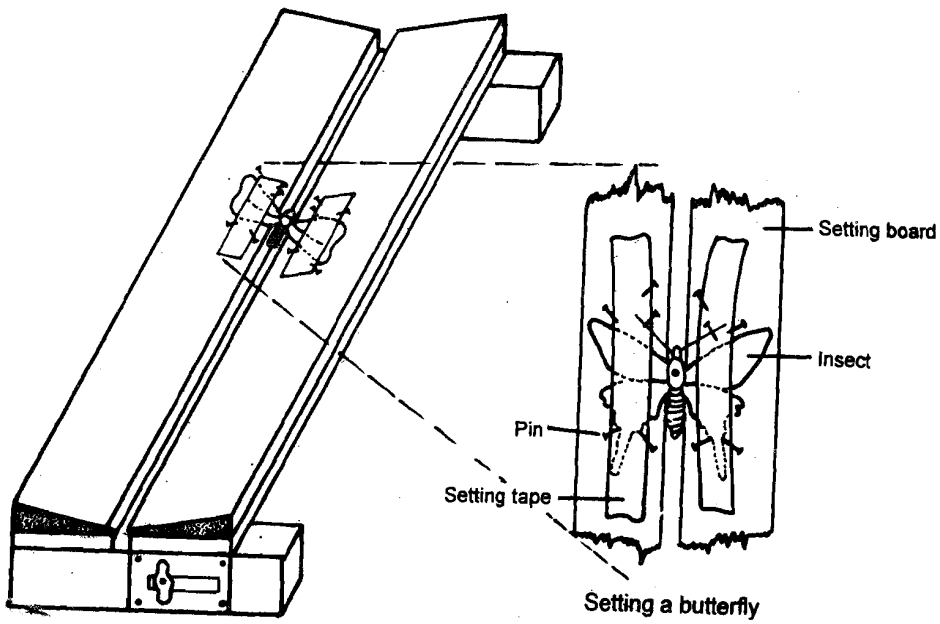


Fig. 4.6: Spreading of butterfly on the spreading board. The body parts of the butterfly are properly spread and set on the board.

Steps

1. The insect is placed in such a way that the body and thorax of insect rest in the groove of the board.
2. One end of a narrow strip of setting paper is pinned at the front end on each side of the insect body.
3. The fore wings on the back are drawn forward and each pinned on either side with a fine pin inserted behind one of the strong veins in the wings.
4. The hind wings are also spread like this and pinned.

5. When the wings are correctly placed the paper strips can be taken over the wings and their other end is pinned on the back of the insect body so that both the wings are held by paper strips and setting pins.
6. The antennae are also spread symmetrically and pinned under the narrow strip.
7. Legs (appendages) are also spread and pinned on both sides under the strips. Care should be taken that while spreading, the joints and the shape of the appendages remain intact.
8. If the abdomen is inclined to fall into the groove it can be supported by crossed pins placed beneath it.

After the pinning and spreading the specimens are dried for few weeks in the open or in drying chambers and stored (see Fig. 4.6 for spreading the insect butterfly).

4.4.3 Displaying

Once the specimens are collected and spread, they should be given permanent labels. Proper mounting, spreading and displaying are necessary for taxonomic studies such as the identification and study of external morphology of the insects. These labels should be small and made of white card. The following information should be there on the label of each specimen:

1. Name of the insect.
2. Host plant, crop or the area from where it is found.
3. Locality from where it is found.
4. Date
5. Collector's name (See Fig. 4.7)

The ink used for writing should be permanent and not spoiled when in contact with any type of liquid.

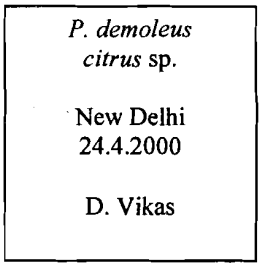


Fig. 4.7: Label for display of insects.

The spread board along with spread insects with labels should be displayed in wooden boxes with glass tops. The mounted insects should also be stored in closed boxes. You must keep napthalene balls in the storing or displaying boxes used for insect specimens. In case these precautions are not taken the specimen insects can get spoiled or eaten by other insects/small animals.

4.5 PRESERVATION OF ANIMAL MATERIAL

Once you have purchased the animals from the dealers or collected them yourselves, you might end up having material in surplus. In such a situation you will need to preserve these animals for further use at a later time. In this

section you will study the way to preserve certain animals that are normally used in the school or college lab.

4.5.1 Preservation of Specimen Animals

Material Required

Animal specimens
40% formalin
Glass jars with lids
Big containers with lids

Steps

1. All the museum specimens are stored in 40% formalin in glass jars or containers with lids.
2. Earthworms, scolidon, rat and pigeon heads, *Mystus*, prawns, *Sepia*, *Pila* etc are also stored in 40% formalin in the containers.
3. For *Pila* you need to make a hole in the shell before keeping it in the formalin so that formalin enters the mantle cavity and internal tissues are preserved.
4. Use different containers for different types of animals e.g., all earthworms in one container, scolidons in another container and so on.

All these preserved animals can stay fresh for 3-4 years.

4.5.2 Preservation of Live Animals

The live animals cannot be preserved for long. The methods for keeping animals alive differ for different animals and are as follows. The material requirements for specific animals are written beside them.

- **Leeches** are kept in glass containers filled with fresh water. Water should be changed everyday or on alternate days. The water used should be clean and free of dirt.
- **Cockroaches** are kept in a plastic or metal jar. The jar must have many small holes so that air is available for their respiration. For feeding the insects small pieces of paper are put in the jar. It is advisable that fresh paper pieces are put in everyday.
- **Rats** are kept in rat cages. For feeding they need to be given bread pieces. If the rats are to be kept for a longer period i.e., few days, they should be kept in separate cages, otherwise they will hurt each other and die. Prior to dissection the rats are killed with chloroform and treated with disinfectants like phenyl, dettol solution etc.
- **Frogs** are kept in a sink that is covered with a wooden or plastic plank having a few holes. The water tap should be adjusted to allow the water to fall into the sink drop by drop only. This keeps the skin of the frogs moist for respiration. (In some institutions a cemented tank that has water and certain aquatic plants is built. The cover of the tank has big holes for air circulation. The frogs are kept in this tank. Here they live and breed. Within the tank area a few cemented elevations of different heights and flat top surfaces are also made. So when the frogs need to be out of the water they can jump and sit on these elevations).

4.6 IDENTIFICATION OF MALE AND FEMALE ANIMALS

In the theory part of this course you have already read about the general characters of the animal phyla one generally handles in the lab. In this experiment you are going to identify and make a distinction between males and females of certain types of animals. You must be aware that lower invertebrates generally do not exhibit external variations in males and females. Also a few of them have both male and female sex organs. Let us now study some of the examples.

- **Earthworm and Leech:** Both are hermaphrodite i.e., the animal has both male and female sex organs.
- **Cockroach:** The female cockroach has two processes i.e., anal cerci at the abdominal end. The male has four processes at the abdominal end: two anal cerci and two anal styles.
- ***Pila, Sepia* and Prawn:** Males and females of *Pila* and *Sepia* cannot be distinguished externally. However, male prawns show appendix masculine in the pleopods (swimmerets i.e. abdominal appendages).
- ***Scolidon*:** In males pelvic claspers are present. However, it is not easy to distinguish between male and female fish.
- **Rats:** The male rat has one external opening and possesses scrotal sacs. The female rat has two openings – vaginal and anal opening and no scrotal sacs.
- **Frogs:** Male and female frogs cannot be distinguished externally.

4.7 SELF ASSESSMENT QUESTIONS

Now that you have performed various activities of this experiment, you will be confident in doing these activities as and when they are part of your assignment. Attempt the following SAQs to recapitulate your knowledge.

1. Write a purchase order to buy two specimen animals from the dealer.

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2. What type of light source is best for trapping insects?

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3. Differentiate between direct and point mounting procedure used for insect specimens.

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4. Take any two animals and write the distinguishing characteristic of the male and females of these animals.

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5. Write the preservation method used for one live and one dead animal.

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EXPERIMENT 5 SETTING OF DEMONSTRATIONS OF PHYSIOLOGICAL PROCESSES IN PLANTS

Structure

5.1 INTRODUCTION

Certain experiments require special equipment and are tedious to set up. Instead of asking students to perform such experiments individually, demonstrations are set up for the class in such a way that each student can record, interpret and analyse the readings and present the results. The job of demonstrating experiments is assigned to a technician. In this exercise, you will learn to set up demonstrations for the following three physiological processes:

1. Transpiration
2. Respiration
3. Photosynthesis

Objectives

After doing this exercise you should be able to:

- list and procure equipment and materials required to set up a given demonstration,
- set up the demonstrations for transpiration, respiration and photosynthesis,
- improvise suitable alternative equipment and material for these demonstrations, if need be,
- identify faults in the set-up and correct them and
- help students in recording the readings.

5.2 TO SET A POTOMETER

The loss of water by a plant through stomata or other pores is called transpiration. The instrument used to show the process and measure its rate is called a potometer. In this exercise you will set up a potometer.

5.2.1 Materials Required

Potometer
250 ml beaker
Iron stand with a clamp
2 ml graduated pipette
Razor, sharp knife or scissors for cutting a branch
Rubber bung
A piece of rubber tubing (about 6 cm)
Stop watch
Eosin or any other water soluble dye

5.2.2 Method

Potometer is generally available in most labs. If it isn't, you can assemble one as shown in Fig. 7.1. To set up the equipment you should follow the steps given below:

1. Bring a small branch of a soft-leafed plant from the campus of your college. It is important to see that the plant material used is in healthy condition; therefore while you cut the branch minimize the damage done. It is best to cut the part to be used for the experiment under water. So keep a water container ready with you and place the cut end of the twig quickly in it. Also remember to make a second cut above 2 cm or more from the end of the twig, while under water before you fix it in the rubber bung.
2. Fit the lower end of the branch into a non-flexible rubber tube and insert it tightly through the hole in the rubber bung. If you need to drill a hole use a cork-borer. Both the bung and the cork-borer must be kept wet all the time. You can drill better if water plus a trace of *surfactant* is used.
3. To set up a potometer choose a place on a clean bench close to a window, clamp it to the stand and assemble it carefully as shown in Fig. 7.1. The essential part of a potometer is a *graduated capillary tube which shows very small losses of water by transpiring leaves.*

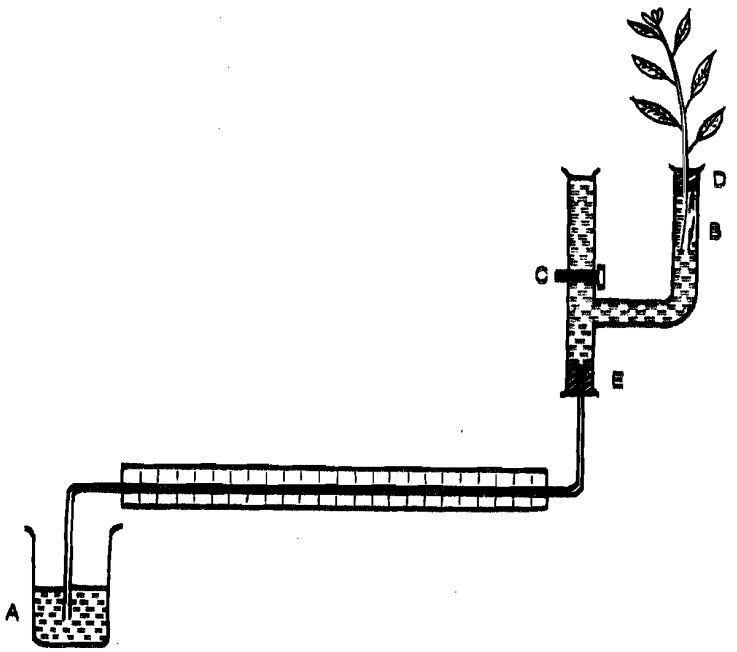

Fig. 5.1: A Potometer.

4. Pour eosin coloured water into the beaker (A). You must ensure that when the capillary is dipped in the beaker containing coloured water there isn't a trapped air bubble. Fill the tube B with tap water and then close the tap C. Also make sure that the two rubber bungs (D and E) are sealed well.
5. Now lift the capillary end out of the beaker carefully and hold it till a bubble of air is introduced. Then re-immerse it so that the bubble of air is caught. Since the twig is transpiring the bubble will gradually travel along the capillary followed by coloured water. When the bubble proceeds beyond the marked area it may be driven back by opening the tap C.

Now your potometer is ready, and you can demonstrate to the students transpiration of water from leaves, and they can measure its rate. *The rate of transpiration is measured as distance moved by the bubble per unit time.* To compare the rates under different environmental conditions you should place the apparatus under the fan, in light and dark.

5.3 TO SET A RESPIROMETER

Respiration involves gaseous exchange of O_2 and CO_2. Warburg devised a manometer (*a manometer is a device for measuring gas pressure*) to measure this exchange, therefore the instrument available commercially is called Warburg manometer. Since it is an expensive instrument, a simple manometer also called respirometer is assembled in most labs for routine demonstrations to the students. In this exercise you will learn to assemble a respirometer to measure respiration rates.

5.3.1 Materials Required

500 ml conical flask
25 ml test tube
T – tube
Pinch clip
A rubber bung
A piece of rubber tube
1 mm diameter graduated pipette
KOH pellets
Stop-clock
Wire gauze
Thermometer
Sprouted mungbean

5.3.2 Method

1. You can assemble a simple respirometer as shown in figure 7.2. Take a T – tube, fix its one end in the rubber bung and slip a piece of rubber tubing and pinch clip to the other end.
2. Now fix the end with the rubber bung to a test tube.
3. Wrap 2 pellets of KOH in a piece of wire gauze and place them at the bottom of tube. Add a few drops of water.
4. Now insert carefully a wad of cotton to partition KOH from the specimen (see Fig. 7.2). Make sure that the cotton is well above the wire gauze.
5. Weigh 20 germinating mung bean and place them on the cotton wad.
6. Join a 2 ml graduated pipette to the central arm of the T with a piece of rubber tube.

7. Assemble the apparatus and place it in a 500 ml conical flask containing water at room temperature or in any other suitable water bath. Place the thermometer in it to record the temperature. Observe the marking on the 2 ml graduated pipette of manometer and calculate in µl the minimum volume change it indicates. If 2 ml is divided into 20 fractions each fraction will be equal to 100µl (1 ml = 1000 µl).
8. Loosen the pinch clip and leave it to equilibrate for 5 to 10 minutes. Then tighten the pinch clip.
9. Insert a drop of colour solution (eosin) in the pipette. It will start moving to the left. Adjust it to extreme right upto a certain mark by carefully loosening the pinch clip.
10. Now the student can start the stop-watch and take the reading at zero and after an interval of 5, 10, 15, 20 and 25 minutes and they can calculate the volume changes (distance moved by the colour drop) in µl/minute.

The unit should be air-tight. You must ensure that when you place an organism (seeds or insects) it must not be in contact with KOH as it is corrosive.

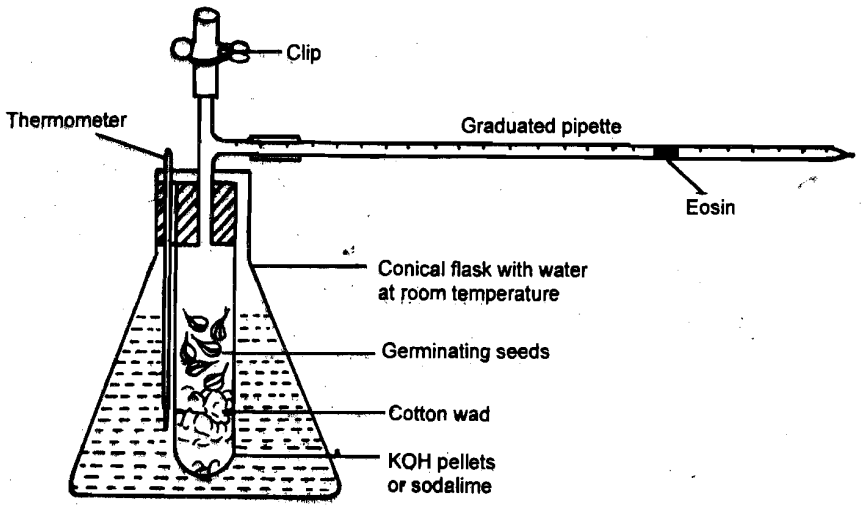

Fig. 5.2: A simple pipette manometer.

5.4 TO SET UP DEMONSTRATION EXPERIMENTS FOR PHOTOSYNTHESIS

Photosynthesis is a process by which green plants make carbohydrates, sugar and starch from CO_2 and H_2O using light energy absorbed by chlorophyll. The process can be demonstrated by evolution of gas bubbles from a leaf or the water plant *Hydrilla* in the presence of sunlight. You may have seen this demonstration in your school. In this exercise you will set up this demonstration and also learn how to improvise it for measuring the effect of quantity of light.

5.4.1 Materials Required

250 – 500 ml beaker
15 – 25 ml test tube
Leaves
Cork borer
Table lamp with 100 W bulb
Iron stand with a clamp

Stop watch
2% $NaHCO_3$
Green, blue and red cellophane paper

5.4.2 Method

The simple pipette manometer used for measuring the rate of respiration can be used for the demonstration of the following experiments on photosynthesis:

A Measurement of Rate of Photosynthesis

1. Clean the manometer thoroughly and dry it before use.
2. Using a dropper pour 2 ml of 1% sodium bicarbonate solution into the tube. Make sure that it does not touch the sides of the test tube. Now insert a cotton wad into the tube to partition bicarbonate solution.
3. Cut several discs of leaves with 1 cm diameter cork borer and keep them moist in a petridish in the dark. Insert one piece in the tube and place it vertically on the cotton wad.
4. Assemble the apparatus and place it in a 500 ml flask containing water at room temperature. Cover the flask with black paper thoroughly. Let it equilibrate for 5 to 10 minutes.
5. Introduce a drop of eosin dye in the pipette and adjust it towards a mark to extreme right.
6. Start the stop-watch and note the readings on the pipette immediately and after an interval of 5 to 10 minutes.
7. Now adjust the black paper to make a vertical slit so that the leaf can be exposed to the light source. Keep the light source 10 cm away from the apparatus.
8. Again adjust the marker drop to the extreme left upto a mark.
9. Readings can be taken at zero time and after an interval of 5, 10, 15, 20 and 25 minutes.

B Effect of Light Quality on the Rate of Photosynthesis

1. Cover the light source with green, blue or red cellophane paper. Readings are taken as in the previous experiment.

5.5 SELF ASSESSMENT QUESTIONS

1. Why are KOH pellets kept in the boiling tube of a pipette manometer?

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2. Why is a drop of coloured solution introduced in the pipette?

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3. What is the purpose of a pinch clip?

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4. What would happen if the rubber tube is sealed at the top?

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5. Why is sodium bicarbonate solution put in the test tube?

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6. Why is the marker dye positioned at the right extreme of a pipette during respiration and at the left extreme during photosynthesis?

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EXPERIMENT 6 SETTING UP APPARATUS FOR DEMONSTRATING PHYSIOLOGICAL ACTIVITY IN ANIMALS

Structure

6.1 INTRODUCTION

In the earlier experiment you learnt how to set up an apparatus to show physiological processes in plants. In this experiment you will learn how to set up two apparatuses to demonstrate physiological activity in animals. You will set up the kymograph apparatus to show physiological activity in the muscle and heart of a frog, and set up an experiment to observe the capillary blood flow in the webbed foot of a frog. These experiments are usually performed at undergraduate as well as graduate level and it is the lab technical staff who are expected to arrange all the equipment required for them. The students are expected to do the dissection and make the actual recording. The supervisor/faculty in charge of the lab usually demonstrate these experiments to the students but it is the technical staff present who give assistance where the students require it.

Objectives

After doing this experiment you will be able to:

- set up a kymograph apparatus to measure the heart rate and muscle activity in frog,
- set up an experiment to show the capillary blood flow in a frog, and
- immobilize a frog by pithing.

6.2 SETTING UP A KYMOGRAPH APPARATUS

A kymograph apparatus consists of a vertical cylindrical drum which can be made to rotate at a variety of speeds by an electric motor. It is used for recording movements such as contraction of muscle, contraction of heart, etc. The speed of rotation can be adjusted as required. Movement can be recorded by either a pen filled with suitable ink, writing on white paper, or a writing stylus marking on a writing paper which has been blackened evenly. This paper is pasted on the drum.

If the kymograph recordings are to be of any use, it is important to provide a time scale. In most kymographs, the speed at which the drum rotates is given and a time scale is worked out from this information.

An electrical stimulator is usually made by attaching wires on each side of a 1.5 volt dry cell. The ends of these wires are fitted with simple electrodes.

6.2.1 Materials Required

1. Kymograph Recording System Consisting of:
   Kymograph apparatus
   1.5 volt dry cell (stimulator) with
   electrodes attached to it.
   Muscle lever
   Double hook
   Femur Clamp and Stand
   5 gm weight
   Recording stylus
2. Dissection tray, Dissection instruments
3. Bone cutter
4. Frog Ringer solution, 20% urethane
5. 50 ml beaker
6. Cotton, Thread
7. Injection syringe and needle
8. Live Frog

6.2.2 Procedure

Blackening of kymograph paper.

1. Cut a strip of recording paper to the height of the drum and paste it on the drum. Blacken it evenly by holding it over a kerosene / zylene flame.
2. When the drum is ready for recording, mount the drum on the instrument taking care not to smudge the blackening.

Setting up the instrument for recording the muscle twitch

1. Fit the femur clamp to the stand. This will hold the nerve muscle preparation vertically directly above the point where it will be attached to the muscle lever (dissected muscle preparation will be provided by the counsellors Fig.6.1).
2. Hook the thread to the lever with a double hook and to the other end of the hook tie a 5 g weight. This arrangement keeps the lever in a horizontal position and the muscle and thread in a vertical position.
3. Clamp the muscle in such a way that the distance between the fulcrum and the tendon attachment raises the lever by about 2 inches for every contraction of the muscle. Keep the preparation moist by using a cotton soaked in frogs Ringer solution.
4. Attach the writing stylus to the horizontal lever in such a way that it just touches the drum without affecting its speed. Set the motor at a speed of 32 cm/sec. Now the kymograph apparatus is ready for recording the muscle twitch.

Setting up the instrument for recording heart beat *in situ*

1. Cover the revolving drum with sooted writing paper.
2. Attach the thread to the lever by means of a small piece of plasticine. Position the lever on the stand and counterbalance it with plasticine so that the heart is fairly stretched and the lever is horizontal.
3. You will be provided with a frog with heart exposed. Attach a hook with thread to the muscle of the ventricle. The writing point should move up a centimeter with convulsion of the heart.
4. Be careful to adjust the writing lever in such a way that there is hardly any friction between the writing stylus and paper.

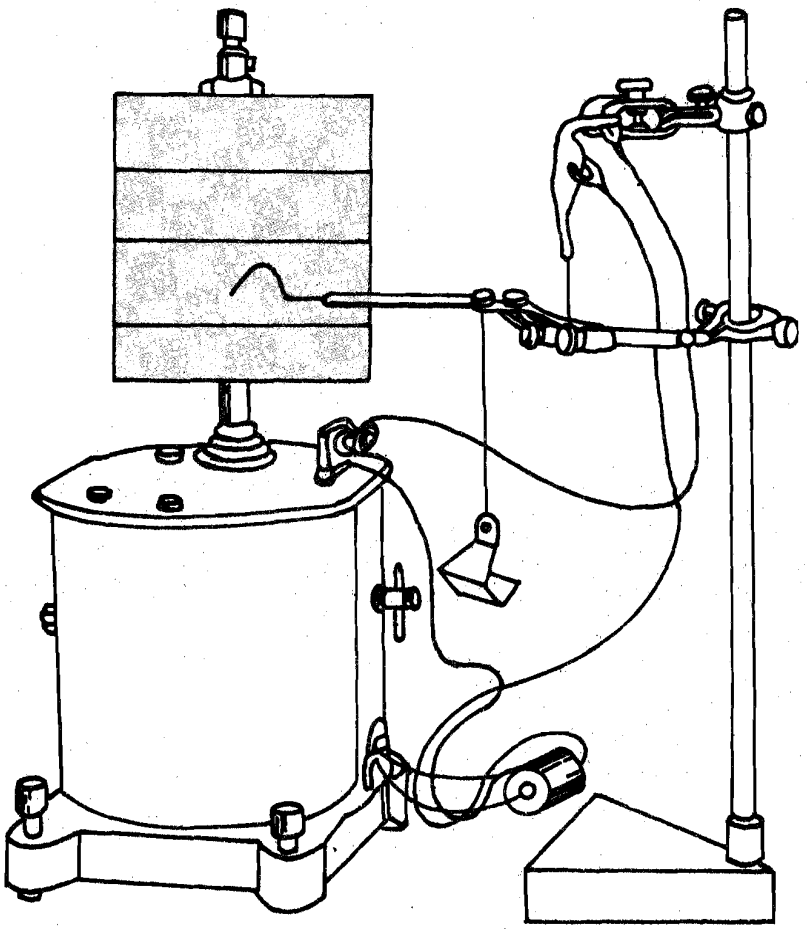

Fig. 6.1: Kymograph instrument arranged to record muscle twitch in frog.

6.3 DEMONSTRATING MICROCIRCULATION IN FROG

This experiment is set up to demonstrate to students circulation of blood through capillaries in the web of a frog's foot.

6.3.1 Materials Required

1. Live frog
2. Compound microscope
3. Medicine dropper
4. Absorbent wet towel
5. Isotonic frog's Ringer solution

6. Board made of soft wood
7. Pins
8. String
9. Adrenaline solution

6.3.2 Procedure

1. Take a wooden board and make an aperture of 2mm about 4cm away from one end.
2. Wrap a moist towel around the live frog tightly enough to prevent it from moving but leave one foot exposed. Tie up the frog on the wooden board placing the web of the foot over the aperture.
3. Pin the web of the foot over the aperture. You will not hurt the frog as long as you don't pin it by the foot. The web does not have any nerves in it, therefore it does not feel any pain on being pinned.
4. Support the plank on the stage of the microscope as shown in Fig. 6.2 and adjust the foot under the low power of the microscope. The demonstration is now ready to be observed by the students.

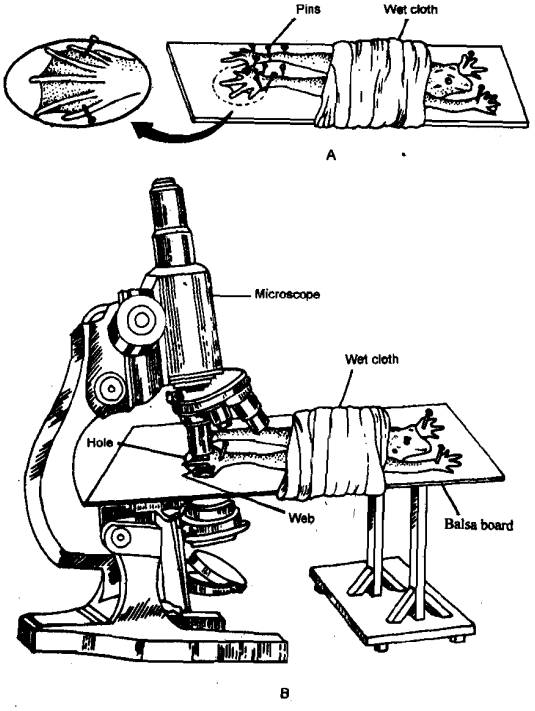

Fig. 6.2: Demonstrating microcirculation in the web of frog.

Caution: Keep the towel wet at all times as it allows the frog to breathe through its skin. Also keep the web of the foot wet by Ringer's solution during the experiment.

6.4 HOW TO PITH A FROG

When using live animals in the laboratory it is important to understand the purpose for which they are to be used. They must always be treated in a humane way. Never cause them unnecessary injury or irritation. Accordingly, if any tissue or organ damage may results from experimentation, first put the animal under an anesthetic or treat the nervous system to make it insensitive to pain. Avoid injuring the animal's tissue or making it bleed; such damage makes the animal less capable of normal reactions.

A spinal frog, one in which the entire brain is destroyed, is prepared by a procedure known as **pithing.**

1. Hold the frog as shown in Fig. 6.3, using the thumb and finger to secure the limbs.
2. With the index finger, press the snout down so that the head is at a sharp angle to the body.
3. Run the dissecting needle down the midline of the head. 2-3mm behind the posterior border of the eardrums, until a depression is felt at the rear of the skull. This indicates the location of the **foramen magnum,** the opening through which the spinal cord emerges out of the skull.
4. Using the dissecting needle, with a sharp movement pierce the skin at this point and insert the needle into the brain through the foramen magnum. Twist and turn the needle to destroy the brain. Halt any bleeding that may result and use the animal.

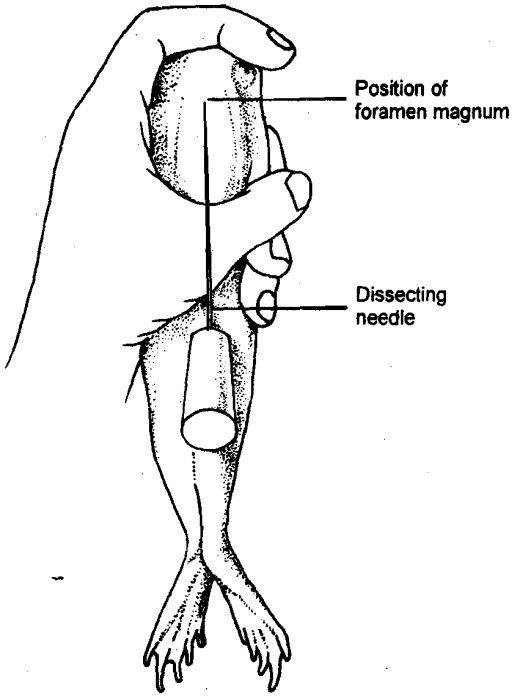

Fig. 6.3: Pithing the brain of a frog.

EXPERIMENT 7 MICROSCOPE HANDLING AND MAINTENANCE

Structure

7.1 INTRODUCTION

Optical or light microscopes are widely and routinely used in all kinds of biological laboratories, right from schools and colleges to hospital pathology and research labs. They are used to examine various kinds of cells, tissues, and microorganisms and also to look at the chromosomes. In order to carry out the above tasks smoothly and effectively, it is essential that one should know how to handle the microscope. Handling the microscope involves familiarity with the instrument and its parts, the methods for its setting-up, the focussing mechanism, and the use of instrument based on the nature of the material to be studied.

This exercise is a composite one that will enable you to have hands on experience in handling microscopes for specific purposes and in different situations, as well as in knowing the nitty-gritty of its care and maintenance. Before you begin your work, it is important that you have a clear understanding and recall the concepts and techniques used in microscopy, that have been discussed in Unit 6 of the LT-02 course. This will help in making this exercise an enjoyable as well as an effective learning experience.

Objectives

After completing this experiment you should be able to:

- set the microscope's light source ensuring optimum direction and intensity of illumination by manipulating mirror and iris diaphragm;
- focus objective/eyepiece upon a slide;
- produce normal illumination by focusing the Abbe condenser upon the light source;
- calibrate an eyepiece graticule against a stage micrometer, and use it to measure various kinds of cells;
- demonstrate the setting up of an oil immersion objective;

- explain the need to clean off immersion oil after use;
- describe the simple maintenance methods for a compound microscope;
- perform simple maintenance tasks of a compound microscope such as cleaning lenses, changing bulbs, and so on.

7.2 SETTING UP A STUDENT MICROSCOPE

In the first part of the exercise you will learn to set up a student microscope using a bench lamp for illumination, so that you can observe a cell preparation on a microscope slide. If you are not familiar with the use of this instrument, you should first understand and then practise the setting up technique following the steps (i-xi) given below. After this, you should practise from time to time without referring to the text.

Requirements

For this exercise you will need the following:

(a) A microscope, preferably one fitted with at least three eyepieces – 5x, 10x, and oil-immersion, a condenser and having a mirror for use with an external light source.
(b) A 40 W or 60 W lamp, preferably with a front cover-field stop, to restrict glare. The bulb should be frosted and ideally without any markings on the lower end.
(c) A well-stained, prepared slide for observation.

A typical student microscope is shown in Fig. 7.1. You are already familiar with this figure, from your study of Unit 6 of this course. Before you begin to setup the microscope remember that *a microscope is a delicate precision instrument, and you must handle it carefully. You must not touch its optical surfaces nor allow them to come into contact with any liquid. You will spend quite some time with a microscope and it is therefore important that you know how to use it correctly.* The following instructions should give you good results, so work through them step by step. The numbers given in parentheses in the following instructions, refer to the parts of a microscope, that has been illustrated in Fig. 7.1.

Method

i) Put the bench lamp, with fitted iris to restrict the light, about 10 cm directly in front of the mirror (13). Point the lamp directly at the plane side of the mirror and switch the lamp on.
ii) Check that the sub-stage iris (9) is open and check that the top lens of the condenser (8) is about 2 mm below the stage.
iii) Rotate the nose-piece (6) to engage the 8× or 10× objective (7) depending on the objectives available on your microscope.
iv) Remove the eyepiece and adjust the mirror until a bright spot of light is seen down the tube. Replace the eyepiece.
v) Put the slide on the stage (18) so that the mounted object is under the objective lens. Use the stage clips (20) or mechanical stage to secure the slide.
vi) View the microscope from one side and use the coarse focus control (16) to lower the 8× or 10× objective to within about 5 mm of the slide.
vii) Look down the eyepiece and use the coarse focus control (16) to raise the objective slowly until the object on the slide comes into focus. Make final

adjustments with the fine focus control (15) and, if necessary, readjust the mirror and condenser.

viii) Move the slide to centralize the object to be observed.

ix) If a higher objective magnification is required, first check that the object is in focus and centralized for the 8× or 10× objective. Then (without altering the focus controls) carefully rotate the nose-piece (6) to engage the 20× or 40× objective lens. Refocus using the fine focus control (15) and move the slide as required. It may be necessary to readjust the mirror and/or condenser to give suitable lighting.

x) You should note that some low power objective lenses such as the Biolam 3.7× objective have a long focal length (about 6.5 cm) and a wide field of view. Therefore you will have to raise the objective with the coarse focus and probably have to remove the condenser and use the concave side of the mirror to direct light onto the specimen. After using the 3.7× objective, replace the condenser and rotate the mirror back to the plane side. Refocus the 8× or 10× objective – see steps (vi) and (vii), before using the 20× or 40×.

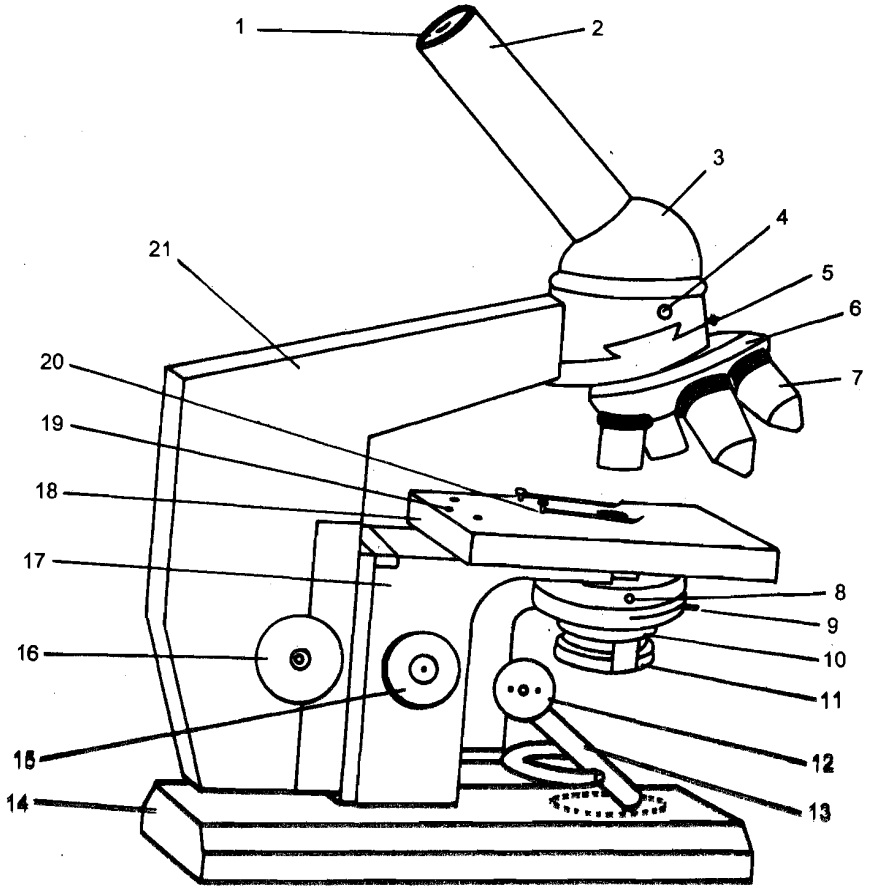

Fig.7.1: Diagram of a typical student microscope.

(1)	**Eyepiece: 7×, 10×, 15×**	**(11)**	**Accessory lens**
(2)	**Tube**	**(12)**	**Condenser focussing**
(3)	**Prism head**	**(13)**	**Gimballed plano-concave mirror**
(4)	**Clamp screw**	**(14)**	**Base plate**
(5)	**Nose-piece centring screw**	**(15)**	**Fine focus control**
(6)	**Nose-piece/turret**	**(16)**	**Coarse focus control**
(7)	**Objective 3.7×, 8×, 20×, 40×**	**(17)**	**Stock (houses focusing mechanism)**
(8)	**Condenser clamp screw**	**(18)**	**Stage (keep clean and dry!)**
(9)	**Condenser iris**	**(19)**	**Mechanical stage mount**
(10)	**Filter holder**	**(20)**	**Stage clip**
		(21)	**Limb**

xi) Many microscopes have three magnifications of eyepiece available – often 7×, 10× and 15×. It is therefore possible, using 3 eyepieces and 4 objectives, to obtain twelve different magnifications, for example, between 26× (3.7× objective and 7× eyepiece) and 600× (40× objective and 15× eyepiece). Oil immersion objectives of 90× magnification are available for examination of, for example, protozoa and blood. Generally, the magnification can be adjusted by (a) changing the eyepiece, or (b) rotating the nose-piece to bring a different objective into position.

7.2.1 Trouble-Shooting

Did you encounter any difficulty in setting up your microscope? Nevertheless, here are some tips for you.

(i) *Not enough light*? Check:

(a) that the iris (9) is open,
(b) that the condenser (8) is in position and properly focused,
(c) that the mirror (13) is properly angled,
(d) that the nose-piece (6) is properly located,
(e) that the sub-condenser filter holder (10) is not in the way.

(ii) *Too much light*?

Reduce the intensity by moving the lamp away from the mirror. Note that the iris should not be used to adjust light intensity.

(iii) *Dirt in the field of view*?

It may be on the eyepiece, objective or slide. Look down the microscope and see if the dirt moves when you:
(a) rotate the eyepiece, or
(b) move the slide.
If neither moves the dirt, then the dirt is on the objective. Clean the lenses very carefully using proper lens tissue paper only.

(iv) *'Cloudy' on high power*?

The previous user (possibly you?) probably has allowed liquid onto the objective. Clean the objective lens (7) carefully – moisten the lens tissue if necessary.

(v) *Dirty stage*?

The previous user (surely not you?) has used a wet slide. So clean the stage carefully.

(vi) *Lenses missing*?

Take a different microscope and report about the missing part(s) of the instrument.

7.2.2 Routine Fault Finding

This section discusses the probable causes of some common problems encountered while setting up the microscope. Identification of the root cause of the problem enables one to take the appropriate corrective measures.

1. Inability to obtain a sharp image

There are two types of causes for it – optical causes and mechanical causes.

Optical causes

(i) Aperture of condenser too wide for objective in use. Reduce it.
(ii) *If hard false 'haloes' present* – condenser aperture too narrow. Increase it.
(iii) Field stop(s) set too wide (causing glare).
(iv) Use of eyepiece of too high a power for the quality of the objective.
(v) Grease, dust or old immersion oil on slide. Clean with lens tissue and polish with Selvyt cloth.
(vi) Lens surfaces not completely clean. Clean them. If the problem remains, try an objective from a different instrument. If this is satisfactory, the original objective is faulty.
(vii) Use of oil immersion objective without oil.

Mechanical causes

(i) Specimen slide upside down.
(ii) Stops wrongly set. The bottom stop should be set to prevent further movement of the coarse drive when the fine focus is at the middle of its range and the image is in focus. Even where stops are not fitted, the fine movement may come to the end of its travel during focusing. This can usually be avoided if the fine adjustment is set at mid-range before the microscope is approximately focused with the coarse drive.
(iii) Jamming of the lower part of a spring-loaded or retractable objective in the body. If *very gentle* attempts to free the lower lens cell are unsuccessful, the objective should be returned.

2. Image moves slowly or suddenly goes out of focus

Its possible causes are:

(i) Body slip or slipping of the stage in stage focusing instruments, caused by poor adjustment of the focusing mechanism.
(ii) Hand pressure on flexible stage.

3. Condenser will not focus the surface of the lamp in the field of view

This may be caused by the following reasons:

(i) Condenser not pushed fully home in mount.
(ii) A stop (if present) set too low.

4. Field of view too bright

The probable causes for this are:

(i) Substage condenser aperture or disc diaphragm aperture too wide.
(ii) Power of source too high.
(iii) (*If variable*) Lamp intensity set too high.
(iv) Neutral density filters needed.

5. Field of view too dull

It may be due to one of the following causes:

(i) Substage condenser aperture or disc diaphragm aperture too narrow.
(ii) Substage condenser not properly focused.
(iii) Neutral density filters (*if fitted*) should be removed.
(iv) Power of bench lamp too low.
(v) (*If variable*) Internal lamp intensity set too low.

6. Specks in field of view

The possible causes of this may be:

(i) Specks ill defined and 'floating' across field of view – in observer's eye.
(ii) Specks normally stationary but revolving with eyepiece – clean eyepiece lens, especially field lens.
(iii) Specks normally stationary and not revolving with eyepiece – clean (in order) condenser lenses, slide, any filters, objective.

7. Objective aperture will not fill with light

It may be due to any of the following reasons:

(i) Substage condenser improperly focused.
(ii) Total condenser aperture inadequate for objective in use.
(iii) Oil immersion condenser (if used) not oiled to underside of slide.

8. Image moves sideways during focusing

This may result because of any of the following:

(i) Slide or specimen not perpendicular to optical axis.
(ii) Stage fitted 'out of square'.
(iii) (*In a stage focusing instrument, if effect very small*) Poorly fitted dovetails, allowing stage to move under hand pressure.

SAQ 1

Did you get a dull field of view in your microscope? List two probable causes for it.

i) .....

ii) .....

SAQ 2

When setting up a microscope, if you find that you are unable to obtain a sharp image, what do you think might be wrong? Write down four possible causes.

i) .....

ii) .....

iii) .....

iv) .....

SAQ 3

If you do not focus the substage condenser of a microscope properly, what effect would this have? Tick the correct answer.

(i) Objective aperture would not fill with light.
(ii) Field of view would be too bright.
(iii) Field of view would be too dull.
(iv) Object would be out of focus.

7.3 SETTING UP A MICROSCOPE WITH AND WITHOUT A CONDENSER

During the course of this practical you will set up microscope in different light conditions, and also learn about the utility of a condenser in this process. You can observe the differences brought about in different set-ups.

You require the following items for exercises 7.3.1 to 7.3.4.

Requirements

Microscope
Illuminator or lamp
Slides
Coverslips
Specimens of materials relevant to the techniques to be studied
Already prepared microscope mounts
Spatulas
Forceps

7.3.1 Illumination without a Condenser

There are two possible sources used for illumination, one an external light source (this is described in a), and the other is the use of daylight (discussed in b).

a) Setting up a Typical Microscope using an External Source

For optimum illumination and resolution the aperture of the objective should be filled (or nearly) with light. This is best achieved by having a condenser with a properly regulated iris diaphragm. In the absence of this, the apertures of medium and high power objectives may be underfilled and resolution will suffer. The vast majority of microscopes used at lower levels are not fitted with such condensers, but this is not serious as long as the limitations are recognised.

The difficulties experienced in using the simpler kind of microscope most often lie in obtaining optimal illumination with the simple controls available. The field may be difficult to illuminate evenly and there may be lack of contrast with excessive glare. The most common form of sub-stage assembly for controlling illumination in simple microscopes is the disc diaphragm. This is a movable metal or plastic disc with a series of circular holes of various diameters. Unlike the iris diaphragm of the more advanced instrument, which

is imaged at the back lens of the objective and is therefore called an aperture stop, a hole in a sub-stage disc diaphragm is used mainly to limit the effective size of the source in order to reduce glare and improve contrast. The holes in a disc diaphragm should be used to limit the area illuminated to the diameter of the field of view – they act as field stops. One should therefore usually select the smallest size of hole that will illuminate the field of view evenly. In fact, on many simpler instruments only the use of the 4× of 5× objective necessitates a change from the use of the smallest hole available.

Occasionally it may prove difficult to illuminate the whole field of view with the 4× or 5× objective. In this case the converging mirror may have to be used and the lamp moved nearer in order to produce an effectively larger source. This is acceptable for low power work but produces a distorted image of the source. On an 'advanced' instrument it may be necessary to remove the condenser. Even if it can be left in place, it may be difficult to focus accurately.

b) The Use of Daylight Illumination

Ordinary daylight is often the most readily available of all sources of illumination; it is used a great deal for routine microscopy. However, there are limitations: daylight is a very diffuse source of virtually unlimited extent, so that light enters the microscope at almost all angles. It is therefore difficult to know when the 'source' is focused on the object and to restrict the illuminated area to the field of view. So there is a greater tendency towards glare with daylight than with the more controllable artificial sources. Apart from this problem of glare, there are few serious objections to the use of daylight, provided care is exercised in properly illuminating the aperture of the objective.

Setting up the microscope in this way is quite straightforward.

(i) Place the microscope near a large window, preferably one without too many glazing bars.
(ii) Focus the 10× (16mm) objective in the usual way using a well-stained slide.
(iii) Remove the eyepiece and inspect the back lens of the objective.
(iv) If fitted, the sub-stage condenser should be racked up and down until a fairly sharp image of the condenser iris diaphragm can be seen at the back lens of the objective, and (when the diaphragm is fully opened), the back lens can be filled with light fairly evenly. With a simple instrument this step is omitted; use the sub-stage disc diaphragm to control glare.
(v) Replace the eyepiece. A reasonably good image should be seen and minor adjustments of the iris diaphragm can be made to reduce glare.

Obviously with daylight the source cannot actually be focussed on the object. However, it can be helpful to hold a pointed article 150-200 mm from the mirror and to focus this in the field of view. This often ensures fairly good illumination of the back lens. Because the source is of almost unlimited size the exact position of the mirror is not critical. The imaging of glazing bars in the field of view can often be avoided by tilting the mirror slightly or by placing the microscope closer to the window.

To put it briefly, while daylight provides a very convenient and useful source, giving adequate illumination when specimens with high contrast are used, the more readily controllable artificial external sources are generally preferred.

7.3.2 Illumination Using an Abbe Condenser

Using an External Source to Obtain Correct 'Nelson' or Source-Focussed Illumination

Use a 10× (16 mm) objective and 10× eyepiece as follows:

(i) Place on the stage a specimen slide. Ensure that the coloured part (that is the material to be seen) of the slide is directly under the centre of the objective.

(ii) Place the external lamp about 150-200 mm away from the instrument and level to it. Ideally, the lamp should have an opal bulb and should possess a circular field stop. Very often such lamps are not available and an ordinary bench lamp has to be employed. If this is the case, then draughtsman's tracing paper is fixed to one side of a hole cut in a piece of blackened cardboard or hardboard. This illuminated area then functions as a secondary light source.

(iii) Focus the slide and manipulate the plane mirror to centre the illumination.

(iv) Focus the microscope condenser. The aim is to focus the image of the source in the object plane. If a field stop is fitted to the lamp, then the condenser may be focused on the edges of this. If there is no field stop, or if, at its narrowest setting, it more than fills the field of view, the condenser can be focused on a pencil point or mounted needle held just in front of the lamp. The position of the condenser should be adjusted by means of its focusing mechanism until the image of the field stop or pointed object is in sharp focus at the same time as the image of the object on the microscope stage. An image of the surface of the bulb (possibly even of the manufacturer's name, etc.) may come into the field of view. To avoid this, the condenser may be slightly defocused.

(v) Change to the 40× (4mm) objective.

(vi) The aperture of the iris diaphragm should now be adjusted to suit the numerical aperture of the 40× objective. This is done by removing the eyepiece and inspecting the illuminated circle at the back of the objective lens. This can be done either by direct inspection or by using a centring telescope focused on the back lens of the objective. As the iris diaphragm is closed, the image of its edge will be seen at the periphery of the illuminated area.

The diaphragm should be closed until the illuminated area occupies two-thirds to three-quarters of the diameter of the back lens of the objective. It is usually recommended that this aperture adjustment be carried out for each objective on each occasion it is brought into use. However, a single setting for the 40× lens will usually also serve adequately for the 10× objective and this saves time and trouble.

Readjustment of the iris aperture in the same way may well be needed when the low power is brought into use.

(vii) **Replace the eyepiece. The microscope is now correctly adjusted and ready for use. If the light is too bright (not usual with a 60 or 100 W lamp) the**

intensity should be lessened by the use of neutral density filters, by changing to a lower power bulb, or by altering the field stop on the lamp if one is fitted. The iris diaphragm of the condenser should *not* be used to control the intensity of illumination, nor should the condenser focusing controls be adjusted. Slight adjustments of these controls may sometimes be necessary to control glare and obtain better contrast, but as a general rule, intensity of illumination should be adjusted at some point in the light path between the lamp and the iris diaphragm of the condenser.

7.3.3 Setting up for Köhler Brightfield Illumination

This is an important technique which is in a sense a foundation upon which other techniques rest. Here we refer to a simplified version, pertinent to a monocular compound microscope of a basic type with an Abbe condenser, using a mirror and an external source of illumination.

(i) Place the lamp about 20 cm in front of the microscope and switch it on. Ensure that the microscope is in a comfortable working position and is angled correctly for the user. Aim the light at the mirror, plane surface, and angle the mirror so that it reflects the light centrally on the base of the substage apparatus.
(ii) Close the iris.
(iii) If the lamp has a field iris, open it wide and focus the condenser so that the light falling on the iris overfills its area slightly. By closing and opening the field iris you can check the centricity of the lamp and the mirror.
(iv) Open the aperture iris and place a slide on the microscope stage.
(v) Focus on this slide using a low power objective. Ensure that the substage is as high as it will go, close down the field iris and look for its image in the microscope.
(vi) Having identified the field iris, focus its image by moving the substage condenser downward. If the field iris cannot be focused, move the lamp towards or away from the microscope until it can be focused; at this point the substage should be a little below the top of its travel. If the substage is not central, it is time to centre it using the image of the field iris as a guide.
(vii) Open the field iris until the whole field of view is just covered.
(viii) Remove the eyepiece and look down the tube. Open and close the iris so that you can identify its image. Now open it wide and then gradually close it until you just see its edges. Leave the aperture iris in this position and replace the eyepiece.
(ix) If the light is too bright the lamp may be dimmed by means of an electrical dimmer or by the use of neutral density filters. Attempting to change the light by moving either iris or by lowering the substage will destroy the Köhler illumination.
The illumination is set up for the critical lighting of the specimen. Changes in objective power will necessitate only the increase or decrease in intensity of light. The microscope nosepiece will ensure the centricity of the other objectives.

7.3.4 Setting Up for Phase Contrast Microscopy

For this exercise, you will additionally need a focusing telescope, also known as a Bertrand lens, for the following series of operations. A Bertrand lens is a supplementary lens system which may be introduced into the tube above the

objective. Together with the eyepiece this forms a viewing telescope for examining the back focal plane of the objective. It is chiefly used when setting up the microscope.

(i) The correct substage annulus must be chosen to accompany the light ring of the objective required. Ensure that the objective turret, if used, is properly placed in its notch so that the objective is in the right place.
(ii) Open the iris diaphragm fully.
(iii) Place a slide of a thin section of animal tissue, e.g., kidney, on the stage of the microscope and critically focus on an edge of the section.
(iv) Close down the field diaphragm and adjust the height of the substage condenser unit. A sharp image of the diaphragm can be seen.
(v) Open out the field diaphragm until it almost fills the field, then centre the image of the diaphragm by means of the substage condenser centring screws.
(vi) Open the field diaphragm until it just goes beyond the edge of the field of view.
(vii) Take out the eyepiece and replace it with the focusing telescope. There should be an adjusting collar on the telescope to enable you to focus the telescope (you may have to undo a locking ring in order to achieve this). Focus the telescope on the image of the light ring on the phase ring.
(viii) The phase ring is axiomatically correctly placed; therefore it is necessary to centre the substage annulus so that the two images may be superimposed. This is achieved by means of a centring mechanism separate from that of the main substage condenser mount. The phase centring device often takes the form of a pair of spring loaded screws which only engage when pushed in. Find these screws and push them in until they engage and by screwing and unscrewing them centre the image of the light ring on the phase ring (see Fig. 7.2).

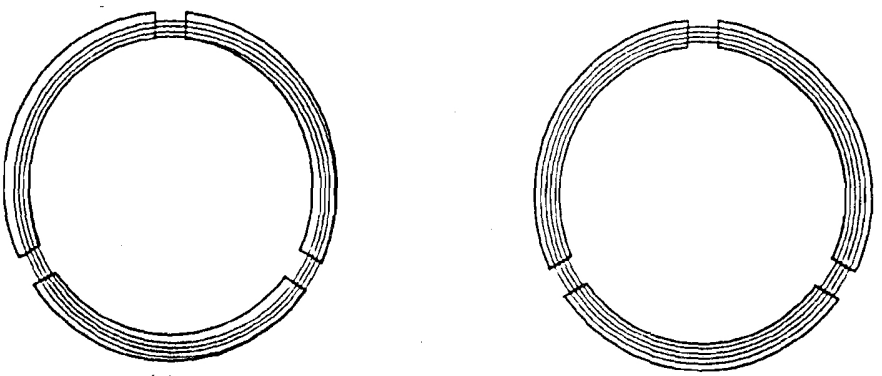

Fig. 7.2: Phase ring and light ring in different positions. (a) off, and (b) centred.

Each combination of annuli and objectives must be set up in this way, but unless the settings are interfered with they should remain set once and for all. There is no written component for this practical upon which you could be directly assessed. You should make notes and diagrams of what you have observed since these may be useful to you at a later date.

Ask your counsellor to give you feedback on your techniques for the above set-ups of the microscope.

7.4 MAKING MICROSCOPICAL MEASUREMENTS

Objects on a slide cannot be measured directly. Instead, they are measured against a pre-calibrated graticule (see Fig. 7.3) which is placed inside the eyepiece of the microscope. A conversion factor is then applied to obtain the actual size of the object. This whole process is known as micrometry.

Requirements

Slide with an object to be measured
Microscope with a graticule* in the eyepiece
A lamp
Slide micrometer graduated in 0.1 mm or 0.01 mm units

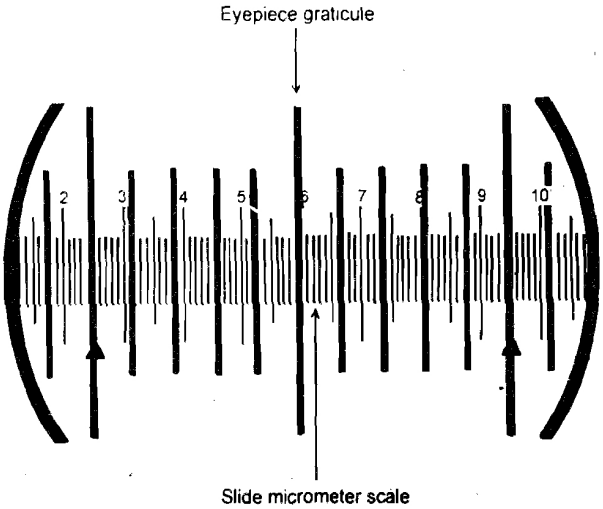

Fig. 7.3: Eyepiece graticule with stage micrometer graticule.

Method

(i) Focus the microscope onto the micrometer slide using the required combination of eyepiece and objective.

(ii) Align the two graticules; read off and note the eyepiece units (**w**) in fractions of a mm (**x**). Use at least half the field of view.

(iii) Calculate the equivalence of one eyepiece unit by **x/w**. This is the conversion factor **y****.

(iv) **Remove the micrometer slide and focus on the slide carrying the object to be measured.**

(v) **Align the object to be measured with the eyepiece graticule. Measure the object in graticule units and note the reading (z).**

(vi) **Calculate the size of the object in mm by multiplying reading z by conversion factor y.**

* The eyepiece graticule consists of a grid or ruled lines etched on a glass or plastic disc. It is inserted by carefully removing the top lens of the eyepiece and dropping the graticule onto the diaphragm. The eyepiece is then reassembled and used in the normal way – the graticule grid or lines are superimposed on the field of view.

** The conversion factor is true only for that particular combination of objective and eyepiece. If micrometry is being performed routinely then a table of conversion factors can be prepared for the usual combinations of eyepieces and objectives used.

A worked out example is being presented below.

Note that 70 eyepiece units (**w**) are equivalent to 10 micrometer units (**x**), i.e., 1 mm or 1000 μm. Therefore:

$$1 \textit{ eyepiece unit} = \frac{1000}{70} = 14.3\ \mu m$$

Suppose you are measuring a plant cell. Assume the cell measures 2.5 eyepiece units by 4 eyepiece units.
Its actual size, therefore, is (2.5 × 14.3) μm by (4 × 14.3) μm or 35.75 μm by 57.2μm.

7.5 THE OIL IMMERSION TECHNIQUE

When we have to examine small objects such as red blood cell (7 μm) and bacteria (1-5 μm), an objective lens of 90x or 100x is required. These lenses have a very short working distance and the diameter of the lower objective lens is very small. To maximize the light gathering ability of the lens, i.e., to raise its NA , medium of high refractive index is included between the objective and the coverglass. The medium normally used is immersion oil, or more rarely water.

Requirements

Microscope fitted with Abbe condenser and usual objectives
Oil immersion objective lens
Good light source
Immersion oil
Lens tissue
1,2-dimethylbenzene (xylene) or 1,1,1-trichloroethane
Suitable stained permanent slide which must have a thin coverglass, recommended material – blood smear.

Method

(i) Check that the oil immersion lens is clean.
(ii) Polish the slide with a lint-free cloth.
(iii) Screw the oil immersion lens into the turret next to the 40x objective (or similar).
(iv) Set up the microscope. Pay particular attention to the condenser settings.
(v) Select an object of interest which is on the slide. Centre it in the field of view and focus on it with the 40x objective. Use stage clips or mechanical stage clips, to fix the slide.
(vi) Using the coarse focus control, rack up or raise the nose piece and swing in the 90x or 100x oil immersion lens.
(vii) Put a drop of the immersion oil onto the slide.
(viii) Rack down until the oil immersion lens enters the oil and is very nearly touching the coverglass.
(ix) Now look down the microscope. Slowly rack up using the fine focus control. The object on the slide should come into view. If necessary, readjust the lamp, mirror and condenser.
(x) The slide can be carefully moved to examine adjacent objects – for example to compare the structure of red blood cells and white blood cells.

(xi) When you have finished, rack up and remove the slide and oil immersion objective.

(xii) The oil is removed from the objective lens with dry lens-tissue. Ordinary tissue is suitable for the slide. Slide and objective require more cleaning, use a tissue paper on which a few drops of xylene have been poured. Caution! Avoid excessive use of the solvent as it is poisonous by skin contact and inhalation. Also it dissolves the content used to fix optical components in place. Now that you are familiar with the oil immersion technique, practice it and get the feedback from your counsellor.

(xiii) Return the clean lens to its storage container and the slide to its tray.

SAQ 4

Under what circumstances would you use the oil immersion techniques?

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SAQ 5

Describe the procedure which you would use to correctly set up and focus an oil immersion objective so as to prevent damage to the objective and slide.

i) .....

ii) .....

iii) .....

iv) .....

.....

.....

SAQ 6

Give two reasons why it is important to use only the minimal amount of xylene (1,2-dimethylbenzene) for cleaning the slides and the objectives?

i) .....

ii) .....

iii) .....

.....

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7.6 MICROSCOPE CARE AND MAINTENANCE

This section would acquaint with the main elements of microscope care and maintenance. First we look up at some of the practices that we must follow, described as Do's, and the ones that are not quite microscope-friendly also listed under the category of Don'ts in the subsection that begins now.

7.6.1 Some Do's And Don'ts

DO'S

Handle it carefully.

Focus correctly.

Keep it clean.

Use the proper lens tissue.
Report problems.
Leave a low power lens in position when finished.

DON'TS

Bang it down on the bench.
Focus high power lenses with the coarse control.
Use wet slides.
Use ordinary cloth or tissue for cleaning lenses.
Put it away dirty.
Remove slide with a high power lens in place.

7.6.2 Microscope Maintenance

In the subsection some descriptions and instructions are given that should enable you to tackle the routine servicing tasks, and also to keep the instruments in good order.

Given below are some of the operations which can readily be carried out

(1) Cleaning of optics;
(2) Cleaning and degreasing of dovetails and nosepiece movements;
(3) Adjustment of some types of coarse focusing mechanism.

Caution: Work which should *not* be attempted includes anything requiring the dismantling of objectives, adjustments to fine focusing mechanism, or, in general, the removal of pinions or pinion bearings.

If a number of instruments are in constant use, or if instruments have complex focusing mechanisms, it may still be necessary to call in a service engineer periodically (from either the makers or a firm specialising in microscope servicing and repairs). However, regular attention to instruments, even if limited, can go a long way towards reducing the frequency of these visits.

Tools and Working Arrangements

Before starting operations, spread out a large, clean sheet of white paper on the bench to one side of the work station and lay out these tools and materials on the other side.

(1) Allen keys (Imperial and metric)
(2) A set of instrument ('watchmaker's) screwdrivers
(3) A range of larger flat-headed and a Philips screwdriver (the former with wide but fine blades)
(4) A rubber hand bellows of the type used by photographers
(5) Camel hair brushes (different grades – finer – medium – coarse)
(6) A good quality hand lens
(7) Lens tissues
(8) 'Selvyt' clothes and dogwood pegs
(9) Clean, lint-free rags
(10) 1,2-dimethylbenzene (xylene)
(11) 1,1,1 – trichloroethane
(12) Distilled water
(13) Lubricants for the main dovetail slides and the pinion bearings
(14) Cotton buds

A few small dishes or boxes are useful for components which are removed. A well shaded adjustable lamp is an asset, especially for the inspection of lens' surfaces.

When working on an instrument, give yourself plenty of room. Work well away from the edges of the bench so that screws and other small parts do not find their way to the floor.

If, possible, work on the instrument should be completed in an unbroken, orderly sequence. If there are interruptions, it is not easy to remember just what has been completed. It is also advisable to work on one instrument at a time to rule out the possibility of parts getting mixed up. (This also has the advantage that if one does make a mistake or come to an impasse, there is only the embarrassment of one instrument in pieces, and not a whole set!)

Working Sequence

The sequence of operations in servicing a microscope can be broken down into a series of steps; these are illustrated in Fig. 7.4. *The broken lines indicate the work sequence in routine maintenance of the optics when there isn't the time or the need to clean and regrease the mechanical components.*

(1) Removal of Optical Components

After removal, these should be placed on the sheet of clean paper to one side of the work station. Whenever screws are removed, take great care to use the correct size of screwdriver. If the blade is not an exact fit, place a piece of paper towel or cloth over the screw slot. If the instrument is relatively new and is being disassembled for the first time, some screws may be hard to loosen because of the presence of a locking compound.

Eyepiece

This should first be inspected to ascertain whether it is free or fixed. Some eyepieces make a snug, push fit in the tube; some are fixed by a grub screw which will require loosening with an Allen key or instrument screwdriver; some are screwed to the tube and must be unscrewed bodily.

Objectives

These can usually just be unscrewed carefully from the nosepiece. Small non-standard objectives without milled collars can be gripped in a clothes peg type test-tube holder or, more simply, by sliding a short piece of rubber tubing over them. On a few instruments objectives have locking screws which must be loosened before the objective itself can be unscrewed.

Mirror and gimbal

These are usually a push fit, the gimbal peg being a split shaft which is housed in a hole at the base of the limb or on the base-top. However, this shaft may well be fixed by a grub screw which will have to be loosened before withdrawing the mirror gimbal. Illuminator disassembly is done in a similar way.

Condenser

Most focusing condensers are held in their mounting by screws. There may be one large clamping screw with a slotted head or several small screws requiring the use of an instrument screwdriver. If the latter arrangement is used, be

careful to loosen only the actual clamping screws and not those holding the condenser components together. Some condenser mounts have centring screws and, if possible, these too should not be distrubed.

A modern fixed-limb microscope with an angled head will also have a mirror or prism in the body tube. *Do not remove this,* as it is difficult to replace it in the correct position.

If the full sequence of servicing operations is not to be followed, but only the optics cleaned, follow the broken line in Fig. 7.4 to the Box no. 6 (in the Figure).

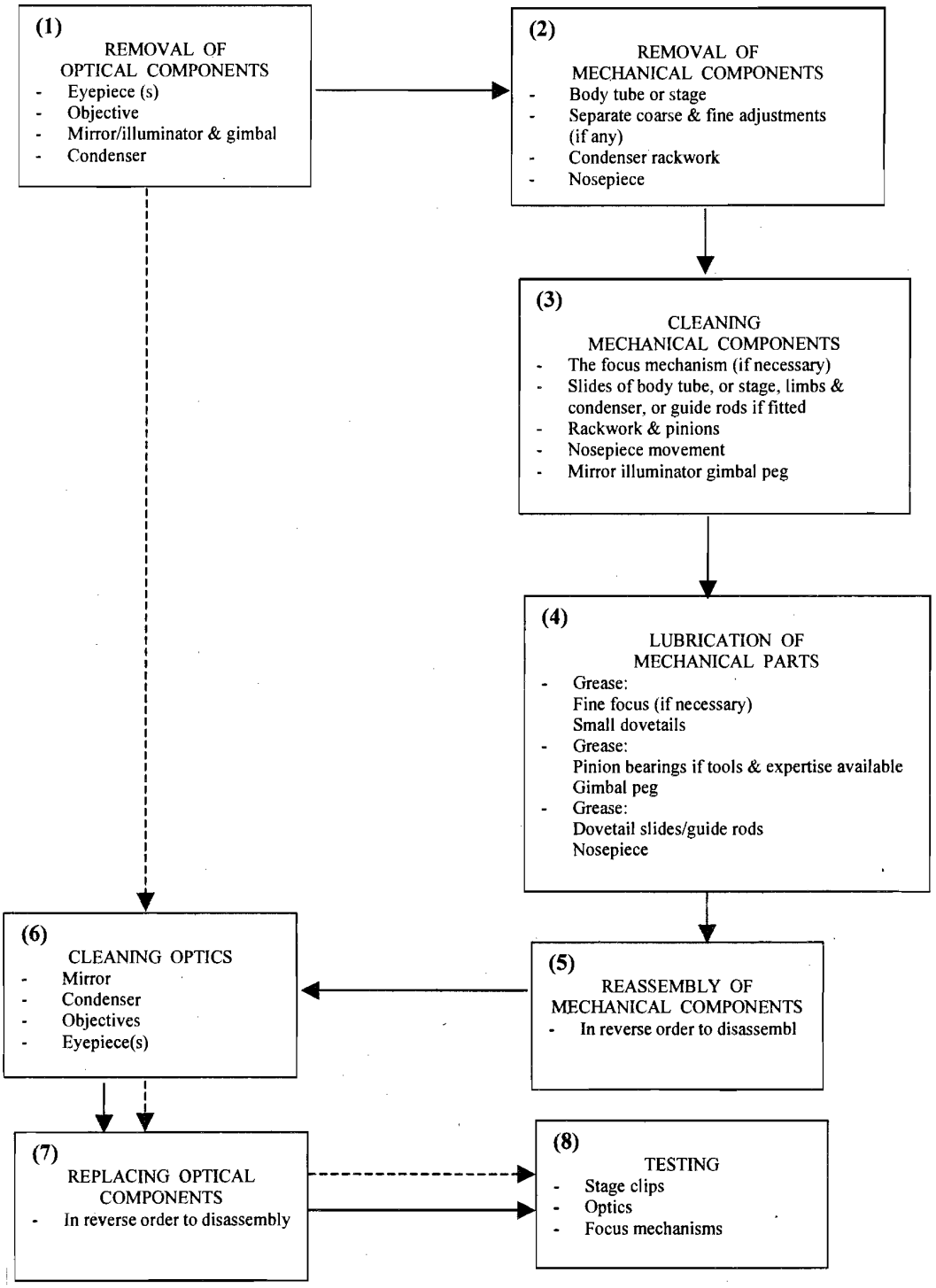

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Fig. 7.4: A Servicing schedule.

(2) Removal of Mechanical Components

Body tube (traditional instruments)

The coarse focus mechanism should first be examined carefully to identify any stops or other features which limit its travel. This movement is almost always derived from a rack and pinion system with diagonally cut teeth (Fig. 7.5).

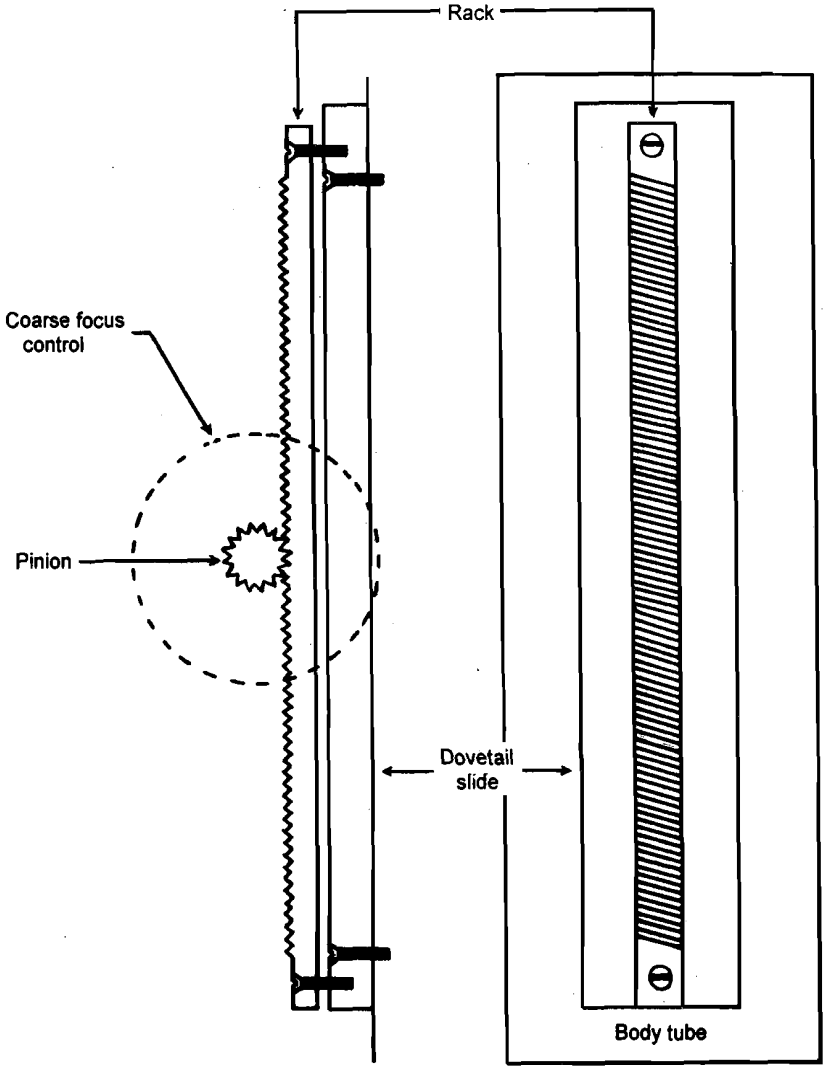

Fig. 7.5: Coarse focus drive – rack and pinion : side (Fig. on the left) and front (see the figure on the right) views.

Most instruments only have a bottom stop, but fixed or adjustable stops may be present at either end of the movement. Any stops which limit the upward travel of the body tube should be removed. In extreme cases, it may even be necessary to remove the stage to take out a lower stop. The movement can then be run apart. This should be done carefully, especially when the end of the rack is nearing the pinion. Any small screws on the limb above the coarse focus spindle should not be removed. They are clamping screws affecting the tension on the pinion bearings and do not interfere with the removal of the body tube.

Stage focusing movements (modern fixed limb instruments and some junior microscopes)

In modern style instruments the base and stage may have to be unbolted before the stage focusing mechanism can be removed. In a few cases, the removal of an upper stop will allow the stage to be racked off upwards.

Substage assembly

Dismantle the substage condenser scroll focusing assembly by loosening the mounting screws, or rack, if of the rack and pinion type. In some rack and pinion condenser movements it may be necessary to withdraw the pinion spindle; this may necessitate the removal of a screw from the plain end of the spindle.

Fine focus movement (if fitted)

The dovetail slides and other parts of a fine focusing movement are hardly ever exposed in use and usually do not require attention. Where the mechanism is a straightforward screw and lever (Fig. 7.6) or other simple type, it can be partly dismantled so that the condition of the grease may be inspected. If necessary, the dovetails are cleaned and regreased as described below. With more complicated movements, it may be wise to seek professional aid.

Access is gained to a screw and lever type mechanism by removing the cover plate at the top of the fine dovetail slide. It is usually held in place by two screws. The plate should be held down while these are removed, otherwise the strong return spring underneath the plate and the plate itself will become airborne.

In most modern stage focusing microscopes the stage support carries the male part of the fine movement dovetail. It may be necessary to remove the stage (if this has not already been done) to gain access to the cover plate.

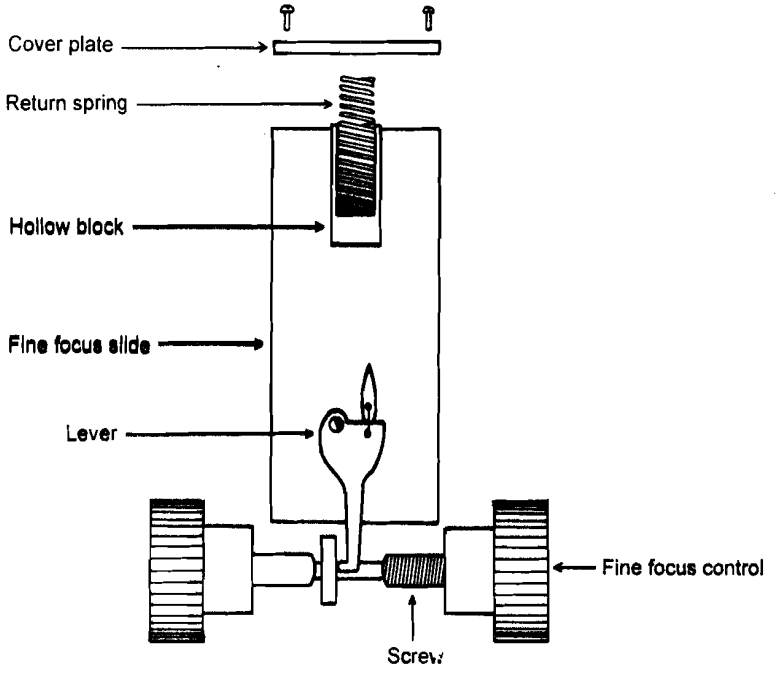

Fig. 7.6: Screw and lever fine focus movement.

In traditional instruments, removal of the cover plate and spring will allow the coarse focus slide with the coarse focus knobs and pinion to be removed upwards as a unit (Fig. 7.7a), exposing the fine focus movement in the head of the limb.

In many 'modern' instruments the male half of the coarse dovetail, the coarse rack, the fine movement and the stage support are usually removed from the limb as a unit (Fig. 7.7 b). In this case, removal of the cover plate and spring allows the stage support, with the male half of the fine dovetail, to be removed, thus leaving the fine focus as a separate unit with the screw and lever exposed.

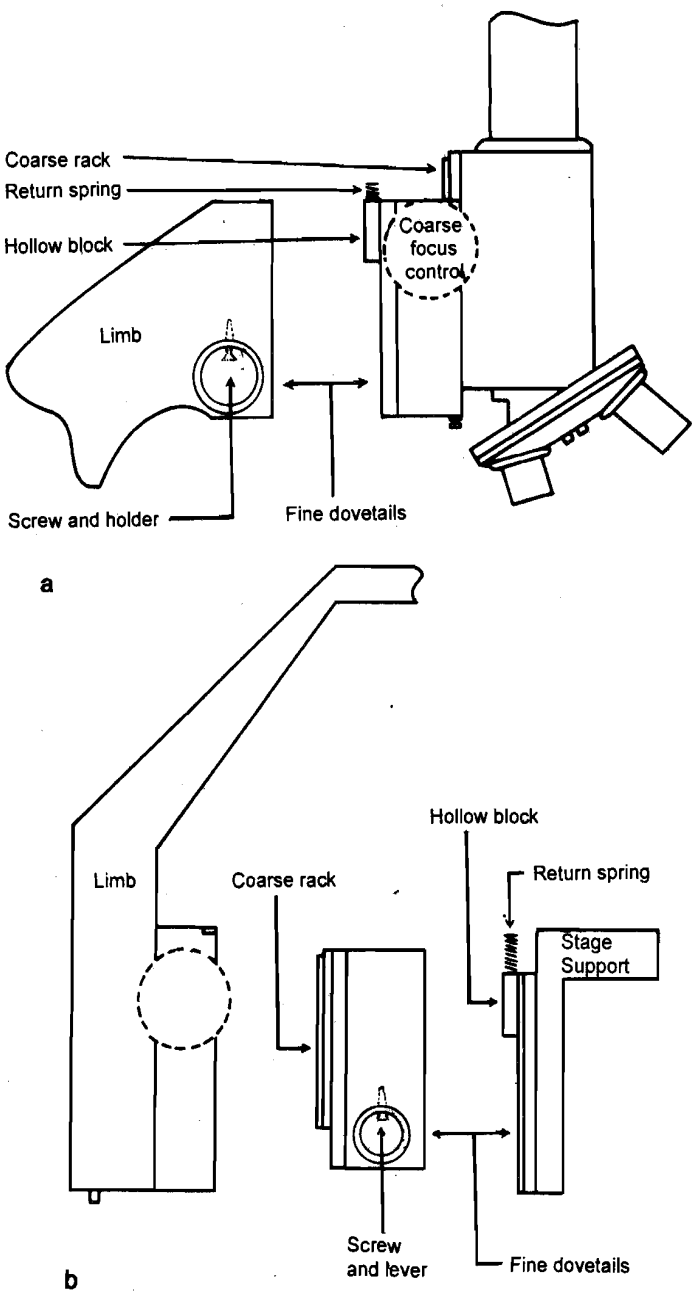

Fig. 7.7: Dismantled focusing mechanisms: (a) traditional stand; (b) 'modern' stand.

Nosepiece

This can usually be removed by undoing a central holding screw (large headed with a narrow slot so that a wide fine bladed screwdriver is needed). For some

instruments a special tool may have to be acquired or made before this screw can be removed. If there are no external indications of the type of nosepiece indexing mechanisms used (Fig. 7.8 a), a ball bearing system is probably present (Fig. 7.8 b). This type of nosepiece should not be dismantled – or great care must be taken to avoid dislodging and losing the balls.

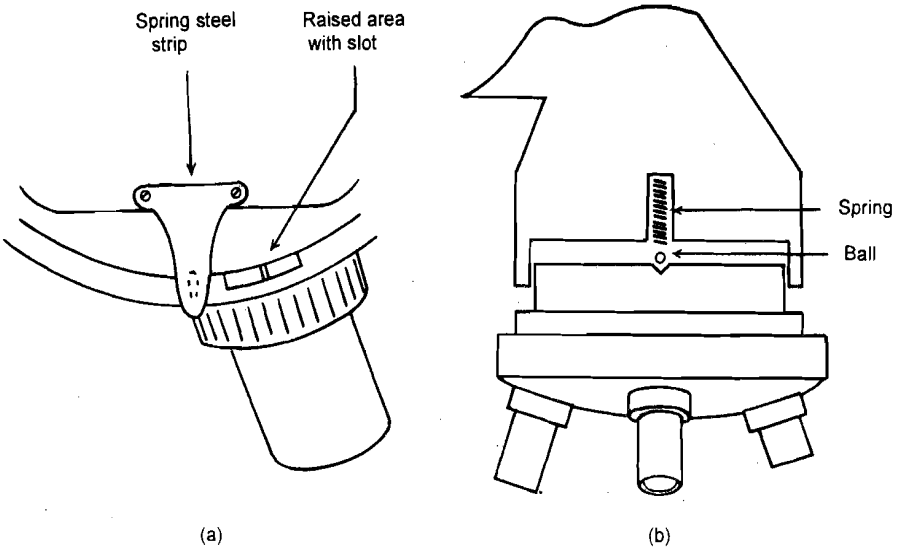

Fig. 7.8: a, b) Nosepiece indexing mechanisms.

Coarse focusing pinion spindle

As a general rule, it is not necessary to remove the coarse focusing pinion. Indeed on some models, special tools and/or a little expertise are required. In some models, although the knobs are screwed on to the spindle in the usual way, locking nuts are used to prevent their relative movement. Special clamps are needed to hold the knobs while undoing the locknuts and again on reassembling. In some instruments, a side and a special C-spanner is required. Unless these tools are available, the pinion spindle should not be removed from these instruments. Systems of tensioning the spindle and adjustment of the coarse focus mechanism are described in the point (5).

(3) Cleaning Mechanical Components

Fine focus mechanism

Normally this requires no attention. Inspect the condition of the grease. It should be clean and soft without discoloration or lumps. If it is dirty and lumpy, carefully remove the worst portions with a clean rag over the end of a dogwood peg. Do *not* use a grease solvent – this might run into the fine focus pinion bearings and affect the grease there. Fresh grease may be applied. Use the brand recommended by the manufacturer. However, if the original grease is in a very poor condition so that most of it needs removing, it is wise to seek professional aid. This applies especially where the movement is somewhat complicated.

Slides, guide rods, etc.

Dovetail slides, guide rods and the bearing surfaces of the nosepiece rims and spindle should have the old, dirty grease removed from them. A clean rag

moistened with a solvent such as 1,1,1-trichloroethane can be used for this. A piece of rag placed over a sharpened dogwood peg will clean the less accessible places such as corner in the dovetails.

Caution: Trichloroethane is less hazardous than the solvents formerly used, but the work area should still be well ventilated and the rag should not be saturated with solvent. Care should be taken not to allow solvent into the fine focus mechanism or on to the pinion, where it may run along the shaft and into the bearings. The bearing surfaces of pinion spindles are under very high loading and they must be kept separated by a continuous layer of grease, otherwise the bearing may seize.

Rackwork and pinions

These should be brushed out with an old toothbrush and the teeth of the rack cleaned out with a sharpened dogwood peg. Similarly treat any substage rackwork which may be fitted.

Mirror or illuminator gimbal peg

This can have the old grease cleaned off with the solvent moistened rag and its aperture in the limb or foot cleaned out with a rag over a dogwood peg.

Other 'mechanical' parts (such as limb, foot and stage)

These can be wiped over with a moist rag and polished with Selvyt cloth. Gross staining or corrosion can be dealt with using appropriate cleaning agents. Frequent changing of the cloth used for a new one *moistened* with the cleaning material is advisable to minimise the risk of damage to other parts of the instrument.

(4) Lubrication of Mechanical Parts

Greases can be applied as a smear with the finger tip, or worked in with a sharpened dogwood peg. All the larger dovetails and any scroll or helical movement of condenser mounted can be regreased. The spindle of the nosepiece and the touching surfaces of the rims should also have a smear of the grease applied to them;. For small dovetails and fine focus movements, use a thin grease developed for high loading. Oils should not be used on slides or most other moving parts of the microscope.

(5) Reassembly of Mechanical Components

The mechanical parts of the stand can now be reassembled, in the reverse order to disassembly, ready to receive the optics, once these have been cleaned.

Take care while replacing rackwork. Slide the mating member down and very gently feed the rack onto the pinion. Do not 'bump' movements together or serious damages may be done to the teeth. The mechanism should be worked a few times: remove any excess grease which appears. While replacing nosepiece movements, tighten the holding screw until the movement is firm without being harsh.

On many instruments it is a relatively simple matter to adjust the coarse focus mechanism at this point. Usually there is some provision for increasing the 'tension' or pressure on the pinion spindle at the bearing point. In some cases

special tools and/or expertise may be required. However, there are a number of instruments where all that is required to effect an adjustment is simple contrarotation of the knobs; this takes up the play by pulling the knobs against a pressure pad.

Where the bearings have to be moved to bring the spindle closer to the rack, trial and error will reveal just how hard to push while retightening screws. If the spindle bearings are clamped by screws on top of the limb these must be tightened alternately by similar amounts until the system is properly adjusted. *Take care that you are adjusting the proper screws. Sometimes the actual adjusting screws lie at the bottom of holes in the top of the limb – the screws which can be seen merely serve to close the holes!*

After adjustments have been made, any locking devices present are retightened and the movement is tested for 'feel'. A smooth, but slightly stiff action is aimed for. A properly adjusted movement should need as much effort to lower the body or stage as to raise it; it should be free from jerks even when operated very slowly.

If it is not obvious how adjustment is effected, consult the supplier. If a complex instrument has noticeable body slip or backlash, it is best to call in an engineer. Resist the temptation to effect a cure by making the teeth of rack and pinion engage more deeply by fitting packing pieces or by bending the rack. This will only make matters worse.

(6) Cleaning the Optics

There are two distinct schools of thought on this subject. Some claim that frequent cleaning can do only harm, because the dust on being repeatedly rubbed across polished surfaces is bound to scratch them. Others claim that dust and grease must be removed regularly if instruments are to give of their best.

Certainly great harm can be done by heavy-handed cleaning and little is achieved by removing dust if a greasy fingerprint is left in its place. Cleaning optical glass needs a light touch and a certain amount of care to achieve best results and avoid damage. Compromise between too frequent cleaning and complete neglect.

If the only 'dirt' present on the glass is dust or other particular matter, it can be removed by blowing air across the surface with a hand bellows or by dusting with a soft brush. The ideal tool is a combination of the two – the photographer's 'blower brush'.

If there are greasy deposits, a gentle wipe with lens tissue may be required. Heavy contamination with grease may mean the use of 1,2-dimethylbenzene (xylene) as a solvent. However, this may attack the mounting compounds used to hold the objective components. Therefore, use enough only to moisten the lens tissue or Selvyt cloth.

Reminding once more

Another good reason for using only small amounts of xylene is its toxicity. It should be used only in a well-ventilated work area for short periods of time

and should be kept off the skin. Objectives should never be immersed in solvents to soak off heavy deposits – they are likely to fall apart!

For contamination by 'aqueous' materials such as sugar solution, blood, glycerine and copper sulphate, use lens tissues moistened with distilled water. Heavy contamination is best removed by repeated gentle rubbing, changing the tissue frequently. Never use a tool of any kind, and avoid the use of tap water (this may leave a deposit of salts after evaporation).

Immersion oil may be removed, using xylene instead of water, in the same way. If the old type of oil has been used and has dried up to a caked deposit, xylene may not remove it. In this case, fresh immersion oil may be an efficient solvent and remove most of the deposit. The remainder can then be cleaned off with xylene.

It is wise to wear gloves, since some older types of immersion oil contain polychlorobiphenyls (PCBs) which, like xylene, are poisonous by skin absroption.

Use a hand bellows to blow off any loose fibres left behind by cloth or tissues.

Objectives

Objectives are cleaned using the techniques described above, choosing the actual cleaning methods according to the degree of contamination of the lens. After cleaning the front lens, hold the objective with this lens uppermost and puff air in strongly to remove any dust from inside. Hold it up to the light and examine the back lens with a magnifier to ensure that no dust remains. In persistent cases a camel hair brush may be used to clean the back lens gently. In no circumstances take objectives apart. If components are displaced, the objectives will have to be returned to the manufacturer.

Eyepieces

Eyepieces are cleaned in much the same way as objectives, except that they can be dismantled for cleaning. If there is likely to be any confusion as to which way a lens should face, mark an edge with a grease pencil or make a sketch of the arrangement of the lenses as they are removed. Most eyepieces are opened up by unscrewing the top half. To minimise the amount of dust settling on cleaned surfaces, the top (eye) lens should be tackled first. While reassembling, take care not to cross the very finely pitched threads.

Condenser and mirror

Dirt on these components is relatively unimportant, though it may sometimes cause glare or loss of intensity. Cleaning is as for lenses, but there should be no need to use solvent. Some condensers can partly be dismantled by unscrewing components; this adds to ease of cleaning. Mirror and condenser can be given a final polish with the Selvyt cloth before inspection with the magnifier for traces of dirt or grease.

Optical head of 'modern' instruments

The prism or mirror in the angled head can be dusted with a blow-brush and gently polished with a lens tissue or Selvyt cloth.

(7) Replacing Optical Components

These are refitted in the reverse order to disassembly – condenser, mirror, objectives, eyepiece. Before replacing the eyepiece, look down the eyepiece tube, using a centring telescope if available, and check the alignment of the components refitted. Small adjustments be required to the mirror or condenser mounts or to the stage, to ensure that all the parts are correctly centred on the optical axis. Many of the threads used in the optical components are fine; care is needed to avoid cross-threading.

(8) Testing

Once it has been reassembled, the instrument should be checked over to ensure that optical components have been thoroughly cleaned and that stops, stage clips and condensers are properly adjusted. The instrument should be set up appropriately.

SAQ 7

When you inspect the fine focus mechanism on one of the lab microscopes, you notice that the grease is discoloured and you decide to replace it. By which one of the following ways you would remove the grease? Tick the correct answer.

1) Wiping the mechanism with a clean rag on a wooden peg;
2) Wiping the mechanism with a clean rag moistened with 1,1,1-trichloroethane; or
3) Flooding the mechanism with a solvent such as that mentioned in (2) and then wiping it clean?

SAQ 8

Based on your experience, write some points in addition to the ones mentioned in Section 7.6 that lists some Do's and Don'ts.

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SAQ 9

What basic 'working rules' should one keep in mind for maintenance operations of a microscope?

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SAQ 10

Pick any five microscopes from your lab, and observe them carefully to prepare an organizer for their servicing.

7.7 ANSWERS

Self-assessment Questions

1. Your answer is correct if you wrote down any of these five points:
   i) Substage condenser aperture or disc diaphragm aperture too narrow.
   ii) Substage condenser not properly focused.
   iii) Neutral density filters (if fitted) should be removed.
   iv) Power of bench lamp too low.
   v) (*If variable*) Internal lamp intensity set too low.

2. Your answer is correct if you wrote down any of these ten points:
   i) Aperture of condenser too wide for objective in use.
   ii) *If hard false 'haloes' present,* condenser aperture too narrow. Increase it.
   iii) Field stop(s) set too wide (causing glare).
   iv) Use of eyepiece of too high a power for the quality of the objective.
   v) Grease, dust or old immersion oil on slide. Clean with lens tissue and polish with Selvyt cloth.
   vi) Lens surfaces not completely clean. Clean them. If the problem remains, try an objective from a different stand. If this is satisfactory, the original objective is faulty.
   vii) Use of oil immersion objective without oil.
   viii) Specimen slide upside town.
   ix) Stops wrongly set. The bottom stop should be set up to prevent further movement of the coarse drive when the fine focus is at the middle of its range and the image is in focus. Even where stops are not fitted, the fine movement may come to the end of its travel during focusing. This can usually be avoided if the fine adjustment is set at midrange before the microscope is approximately focused with the coarse drive.
   x) Jamming of the lower part of a spring-loaded or retractable objective in the body. If *very gentle* attempts to free the lower lens cell are unsuccessful, the objective should be returned.

3. (i)

4. Where a thin specimen with details of diameter <10 μm are to be observed.

5. (i) Raise the objective and adjust in line with the object to be viewed.
   (ii) Place a drop of immersion oil on the slide.
   (iii) Then lower the objective so that it enters but does not quite touch the coverglass.
   (iv) Look into the microscope and raise the objective using the fine focus control until the slide comes into focus.
   You can also refer to Section 7.5.

6. (i) This solvent dissolves the cement used to fix optical components in place.
   (ii) It is poisonous by skin contact and inhalation.

7. 1)

8. Write some Do's and Don'ts that are practiced in your labs, and are not included in the given list.

9. Refer to Subsection 7.6.2.

10. Prepare a brief and crisp table that would clearly convey the precise servicing requirements for each microscope you observe.

EXPERIMENT 8 PREPARATION OF REAGENTS AND STAINS

Structure

8.1 INTRODUCTION

This laboratory exercise is based on unit 8 of your LT-02 theory course which if you can recall, dealt with the nature, theory and preparation of fixatives, alcohol series and stains that are used for the study of whole biological specimens or their histology. In subsections 8.6 of that unit, you were given quite a comprehensive list of the various chemicals reagents and stains that are used in the biological studies and how they are prepared. As mentioned in that unit, you were just asked to go through the various preparation methods and not to memorise them since the list is extensive. You were asked to keep the list as a reference whenever needed.

In this experiment however we will expect you to prepare and remember the method of preparations of a few of the important reagents and stains that are commonly used in the biology laboratories and which are also going to be used by you in Experiment 9 of this course.

Objectives

After this laboratory experiment you will be able to prepare:

- Normal physiological saline for vertebrates
- Frog Ringer's Solution
- Alcohol series or grades of the following strength – 30% – 50% – 70% – 90%
- The plant fixative – Formalin Acetic Acid and the animal fixative – Aqueous Bouin's Fluid
- Stains – Aceto-orcein, Aceto-carmine, Noland's solution, Methylene blue.

8.2 GENERAL RULES FOR PREPARING AND HANDLING SOLUTION

1. All solutions should be kept in clearly and correctly labelled containers.
2. When stock solutions are provided nothing should be poured back to stock bottle. If you take too much solution it is better to discard the excess rather than risk contamination of the entire supply by attempting to conserve a small amount.
3. Distilled water should be used in preparing solutions unless otherwise specified.
4. When diluting acids always add the acid to the diluent, never the diluent to the acid. The acid should be added slowly.
5. Use clean containers for preparing solutions.
6. Strong acids and bases must be handled with care.
7. Avoid inhalation of acid and other fumes, particularly osmic acid or mercuric chloride powder. The laboratory should be well ventilated and volatile fluids such as xylol, toluol, dioxane, chloroform, ether, etc. should be kept covered as much of the time as possible.
8. Flammable solutions should be kept away from open flames and sparks. Be particularly careful with ether and with ether-alcohol-celloidin mixtures.
9. Always wash your hands before smoking or eating after working in the laboratory.
10. Many fixatives and other solutions are poisonous.

Accuracy Required in Preparing Solutions

It should be noted that formulas have been presented with what may appear to be an inconsistent degree of accuracy. Thus, 0.5 ml of acid may be combined with 100 ml (not 100.0 or 99.5) of alcohol. This has been done intentionally, since it indicates the degree of accuracy required in the measurements. The standard practice is to write formulas to the degree of accuracy of the smallest component. Thus, 0.5 ml are combined with 100.00 ml in one formula; 1 ml combined with 100 ml in a second formula; and 100.00 ml combined with 0.24 gram in a third. Although this may appear more consistent in print, it is not realistic. If a volume is given as 100.00 ml it should indicate that the measurement is to be made in a volumertric flask. For most technique purposes (such as preparing fixatives and staining solutions) ordinary graduated cylinder provide the necessary degree of accuracy. Volumes of 1 ml and less should be measured in graduated pipettes. It is well to use a small graduated cylinder for small quantities (thus use a graduated cylinder of 10 ml, rather than a 1000 ml for measurements from 1 to 10 ml, and so on). Most weights have been given to tenths of a gram and the balance used should be accurate to one one-hundredth of a gram. A triple beam balance reading to one-hundredths of a gram is ideal for the technique laboratory. Unless milligrams are specified, an analytical balance is not required for routine preparations in microscopic technique.

8.3 USE OF CHEMICALS

Acids: The acids most commonly employed in technique procedures are acetic and nitric acids in fixatives, and hydrochloric acid in decalcifying and destaining solutions. When percentages are indicated they are based upon

dilutions of glacial acetic acid (99 per cent); concentrated nitric acid (about 70 per cent), and concentrated hydrochloric acid (about 39 per cent). Thus, 1 per cent hydrochloric acid for destaining refers to a solution prepared by adding 1 cc. of concentrated hydrochloric acid to 99 ml of alcohol or water as specified. The exact percentages of the concentrated acids vary slightly with different suppliers and grades and this must be taken into account when a "normal" solution is specified.

Alcohol: References to "alcohol" in technique procedures mean ethyl alcohol unless otherwise specified. Isopropyl alcohol may be substituted for ethyl alcohol in the dehydration and hydration series. Methyl alcohol is preferred for the fixation of smears before staining in Giemsa's.

WARNING: METHYL ALCOHOL IS HIGHLY POISONOUS ON DRINKING.

8.4 PREPARATION OF NORMAL PHYSIOLOGICAL SALINE FOR VERTEBRATES

Physiological saline is used for rinsing blood and debris from tissues before placing them in the fixative. Physiological saline for invertebrates and cold blooded vertebrates is different from that of warm blooded vertebrates because of the proportion of sodium chloride. The physiological saline for various groups within the warm blooded animal vertebrates also vary. In this exercise you will learn to prepare the physiological normal saline for mammals by following the given steps.

8.4.1 Materials Required

Analytical sodium chloride (NaCl) – 0.9 g
Distilled water – 100 ml
Graduated measuring cylinder of 100 ml
Round flask of more than 100 ml capacity
Analytical balance

8.4.2 Procedure

1. Weigh 0.9 gms of analytical sodium chloride and put it in the round flask.
2. Measure 100 ml of distilled water in the graduated cylinder and pour it into the round flask gradually, shaking the flask in order to dissolve and mix the NaCl in the water.

8.5 PREPARATION OF FROG RINGER'S SOLUTION

Ringer's solution is used for moistening tissues which must be kept for some period of time before fixation or for small animals or tissue study which need to be observed in the living state. The ringer solution for invertebrate and cold blooded vertebrate is different from that of the warm blooded vertebrates. In this exercise you will learn to prepare the Ringer's solution for frog by following the given steps.

8.5.1 Material Required

Analytical sodium chloride (NaCl) – 0.65 gms
Potassium chloride (KCl) – 0.025 gms
Calcium chloride ($CaCl_2$) – 0.03 g
Sodium hydrogen carbonate ($NaHCO_3$) – 0.2 g
Distilled water (a little more than 100 ml)
Analytical balance
100 ml Graduated cylinder
Round flask with more than 100 ml capacity

8.5.2 Procedure

1. Weigh each of the following salts separately: 0.65 g of Nacl; 0.025 g of KCl, 0.03 g of $CaCl_2$ and 0.02 g of sodium hydrogen carbonate and put into the round flask.
2. Measure 100 ml of distilled water in the graduated cylinder.
3. Pour the 100 ml of distilled water into the flask containing the weighed salts and gradually dissolve the salts.
4. While pouring the distilled water into the flask, keep shaking the flask in order to mix and dissolve the salts in the water.

8.6 PREPARATION OF ALCOHOL SERIES OR GRADES – 30%, 50%, 70%, 90%

Alcohol is used in various dilutions – 30%-50%-70%-80%-90%-100% (absolute alcohol) for dehydration of tissues and in the reverse (100%-90%-80%-70%-50%-30%) for rehydration of tissues. It is also used as a simple fixative primarily before certain histochemical tests.

Dilutions of alcohols or alcohol grades are usually prepared from rectified spirit which contains approximately 95% or 96 per cent of alcohol and never from the much more expensive absolute alcohol. A simple method of calculating the dilution is to take the number of cubic centimeters of 95 percent alcohol as the percentage required (thus use 70 ml for 70 per cent) and add enough distilled water to bring the final volume to 95 ml. For 50 per cent alcohol, use 50 ml of 95 per cent alcohol and dilute to prepare 95 ml of 50 per cent alcohol. Larger or smaller volumes are prepared on a proportional basis. For example: 700 ml of 95 per cent will provide 950 ml of 70 per cent; 35 ml of 95 per cent will provide 47.5 ml of 70 per cent. For technique purposes the latter dilution may be 47 or 48 ml since the exact percentage in the graded series of solutions for hydration and dehydration is not critical. In this exercise you will learn to prepare the alcohol grades of 30%, 50%, 70%, 80%, 90% by following the given steps.

8.6.1 Materials Required

96% Alcohol (Ethanol or Methanol)
Distilled water
Graduated measuring cylinder of 100 ml
5 – 100 ml bottles with stoppers

8.6.2 Procedure

1. Take a graduated cylinder of 100 ml for preparing the alcohol dilution series.

a) 90% Alcohol

i) Pour 96% of alcohol into the measuring cylinder, upto 90 ml measure mark.

ii) Add distilled water to the cylinder containing the measured amount of alcohol to make the volume upto 96 ml. Keep the 90% alcohol in a tightly stoppered bottle.

b) 70% Alcohol

i) Pour 96% of alcohol into the measuring cylinder, upto 70 ml measure mark.

ii) Add distilled water into the cylinder containing the measured amount of alcohol to make the volume upto 96 ml. Keep the 70% alcohol in a tightly stoppered bottle.

c) 50% of Alcohol

i) Pour 96% of alcohol into the measuring cylinder upto 50 ml measure mark.

iii) Add distilled water into the cylinder containing the measured amount of alcohol to make the volume upto 96 ml. Keep the 50% alcohol in a tightly stoppered bottle.

d) 30% Alcohol

i) Pour 96% of alcohol into the measuring cylinder upto 30 ml measure mark.

iv) Add distilled water into the cylinder containing the measured amount of alcohol to make the volume upto 96 ml. Keep the 30% alcohol in a tightly stoppered bottle.

8.7 PREPARATION OF FIXATIVES

The collected plant material or the animal material is stored in glass-stoppered wide mouth jars containing any general fixative. The purpose of the fixative is to kill the material initially and to preserve the cells and their contents in natural condition as far as possible. Although there are several fixatives in use the best is that which changes the chemistry of the cell to the minimum and which preserves the cells best. In this exercise you will learn to prepare

(a) Plant fixative – FAA and
(b) Animal fixative – Bouins fluid

8.7.1 Material Required

70% ethyl alcohol
Glacial acetic acid
40% formalin
Picric acid (saturated aqueous solution)
Graduated measuring cylinders of 50 ml, 100 ml
Round flask of 150 ml capacity
Bottles with stoppers

8.7.2 Procedure

a) Plant Fixative – Formalin (Formol) Acetic Alcohol (FAA or AFA)

It is a general fixative for plant material and nematodes.

1. Measure 85 ml of 70% alcohol in the graduated, measuring cylinder and pour into the round flask.
2. Also measure 5.0 ml glacial acetic acid in the graduated measuring cylinder and add to alcohol present in the round flask.
3. Again measure 10 ml of 40% formalin in the graduated measuring cylinder and add to the round flask containing the measured amounts of alcohol and acetic acid.
4. Shake the round flask containing all the measured chemicals in order to mix the chemicals.
5. Pour the solution with all the ingredients in a bottle and stopper it.

WARNING: THE VARIOUS INGREDIENTS OF SOLUTIONS SHOULD BE COMBINED ONLY JUST BEFORE USE

b) Animal Fixative – Aqueous Bouin's Fluid

1. Measure 75 ml of saturated aqueous solution of picric acid in the graduated measuring cylinder and pour into a round flask.
2. Also measure 40% of 25 ml formalin in the graduated measuring cylinder and add to the round flask containing the saturated aqueous solution of picric acid.
3. Also measure 5 ml of glacial acetic acid in the measuring cylinder and add to the round flask containing the picric acid and formalin solution.
4. Mix all the ingredients by shaking the flask gently.
5. Pour the mixture solution into a bottle and stopper. This solution will keep indefinitely.

WARNING: PICRIC ACID MAY DRY ON THE CORK AND BOTTLE RIM AND BECOME UNSTABLE DURING STORAGE: WHEN REPLENISHING THE WATER IN THE BOTTLE ENSURE THAT NO SOLUTION REMAINS IN THESE AREAS.

8.8 PREPARATION OF STAINS

Staining techniques are most difficult for the beginner to master because they depend both on method of preparation of stain and the fact that staining effects vary with different tissues. Staining also gets affected by the use of different fixatives, different period of storage and on different types of dyes used.

In this exercise you will learn to prepare those stains which you will use in experiment 9. The stains which you will learn to prepare are:

a) Aceto-orcein
b) Aceto-carmine
c) Noland's solution
d) 1% Aqueous Methylene blue

8.8.1 Material Required

Orcein stain – 0.5-1.5 g
Glacial acetic acid – 90-100 ml
Dry Carmine stain – 0.5 – 1.0 g
Methylene blue – 1.0 gm
Genetian violet (crystal violet) – 0.20 g

Phenol crystals – 6.0 g
40% Formalin – 20 ml
Glycerol (glycerine) – 4 ml
Distilled water – 250 ml
Round flask of 100 ml capacity
Round Corning Flask of more than 100 ml capacity
Graduated measuring cylinder of 100 ml measure capacity
Analytical balances
Burner
Filter paper
4 Stain bottles

8.8.2 Procedure

a) Aceto-Carmine: This is one of the most commonly used stains for chromosomal studies.

1. Weigh 0.5 g of dry carmine stain.
2. Measure 45 ml of glacial acetic acid in a graduated measuring cylinder and pour into a round, corning flask.
3. Measure 55 ml of distilled water in the measuring cylinder and also add to the round corning flask and mix the two.
4. Heat to boiling the corning flask containing distilled water and glacial acetic acid.
5. Add the weighed amount of 0.5 g of carmine stain to the boiling mixture and shake well to mix.
6. Cool the mixture and filter.

b) Aceto-Orcein

1. Weigh 1.0 g of orcein stain and put in a round, corning flask.
2. Measure 45 ml of glacial acetic acid in the graduated measuring cylinder and pour it into the round flask containing the weighed stain powder.
3. Stopper the round flask with cotton wool and heat the mixture to boiling.
4. Cool the mixture in the round flask and add distilled water to it.
5. Filter the prepared stain before use.

c) Aqueous Methylene Blue

1. Weigh 1 g of methylene blue powder and put into a round flask of 100 ml capacity.
2. Measure 100 ml of distilled water in a measuring cylinder and add gradually to the flask containing the methylene blue stain.
3. Shake the flask gently while adding water, in order to dissolve the methylene blue stain.
4. Filter and pour the mixture into bottle for use.

d) Noland's Solution

1. First prepare saturated solution of phenol in distilled water. Weigh 6 gm phenol crystals and put into a round flask. Measure 90 ml of distilled water and add to the flask. Dissolve the crystal in the water by shaking the flask. Pour into a container which is well closed and protected from light.

2. Weigh 0.2 g of genetian violet and put into a clean round flask.
3. Measure 20 ml of 40% formalin in a measuring cylinder of 100 ml capacity and add to the round flask containing the genetian violet.
4. Also measure 4 ml glycerol in a measuring cylinder and add to the flask.
5. Also measure 80 ml of saturated solution of phenol that has been prepared earlier and add into the flask.
6. Shake the flask gently to dissolve the ingredients.

WARNING: DO NOT HANDLE PHENOL CRYSTALS WITH BARE HANDS. DEATH HAS RESULTED FROM AS LITTLE AS 1.5 GRAM ABSORPTION. NEVER APPLY TO LARGE PORTIONS OF BODY SURFACE.

EXPERIMENT 9 PREPARATION OF TEMPORARY SLIDES

Structure

9.1 INTRODUCTION

You have learnt in Block 2, Unit 7 of this course that to observe cells clearly under a microscope the cells need to be spread in a mono layer. This mono layer of cells is then fixed and stained and observed as a temporary preparation or a permanent slide is made which can be stored and observed later.

In this experiment you will learn to make temporary slide preparations of cell suspensions using the smear technique and of soft tissue using the squash technique. You will also learn to make temporary as well as permanent slides of whole mounts of protozoans and phytoplankton.

Before starting your practical you should read Unit 7 of the Biology course LT – 2 again to recollect the methods used for cleaning and labelling slides.

Objectives

After completing this practical exercise you should be able to prepare a

- temporary slide of a given cell suspension using smear technique,
- temporary slide of soft tissue using squash technique,
- whole mounts of *Paramecium, Volvox, Chlamydomonas.*

9.2 GENERAL METHOD OF MAKING A TEMPORARY PREPARATION

A temporary preparation of cells is usually observed as a wet mount. This is prepared by placing a cell suspension in liquid on the slide or if the material to be studied is dry, by placing it directly on the slide and adding water, glycerin or stain to it. The material is then covered by a cover slip. You have to be careful not to trap air bubbles while lowering the cover slip over the material as it will cause interference in observing the material. The procedure is shown in Fig. 9.1.

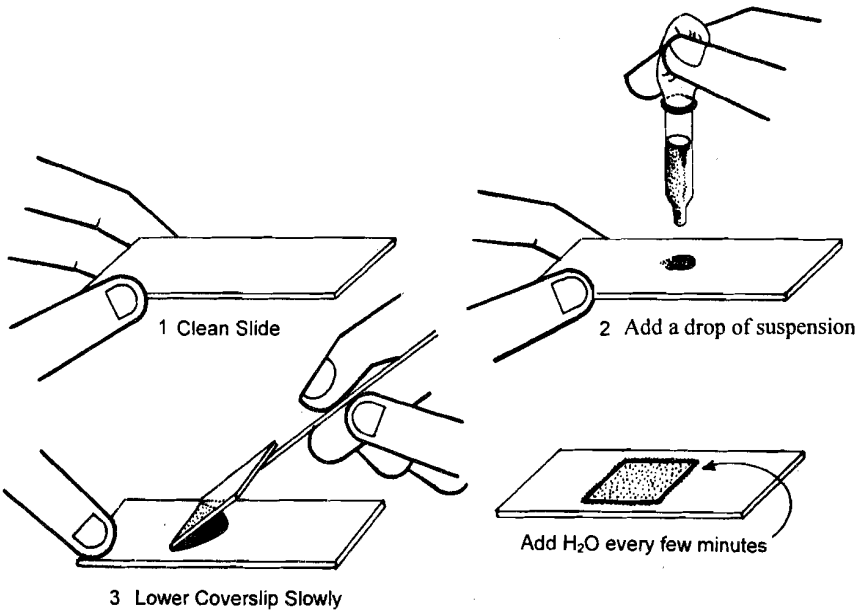

Fig. 9.1 : The technique for lowering the cover slip over the slide.

9.3 SMEAR TECHNIQUE FOR CHEEK SCRAPINGS

In this exercise you will learn to make a temporary mount of cheek epithelial cells by following the given steps.

9.3.1 Materials Required

1. Cover slips, slides, slide labels
2. Disposable spatula or tooth pick
3. 9% NaCl
4. Methylene blue stain
5. Filter paper

9.3.2 Procedure

1. Rinse your mouth well with water.
2. Gently scrape the inside of your cheek with the broad end of a clean tooth prick or a sterilised / disposable spatula.
3. Spread the cells on a clean slide. Add a drop of 0.9% NaCl or physiological saline and a drop of methylene blue (You have learnt how to make physiological saline for mammals as well as for amphibians in Experiment-8)
4. Cover with a cover slip and gently press it to flatten the cells. Alternately you can introduce the stain by irrigation method (see Fig. 9.2)
5. Put the slide under high power in a microscope. Make sure that your counsellor sees the slide. He/She would wish to assess its quality to award marks for it.

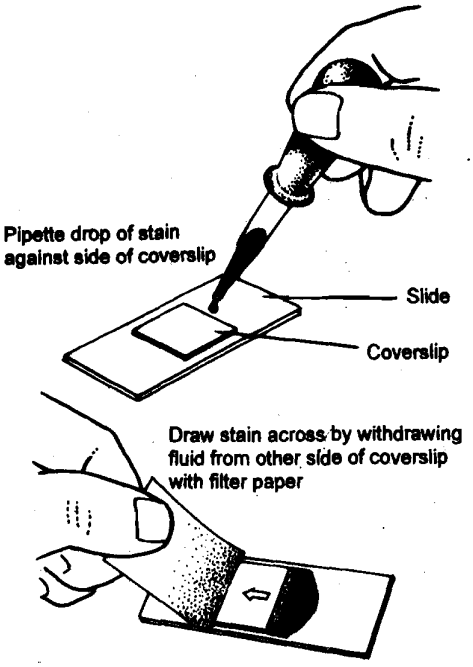

Fig. 9.2 : Technique of Irrigation.

9.3.3 Observations

Locate a single cell under high power. Many of the cells will be crumpled and irregular in outline because the cell membrane is extremely thin and delicate. The nucleus will be stained dark blue in the centre of each cell..

If you don't let in too much light through the microscope you will be able to observe the cell structures better. Compare what you see with Fig. 9.3. In epithelial cells obtained from females a distinct darkly staining body attached to the nuclear membrane can be seen. This is known as Barr body.

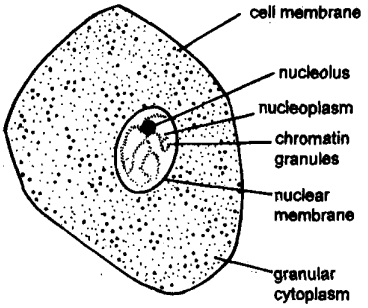

Fig. 9.3: A check cell as seen under the high power after staining.

CAUTION: **Certain infectious diseases can be transmitted through saliva. Avoid any contact with another person's saliva. Do not share a spatula with anyone else.**

9.4 SQUASH TECHNIQUE FOR ONION ROOT TIP

Squash technique is a simple method widely used for the study of chromosomes. This technique consists of applying gentle pressure on a small piece of stained tissue to spread the chromosomes in the cells. This technique is used to study dividing cells showing either mitosis or meiosis and suitable tissues for this are onion root tip, grasshopper testis or anther buds.

In this exercise you will learn to prepare a temporary slide of onion root tip showing mitosis, which can later be made in a permanent slide. You have learnt how to prepare the material in this case onion root tip in Experiment 3.

The onion root should remain in the acetic alcohol solutions for 12 – 24 hours. After fixation the tissue can be transferred to 70% alcohol.

9.4.1 Materials Required

1. Onion root tips
2. 70% alcohol
3. Forceps and dissecting needles
4. Pipette, glass dropper watch glass beaker
5. 2N Hydrochloric acid
6. Acetocarmine/aceto-orcein stain
7. Slides, coverslips,
8. Acetic acid
9. Filter paper
10. Nailpolish

9.4.2 Procedure

1. Transfer the roots from the fixative or storage solution (70% alcohol) in a watchglass.
2. Wash in water until the roots sink.
3. Drain off the water using a pipette and add a few drops of 2N HCl and leave for 10 minutes at room temperature or for a minute over a spirit lamp. While hydrolysing over the flame be careful not to overheat by moving the watchglass. Alternately you can warm the watchglass over a beaker of boiling water till the liquid steams.
4. After hydrolysis drain off the HCl and wash root tips in water.
5. Remove water and add 1% acctocarmine or aceto – orcein to the root tips for staining. The staining requires 10 – 15 minutes.
6. Transfer 2 –3 root tips on a slide, cut above 2 mm from the pointed end. Discard the rest.
7. Place a drop of 45% acetic acid on the root tip and carefully place a coverslip. Remove excess acetic acid by the edge of a filter paper.
8. Place the slide between 2 layers of filter paper and gently tap the coverslip by the back of a pencil to get an even spread of chromosomes.
9. If air bubbles get trapped in the coverslip add a few drops of glycerol or acetic acid to the edge of the coverslip.
10. Seal the edges of the coverslip by applying nail polish so that the fluid evaporation is minimum and observe the slide under a microscope.
11. Label the slide appropriately and show to your counsellor.

Fig. 9.4 : Stages of mitosis from an onion (*Allium*) root tip.

9.4.3 Observations

In a squash preparation of the onion root tip you will find that a majority of cells will be in interphase (non-dividing cells). The interphase nucleus will appear as a darkly stained body with acetocarmine or acetoorcein. The nuclear membrane is intact and the chromosomes form a fine network. You will have to look for dividing cells if you use acetoorcein as only the nucleus and chromosomes will be stained. Acetocarmine will stain the whole cell, use a textbook or the illustration of Fig. 9.4 to identify different phases of division. You could also consult your counsellor for more information.

9.5 WHOLE MOUNTS OF UNICELLULAR ORGANISMS

A unicellular organism has to carry out within one cell all the functions that in a multi-cellular organism are performed by different cells and tissues. Accordingly it possesses a high degree of internal organisation, which can be observed by making a whole mount of the organism. In this exercise you will learn to make whole mounts of three protistans. *Paramecium* an animal like protistan, *Chlamydomonas* an autotropic protistan and, *Volvox* an example of a colonial protistan.

9.5.1 Materials Required

1. Paramecium culture, *Volvox*, *Chlamydomonas*
2. Methyl cellulose
3. Methyl green in ethanoic acid/acetocarmine
4. Acetic acid
5. Albumin glycerin
6. Petridishes, Beakers, Slides, Coverslips, Slide labels
7. Alcohol series for dehydration
8. Xylene
9. DPX mountant
10. Noland's solution
11. Iodine solution

9.5.2 Procedure

A) Temporary whole mount of *Paramecium*

Put a drop of *Paramecium* culture on a clean slide and cover with a coverslip. Since paramecia move very rapidly their movement can be slowed down by adding an equal amount of methyl cellulose to a drop of the culture. Irrigate your slide with either 1% methyl green in ethanoic acid or acetocarmine. Both fix the organism and stain the nuclei green in case of the first and red in the case of the second. Then observe under high power in the microscope. Label and show the slide to your counsellor.

B) Preparation of Permanent Mount

It is quite possible that some of you may be expected to make a permanent preparation of some protozoan like *Paramecium*. It is a fairly simple procedure and good results can be obtained if done carefully. The procedure is given below:

1. Take a clean dry slide, put a small drop of albumin glycerin in the centre of the slide and with the tip of your forefinger spread it in the form of a thin film.
2. With a glass dropper add 1-2 drops of *Paramecium* culture on the slide and observe under low power of the microscope. You will see a large number of paramecia moving rapidly in the culture medium.
3. Let the culture become dry you can put the slide under an electric lamp.
4. Open a bottle of acetic acid and quickly pass the slide right side down over the mouth of the bottle. This will fix the protozoan.
5. Keep this slide in a petridish of 6" diameter with the right side up. Add a few drops of the stain acetocarmine so as to fully cover the culture.
6. Stain the slide for about 5 – 7 minutes.
7. Drain off the excess stain by a blotting paper.
8. Wash the slide with 30%, 50%, 70%, 90% alcohol in ascending order. Always keep the petridish covered by another petridish. This will gradually remove the water from the culture.
9. Now wash the slide twice with absolute alcohol. This completes the dehydration process. Be careful that the petridish is covered tighly so that no atmospheric moisture gets in.
10. Remove the alcohol and add a few drops of xylene on the slide. This will *clear* the protozoan so that it is visible more clearly under the microscope.

    Any turbidity with the addition of xylene shows that the dehydration process is not complete. Repeat the step with absolute alcohol and then xylene again.

11. Put a drop of DPX mountant over the culture, and lower a coverslip carefully over it so as to trap no air bubbles.
12. Label the slide and keep the slide overnight in an incubator. Your permanent mount is ready.

C) Whole mount of *Chlamydomonas* and *Volvox*

Chlamydomonas and *Volvox* are phytoflagellates that are found in freshwater ponds and ditches. Sometimes they are so abundant as to produce a green scum on ponds. *Chlamydomonas* are single-celled green flagellates while *Volvox* is an example of colonial phytoflagellate that forms a hollow spheroid with several thousand cells embedded in its mucilaginous wall. You would be supplied with specimens of these phytoplagellates collected from ponds and ditches or cultured in the laboratory. The method of preparing a temporary mount is the same for both species.

1. Place a drop of culture or a drop of water containing the flagellates on a clean slide and add a drop of 10 per cent methyl cellulose which will restrict the movement of the organism and allow you to study the organism.
2. Place a coverslip on the drop and observe under a microscope. By cutting down the light you will be able to observe the flagellar movement better.
3. You could stain the slide by the irrigation method using acetiocarmine to see the nucleus, Noland's solution to see the flagella and iodine solution to see the starch grains.
4. Show your slides to your counsellor.

9.5.3 Observation

Under high power of the microscope try and observe as much of the internal organs. In *Paramecium* you will find it difficult to see the micro nucleus as it is generally covered by the macro nucleus. You may be able to see the contractile vacuole discharging before you stain and fix the mount. Use the help of the given diagram (Fig. 9.5) to identify the structures in the *Paramecium*.

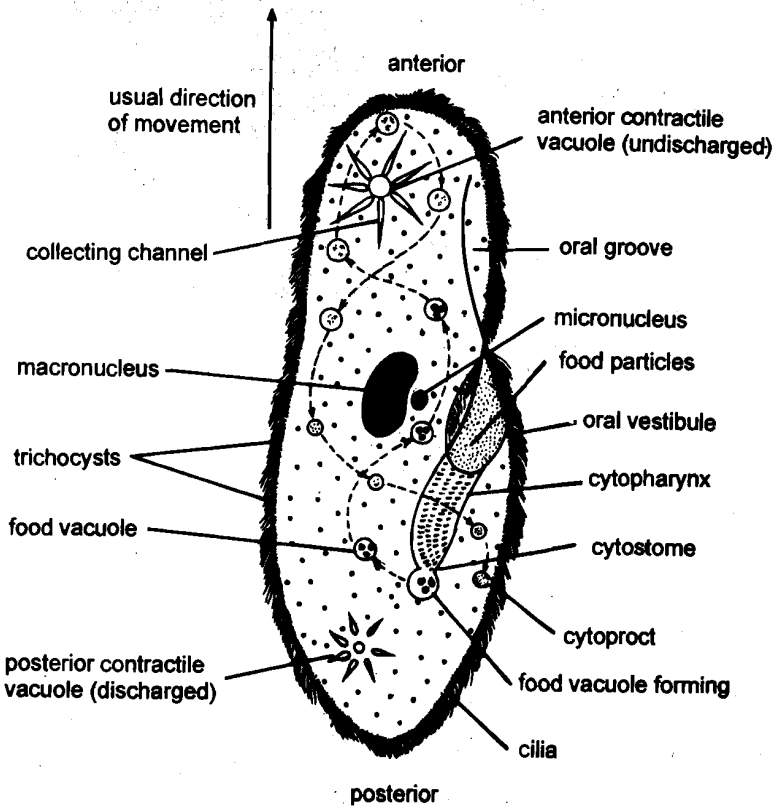

Fig. 9.5 : *Paramecium*. By adjusting the light in the microscope all the structure can be seen.

Under high power you will observe that *Chlamydomonas* has two flagella, cellulose cell wall, two small contractile vacuoles, cytoplasm and cup shaped chloroplast, pigment spot and pyrenoid. You can compare your slide with Fig. 9.6.

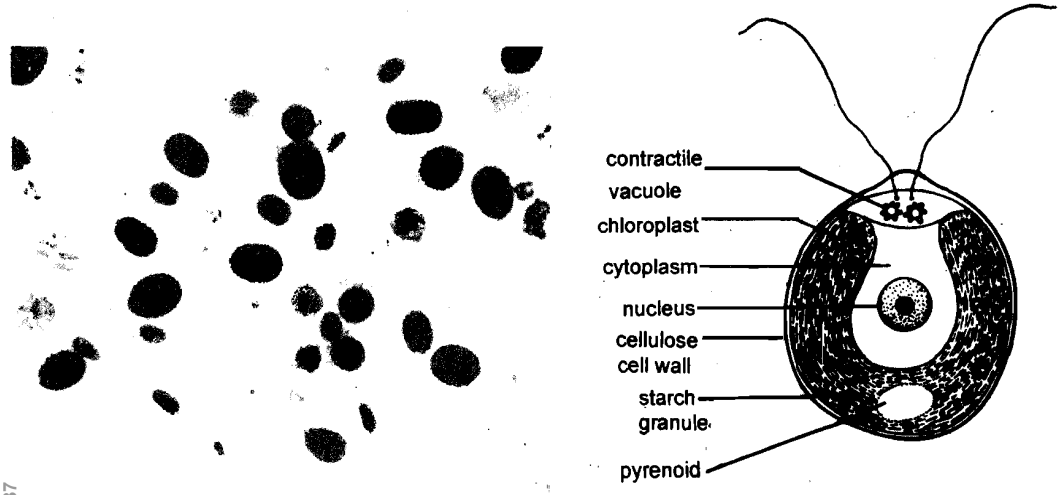

Fig. 9.6 : *Chlamydomonas*, a common green unicellular algae.

In colonial flagellate *Volvox*. Notice that the flagellar beat of the individual cells is coordinated with each other so that the colony moves in an oriented fashion. Compare your slide with Figure 9.7. You may also observe daughter colonies inside individual colonies.

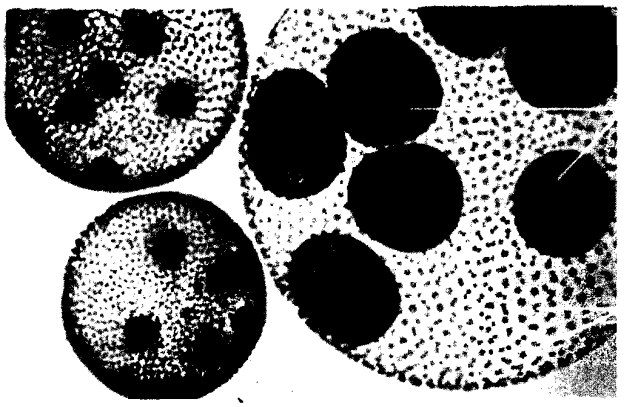

Fig. 9.7 : *Volvox* with daughter colonies. Each cell of the colony looks very much like the single cell of *Chlamydomonas*.

EXPERIMENT 10 PREPARATION REQUIRED FOR DISSECTIONS

Structure

10.1 INTRODUCTION

Dissection is a major component of the biology experiments. The meaning of dissection is to cut open the animal in order to ascertain the structure of its parts. The object of dissection is to separate the several parts from one another, so as to define their boundaries and display clearly their mutual relations. Dissection consists mainly in removing the connective tissue which binds the several parts together. Dissection requires lots of preparations before hand.

Objectives

After doing this experiment you should be able to:

- prepare dissection trays/petriplates,
- anaesthesize the animals correctly,
- dispose dissected animals safely.

10.2 MATERIALS REQUIRED

Dissection trays
Petridishes
Dissection kit
Chloroform
Ether
Formalin
Urethane
Microscope
Table lamp
Animals

10.3 PROCEDURE

In schools, colleges and universities, dissection of chordates/non-chordates is one of the major experiments. A lab technician has to perform several tasks before and after dissection.

10.3.1 Procuring Animals

Orders for animals should be placed with an animal supplier according to the number of students in a class. This exercise should be done two-three days before dissection is to be performed.

10.3.2 Anaesthesizing the Animals

Before dissection animals are given anaesthesia. Chloroform and ether are used as anaesthesing agents. Rats, frogs, and pigeons are generally freshly chloroformed for the dissection, though for some of the dissections e.g. cranial nerves in rat and internal ear in frog, preserved specimens are required. Similarly scoliodon is also preserved after anaesthesizing them. Preservatives commonly used are formalin (5%, 8% or 10%) and 70% alcohol.

10.3.3 Theoretical Knowledge

Keep a well-labelled diagram of the dissection to be done in the classroom. You should have some theoretical knowledge of the dissection to be performed e.g. if students have to dissect male or female reproductive organs of rat, frog or other animals, then you should be able to differentiate them morphologically, just by looking at them externally.

10.3.4 Setting up of dissection trays

Large animals like frog, rat, fish, pila, leech and other animals are dissected in dissection trays whereas small animals such as cockroach and small insects can be dissected in petriplates.

It should be seen that wax is spread over trays uniformly and water does not drip from the trays. The trays should be half filled with water so that the animal can be fully immersed in it. The trays should not be completely filled with water.

One set of dissection kit (about which you have studied in detail in Experiment 1), dissection tray, a microscope and a table lamp should be arranged for the teacher/instructor. Similar sets should be arranged for students. It should be seen that dissection instruments are clean and sharp.

Water should be kept clean in the dissection tray during dissection by changing it whenever stained with blood etc. so that visibility remains the same. If any animal is bleeding profusely, alcohol dipped cotton should be applied to the affected organ/blood vessel. See that every waste of dissection is put in the petridish. It should not be thrown carelessly on the top of table.

10.3.5 Pithing

Some of the experiments like muscle twitch and heart profusion in frog do not require chloroform-anaesthesized animals. Before dissection is started, you have to anesthetise the frog by injecting 2.5 ml of 20% urethane intramuscularly. This would quieten the frog. Alternately you could immobilize the frog by pithing. You have learnt how to pith a frog in Exp. 6 of this course.

10.4 FLAG LABELLING

Frequently, the students are asked to dissect some organs, blood vessels or nerves etc. and flag label them. For flag labelling, small pieces of paper (2.5x0.7 cm) are prepared and a needle is passed through each close to one end.

The names of organs, blood vessels or nerves are written on these flags and each flag is inserted in the dissecting trays close to the organ, blood vessel or nerve bearing the name on the flag.

Some of the dissections require black-papering. You have to cut black paper into small and thin strips which can be inserted underneath blood vessels and nerves.

10.5 SAFE DISPOSAL OF DISSECTED ANIMALS

You have already studied in Unit 14 and Exercise 13 of LT-1 course that the dissected animals should be buried deep in the soil. Simply placing the material in a plastic bag and putting it in the dustbin is not good enough.

10.6 SELF ASSESSMENT QUESTIONS

1. Which are the commonly used anaesthesing agents?

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2. How do you pith animals?

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3. How will you display the dissected specimens?

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4. Which is the safest way to dispose off a dissected animal?

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EXPERIMENT 11 TECHNIQUES FOR MICROBIAL CULTURE AND GRAM'S STAINING

Structure

11.1 INTRODUCTION

Cultivation of micro-organisms involves the provision of the correct nutrients and physical conditions in the laboratory so as to enable the organisms to grow in an environment similar to their natural one. In the theory part of this course we have discussed the nutritional requirements of the micro-organisms in general. In this practical you will practise growing micro-organisms in the laboratory.

On the basis of their response to Gram's stain, bacteria can be categorised as Gram positive or Gram negative bacteria. You will use Gram's staining technique to identify the type of bacteria. Wear gloves while carrying out these experiments.

Objectives

After performing this experiment you should be able to:

- prepare the medium required for culturing the microbes,
- sterilize the medium and glassware in order to minimize the contamination,
- describe the ways of streaking the inoculum to get the isolated colonies,
- list the stains, used in Gram's staining method, and
- prepare and stain the bacterial smear for categorization as Gram positive or Gram negative bacteria.

11.2 FUNGAL CULTURE

Most of the fungi can be grown on various kinds of media that are also used for culturing the bacteria. You have already read in Unit 5 that media can be of two types: synthetic and non-synthetic. Synthetic media i.e. Defined media are those whose constituents and the chemical composition of the constituents are known. Czapek's Dox medium, Richard's solution, Sabourand agar, Starch Casein agar are some of the synthetic media that are used for growing fungi in the lab. Non-synthetic media i.e. Complex media consist of complex natural products whose exact composition is not known. These media are of plant or animal origin and include extracts of fruits, vegetables, milk, egg, blood, meat,

yeast, malt etc. and some known chemical substances. Since these media are of complex nature, they can support large varieties of microbes especially fungi and are useful for routine laboratory cultures. In this practical we will use non-synthetic solid medium made from potatoes for fungal culture. You can always use the specific medium requirements for the specific microorganisms whenever you need to cultivate them in your lab.

11.2.1 Materials Required

500 ml Beaker
500 ml Erlenmyer flask } Heat Resistant Glassware
Petriplates
Test tubes
Metal loop
pH meter/pH paper
HCl 1 Normal
NaOH 1 Normal
Electronic Balance
Media components:

Pealed potatoes	-	50 gms
Dextrose	-	4 gms
Agar	-	3.75 gms

Distilled water

11.2.2 Procedure

1. Cut the pealed potatoes into small pieces, boil in 100 ml distilled water in the beaker to make a thick paste.
2. Add some water (about 50 ml) to the paste and filter through muslin cloth. Put the filterate in 500 ml flask.
3. Weigh required amount (4 gms) of dextrose and add to the filterate.
4. Add distilled water to make the volume upto 250 ml.
5. Adjust the pH of the medium to 6 – 6.2. (Check the pH of the medium by using pH meter or pH paper. If need arises adjust the pH by using 0.1N HCl or IN NaOH as the requirement may be).
6. Weigh required amount of agar (3.75 gms – 1.5%) and add it to the medium.
7. Cover the flask with cap or cotton plug, wrap the foil around the cap and autoclave the medium for 15 min at 15 lb pressure.
8. When the pressure of the autoclave drops to zero, sterilization is complete. Wear the gloves, take out the medium and place in the water bath set at 50°C. This temperature will enable the medium to cool but prevent it from setting. (You can also keep the medium outside and let it cool till you can hold it in your hands. Medium kept in the flask beyond this time will solidify.)
9. Pour 30 ml of medium in each petriplate. For this, first pour the medium from the flask into a graduated test tube and then from the tube into the petriplate (Fig. 11.1).
10. Let the petriplates be cooled and medium be set.
11. Take into the loop the spores from the fungal stock culture and streak on the medium surface as shown in fig. 11.2. You can streak several plates from the same stock culture. Streaking is done to gradually dilute the concentration of the inoculum spore so that isolated colonies growing from a single spore can be obtained. You can do the streaking in the following way:
    a) As shown in Fig. 11.2 (a) and (b), take and spread a loopful of culture over area A without touching the sides of the plate. (Before this inoculation process, ensure that the agar surface is completely set).

b) Area A will contain the highest concentration of bacteria and is referred to as the pool. As you can see the directions in the fig 11.2 (b) streak the culture over area B,C, D and E subsequently one after the other. Before each step sterilize the loop over a hot flame and cool it prior to use. This streaking method effectively dilutes the concentration of spores in a stepwise manner and it becomes possible to produce isolated colonies. You can streak in different patterns as shown in Fig. 11.3. You must streak by moving the loop in the forward direction only. Never make backward movements with the loop otherwise you will not be able to get isolated colonies, as mixing of spores will occur.

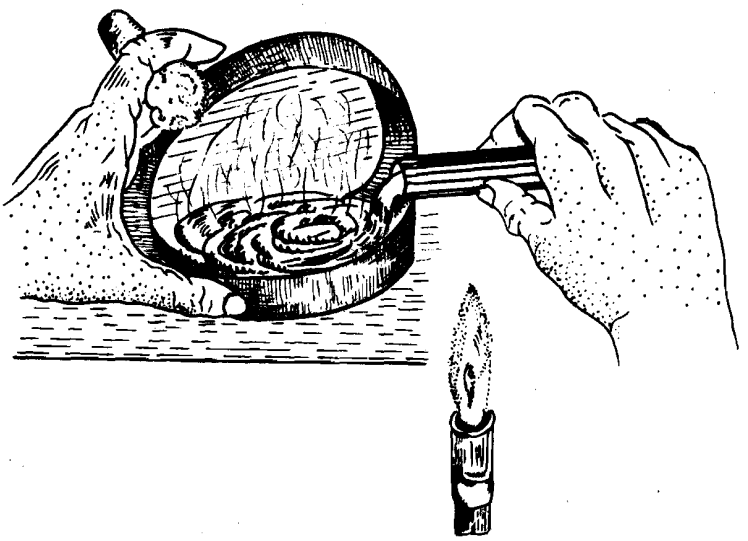

Fig. 11.1: Pouring medium into petriplates.

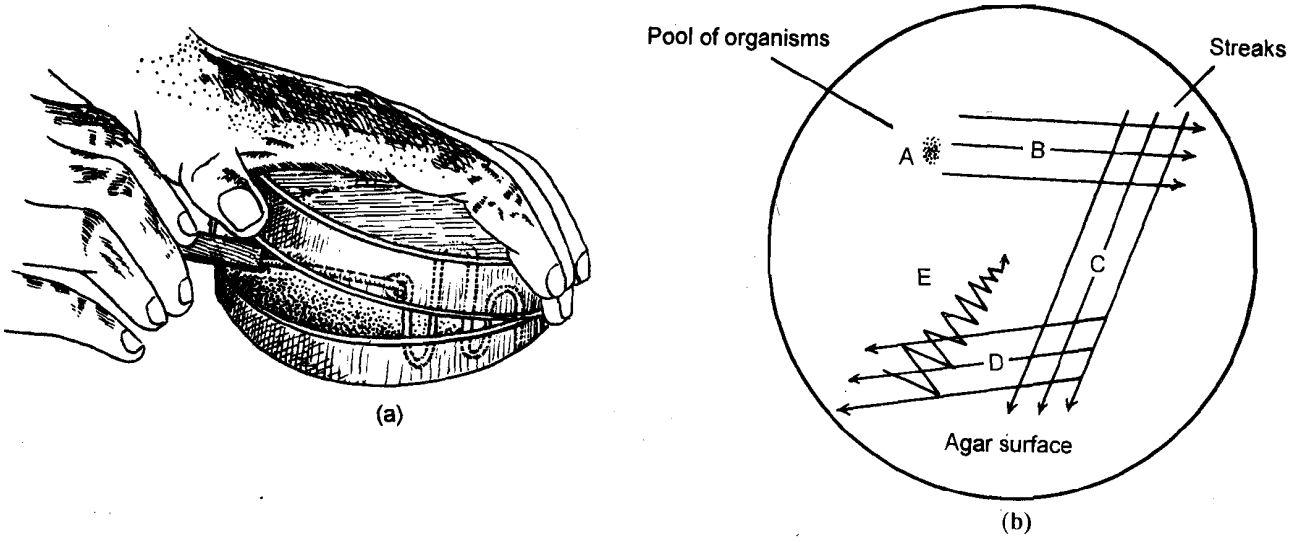

Fig. 11.2: Streaking method for culturing microbes on the solid medium: (a) use of metal loop for streaking the inoculum (fungal spores), (b) streaking directions on agar plate.

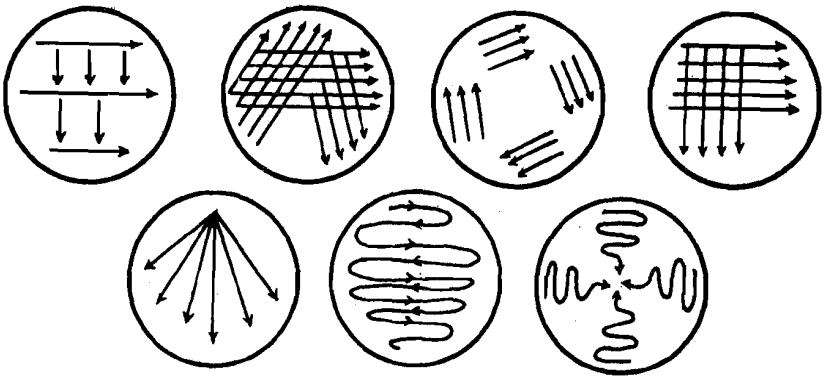

Fig. 11.3: Different ways of streaking on solid medium.

12. Keep the plates in an inverted position in the incubator at 37°C for 3 to 4 days.
13. Observe the plates after 48 hours and see the separated colonies growing. These are isolated colonies and each of them has grown from a single spore. You can reculture these isolated colonies on other solid medium plates and store as stock culture.

11.2.3 Precautions

1. All the glassware and the loop must be thoroughly washed, dried and sterilized before use.
2. The medium should be poured and culture should be streaked on the laminar flow bench. You have already read about how to operate the laminar flow in the earlier practicals. In case you don't have the laminar flow bench in your lab, perform these activities on a normal bench. The bench should be first cleaned by spirit or any other disinfectant. Light the spirit lamp and keep it on the bench before you start your work. (Preferably use two spirit lamps, one on each side on the bench). A spirit lamp is used to sterilize the surrounding air.
3. Operate the autoclave, oven and incubator as you have already learnt in earlier experiments.
4. Thoroughly wash your hands with soap and clean with disinfectant before and after doing the experiment.
5. Clean the bench with disinfectant after finishing your experiment.
6. Mark the plates on the back of the petriplate for the culture identification. To mark, write the name of the fungus cultured and the date of the culturing. Preferably use a permanent marker pen for writing.
7. Dispose off the culture material and sterilize the glassware as you have read in LT-1 course.

11.3 *PARAMECIUM* CULTURE (HAY CULTURE)

11.3.1 Materials Required

500 ml Erlenmyer flask
Petriplate
} Heat resistant glassware
Wheat grains
Hay

Tap water

11.3.2 Procedure

1. Collect some water from a pond having submerged leaves and dead organic matter and *Paramecium.*
2. In the flask take 450 ml of tap water, add 20-25 grains of wheat and some hay.
3. Boil this mixture for 5 to 10 mins. and allow it to cool down. This is the culture solution.
4. In a petriplate pour 20-25 ml. of culture solution and add 5 to 10 ml. of water taken from the pond and cover this petriplate.
5. Keep this petriplate in the incubator. The optimum temperature for culturing *Paramecium* is 22°C - 25°C.
6. You will see that *Paramecium* has appeared in the culture within 4 to 5 days. However, you may also observe the growth of certain bacteria, when you make a slide of the culture and see it under the microscope. These bacteria come along with pond water.

11.3.3 Observation

Study the structure of *Paramecium* by making a temporary mount of this organism. Put a drop of culture on a slide, cover it with the cover slip and see the slide under the microscope.

11.4 GRAM'S STAINING OF BACTERIA

11.4.1 Materials Required

Reagents for Gram's Staining:
Crystal violet, Gram's iodine, Ethyl alcohol or Acetone, Safranin
Slides
Distilled water
Wash bottle
Pasteur pipette
Inoculating loop (metal loop)
Cultures of nonpathogenic bacteria like *Pseudomonas*, *E.coli* etc.

11.4.2 Procedure

1. Transfer a loopful of bacterial culture on a clean slide. Before it is used, the inoculating loop is sterilized by heating in the flame (Bunsen burner) and cooled. The loop should again be sterilized before it is set down after use. (Ask for the bacterial culture from your counseller).
2. To make the smear, spread the bacterial culture on the slide with the help of an other slide and allow it to air-dry. This dry smear is passed through the flame 4 to 6 times to heat fix the bacteria. Because of heat fixing the bacterial enzymes are denatured and autolysis is prevented. Also the bacterial cells adhere to the slide because of heat exposure.
3. To the fixed smear apply crystal violet (called primary stain) solution by pasteur pipette. All bacteria are stained purple.
4. Next apply iodine solution (Gram's iodine also called mordant). This intensifies the ionic bond between the crystal violet stain and the bacteria.

5. Apply ethyl alcohol or acetone (decolourizing agent). The primary stain i.e. crystal violet can be washed out of bacterial smear (decolorization) or the smear can remain unaffected.

Note: The bacteria are gram positive bacteria if the smear is not decolourized and retains the deep violet colour due to crystal violet stain. The decolourized smear contains gram negative bacteria and should be processed as follows:

6. Apply to the decolourized smear safranin stain (secondary stain or counter stain).

Note: The decolourised bacterial smear will be stained red due to counter staining.

11.5 SELF ASSESSMENT QUESTIONS

1. What is the difference between synthetic and non-synthetic media?

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2. How can you obtain isolated colonies of fungal culture on solid medium?

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3. List four important precautions you should take while working with microbes.

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4. **On the basis of staining, how can you differentiate between gram positive and gram negative bacteria?**

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Course Name: **Laboratory Techniques in Biology**
Course Code: **LT- 02**
Credits: **02 Credits**

Experiment 1: Handling Common Laboratory Equipment

Experiment 2: Laboratory Organisation

Experiment 3: Procuring Plant Material

Experiment 4: Procuring Zoological Material for Lab Exercises

Experiment 5: Setting of Demonstrations of Physiological Processes in Plants

Experiment 6: Setting Up Apparatus for Demonstrating Physiological Activity in Animals

Experiment 7: Microscope Handling and Maintenance

Experiment 8: Preparation of Reagents and Stains

Experiment 9: Preparation of Temporary Slide

Experiment 10: Preparation Required for Dissections

Experiment 11: Techniques for Microbial Culture and Gram's Staining

Lab Requirements for LT– 02

1. Autoclave
2. Pressure cooker
3. Microtome knives
4. Dissecting kit
5. Hot air oven
6. Incubator, Water bath, Centrifuge
7. Scissors
8. Coplin jars or wide nouth bottles
9. 100 ml beakers
10. Scalpel
11. Nylon nets
12. Large clean jars or buckets
13. Shallow white pans or papers
14. Trowel
15. A bucket
16. Flashlight torch (for night collection)
17. Blunt-end forceps
18. Insect-collecting net
19. Killing jar
20. Light sources such as an electric bulb (~200 W) or a lantern lamp.
21. A transparent vial made of glass or plastic
22. Rubeer stopper with two holes
23. Two glass tubes each with a bend
24. Rubber tube
25. Small piece of muslin cloth
26. An empty glass bottle with an air-tight lid
27. Potometer
28. Stop watch
29. 500 ml conical flask
30. 25 ml test tube
31. T – tube
32. Pinch clip
33. 1 mm diameter graduated pipette
34. Wire gauze
35. Thermometer
36. 250 – 500 ml beaker
37. 15- 25 ml test tube
38. Cork borer
39. Kymograph Recording System Consisting of:
    Kymograph apparatus
    1.5 volt dry cell (stimulator) with
    Electrodes attached to it
    Muscle lever
    Double hook
    Femur Clamp and Stand
    5 gm weight
    Recording stylus
40. Injection syringe and needle
41. Board made of soft wood
42. Pins
43. String
44. Compound Microscope and Dissection Microscope
45. Illuminator or lamp
46. Slide micrometer graduate in 0.1 mm or 0.01 mm units
47. Microscope fitted with Abbe condenser and usual objectives
48. Oil immersion objective lens
49. Analytical balance
50. 100 ml Graduated cylinder Round flask
51. Graduated measuring cylinders 50 ml, 100 ml
52. Round flask of 150 ml capacity
53. Burner
54. Cover slips, slides, slide labels
55. Disposable spatula or tooth pick
56. Pipette, glass dropper
57. Methyl green Acetocarmine
58. Petridishes, Beakers, Slides, Coverslips, Slide labels
59. Dissection trays
60. Petridishes
61. Dissection kit
62. 500 ml Beaker
63. 500 ml Erlenmyer flask
64. Petriplates
65. Test tubes
66. Metal loop
67. pH meter/pH paper
68. Electronic Balance